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SHORT COMMUNICATION

Self-assembled monolayers as a base for immunofunctionalisation: unequal performance for protein and bacteria detection

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Abstract Biosensor development strongly depends on the optimisation of surface functionalisation strategies. When gold surfaces are considered, immunofunctionalisation by modification of self-assembled monolayers (SAMs) is one of the preferred approaches. In this respect, SAM-based antibody (Ab) incorporation has shown better performance than Ab physisorption for the detection of proteins and small targets. Reports on bacteria detection are less frequent. In this work, we assess the performance of various SAM-based gold immunofunctionalisation strategies, currently applied to protein detection, in the field of bacteria determination. We present the results for Ab chemical conjugation on mercaptopropanoic acid and mercaptoundecanoic acid SAMs, as well as on a dextranized cysteamine SAM. All the modified surfaces studied were shown to be appropriate for the direct detection of an enzyme-labelled protein, but none succeeded in detecting a bacterial target in a sandwich assay format. Conversely, gold functionalised by Ab physisorption allowed *E. coli* detection when a sandwich enzyme-linked assay was carried out. The implications of bacteria size and wall complexity are discussed. These results indicate that immunofunctionalisation strategies appropriate for protein detection are not necessarily transferable to work with more complex targets such as bacteria. In this respect, Ab physisorption appears to be a suitable alternative to SAM-based gold functionalisation for bacteria detection.

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Introduction

Surface functionalisation is perhaps the most important step in biosensor development, as it conditions the efficiency and selectivity of the detection event. Traditional methodologies, such as enzyme-linked immunosorbent assay (ELISA), usually exploit the nonspecific adsorption of the capture biocomponent onto a relatively big surface. On the other hand, the much smaller size of microfabricated biosensors forces the researcher to base biosensor performance on oriented/directed anchoring instead in order to maximize efficiency. Many strategies and components have been employed for this purpose, such as the incorporation of protein A/G or (strept)avidin for immunoglobulin attachment [1–5]. The ultimate success of the approach depends as much on the functionality of the biorecognition material as it does on the nature of the transducer surface and the chemistry used to combine them. When gold surfaces are considered, one of the preferred strategies consists in the incorporation via self-assembled monolayers (SAMs) [6–8]. SAMs have been reported to decrease nonspecific biomolecule adsorption compared with bare gold [9, 10], while covering the gold surface in a relatively homogeneous manner and providing it with suitable reactive groups for the directed conjugation of capture biocomponents [6, 11]. An important number of works have been published reporting the incorporation of biomolecules (either Ab or and Ab-capture protein) onto

SAM-coated surfaces. Some authors, on the other hand, have applied this strategy to bacterial detection. Despite the successful reports in which a SAM-based immunosensor enables the detection of bacteria, the fact is that few of those works provide the negative controls required to guarantee the specificity of their assay [12–16].

In this work we present the results of a comparative study. We applied several surface functionalisation protocols, which are based on Ab incorporation onto SAM-modified gold surfaces, for the detection of both proteins and bacteria. As will be discussed, our data show that immunofunctionalisation strategies appropriate for protein detection are not necessarily transferable to work with more complex targets such as bacteria. This has important implications in biosensor development.

Materials and methods

Reagents and biocomponents

Unless otherwise stated, reagents and molecules were purchased from Sigma (Barcelona, Spain). As enzyme substrate, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) liquid substrate and ABTS-Enhancer were mixed in a 11:1 proportion and incubated in the dark for 1 min before use. Phosphate-buffered saline 0.01 M (PBS) was obtained in tablets from Invitrogen, and reconstituted as recommended by the provider. PBST indicates PBS containing 0.05% (v/v) Tween. Unlabelled and horseradish peroxidase-labelled anti-*E. coli* polyclonal antibodies (developed in rabbit) were provided by AbCam (Cambridge, UK). An anti-rabbit polyclonal Ab (developed in goat) was provided by Sigma (Barcelona, Spain). For protein detection, the unlabelled anti-*E. coli* and anti-rabbit antibodies were respectively immobilised as negative and positive control Ab, according to their ability to capture the anti-*E. coli* peroxidase-labelled Ab used as a protein target (Ab-HRP). For bacteria detection, the unlabelled and peroxidase-labelled anti-*E. coli* Ab were respectively used as immobilised capture Ab and detector reagent.

Escherichia coli K12 (ATCC 10536) from the American Type Cells Collection, was cultured overnight at 37 °C in Agrobacterium broth (AB) minimal liquid medium. The culture was spectrophotometrically quantified (550 nm), serially diluted and agar-plated to obtain the viable counts. The culture was aliquoted into 1.5-mL Eppendorf tubes, centrifuged for 10 min at 12,000 rpm, the supernatants discarded and the pellets stored at −20 °C. Prior to reconstitution, each *E. coli* pellet was warmed to room temperature for 1 min, completely resuspended in 50 μL PBS, and dissolved in PBS to the desired concentration.

Gold immunofunctionalisation

Gold wire of 0.5-mm diameter (Sigma; Barcelona, Spain) was cut into 4-mm-long regular rods. The alternative use of gold foil, which is much more fragile and soft, complicated manipulation, increased background signals and decreased the measurable differences between treatments. The gold rods were pretreated, in order to obtain clean and homogeneous gold surfaces, by immersion for 3 min in a hot piranha solution (3:1 v/v H_2SO_4/H_2O_2), followed by successive washes with distilled water, isopropanol, ethanol and deionised water. (Warning: piranha solution is highly reactive and extreme precautions must be taken at all times). The gold rods were then transferred to 1.5-mL Eppendorf tubes. Each tube contained no more than three rods to prevent them from touching each other and scratching their surfaces, and to facilitate surface coverage.

The thiolated molecules used were dissolved to 10 mM in ethanol and self-assembled on the gold surfaces for 1 h at room temperature, followed by two washes with ethanol and other two with PBS. Dextranized surfaces were generated by conjugating CM-dextran, 8 mg mL^{-1} in PBS containing 100 mM of both *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (NHS), for 30 min on a cysteamine hydrochloride SAM.

Antibodies were conjugated via their –NH groups by incubating the Abs, 10 μg in PBS, for 1 h at 37 °C on either mercaptopropanoic acid (MPA) or mercaptoundecanoic acid (MUA) SAMs or on dextranised surfaces, previously activated with EDC/NHS and washed as described above. The surfaces were then chemically blocked by treating the remaining unreacted groups with ethanolamine. Abs were alternatively conjugated through their terminal –COOH groups. In this case, the dextran-modified gold rods were incubated for 1 h at 37 °C in a 1 M solution of an ethylenediamine/ethanolamine mixture of molar ratios 10:1, 1:1 or 1:10, followed by four washes with PBST. In parallel, the Ab were preactivated for 15 min at room temperature in a 4-morpholineethanesulfonic acid (MES) buffer, pH 5.3, containing 100 mM EDC/NHS, incubated with the gold rods for 1 h at 37 °C, followed by chemical blocking for 15 min with 1 M ethanolamine.

Protein detection

The functionalised gold surfaces were incubated for 1 h at 37 °C in PBS containing 0.5 μg mL^{-1} of the Ab-HRP, washed four times with PBST and incubated with 100 μL of enzyme substrate for 45 min at room temperature in the dark. The reaction was stopped with 50 μL $well^{-1}$ of 1% (w/v) sodium dodecyl sulfate (SDS), and colour development recorded at 405 nm using a ThermoElectron Multiskan plate reader. Unmodified bare gold rods incubated with Ab-

HRP served as control for the level of nonspecific adsorption denoted 100%. Each experiment was independently repeated three times on different days and using freshly prepared reagents. The values shown correspond to the average from at least three replicates manipulated in parallel.

Bacteria detection

Gold rods functionalised with unlabeled anti-*E. coli* Ab or bovine serum albumin (BSA) were incubated with different concentrations of *E. coli* for 30–60 min at 37 °C, washed three times with PBS, incubated with HRP-Ab for 1 h at 37 °C and washed four times more with PBST. When required, the functionalised surfaces were submitted to physical blocking by incubating for 2 h at 37 °C in BSA 2% (w/v) prior to bacteria capture. The rods were developed with enzyme substrate as described above. Each experiment was repeated three times. The values shown correspond to the average from at least three replicates.

Results and discussion

In an attempt to produce biorecognitive surfaces for the detection of bacteria, we conjugated Ab via different strategies to either a SAM of mercaptoundecanoic (MUA) or mercaptopropanoic acid (MPA), or to a dextranized surface. The performances of the different surfaces were assessed by comparing the capture of a protein target (rabbit Ab-HRP) on surfaces functionalised with either a positive or a negative control Ab. In addition, we confirmed that changes in signal were related to changes in Ab specificity for the target, and not to the amount of Ab immobilised. This was done by incubating the two immunofunctionalised surfaces with a second HRP-labelled Ab, which this time recognises the Ab on the surface. Figure 1 (inset, left) shows the results for detection of the target Ab-HRP on dextranized surfaces of slightly different composition, and containing Ab conjugated through either –NH or –COOH groups. The signals recorded on surfaces functionalised with the positive control Ab

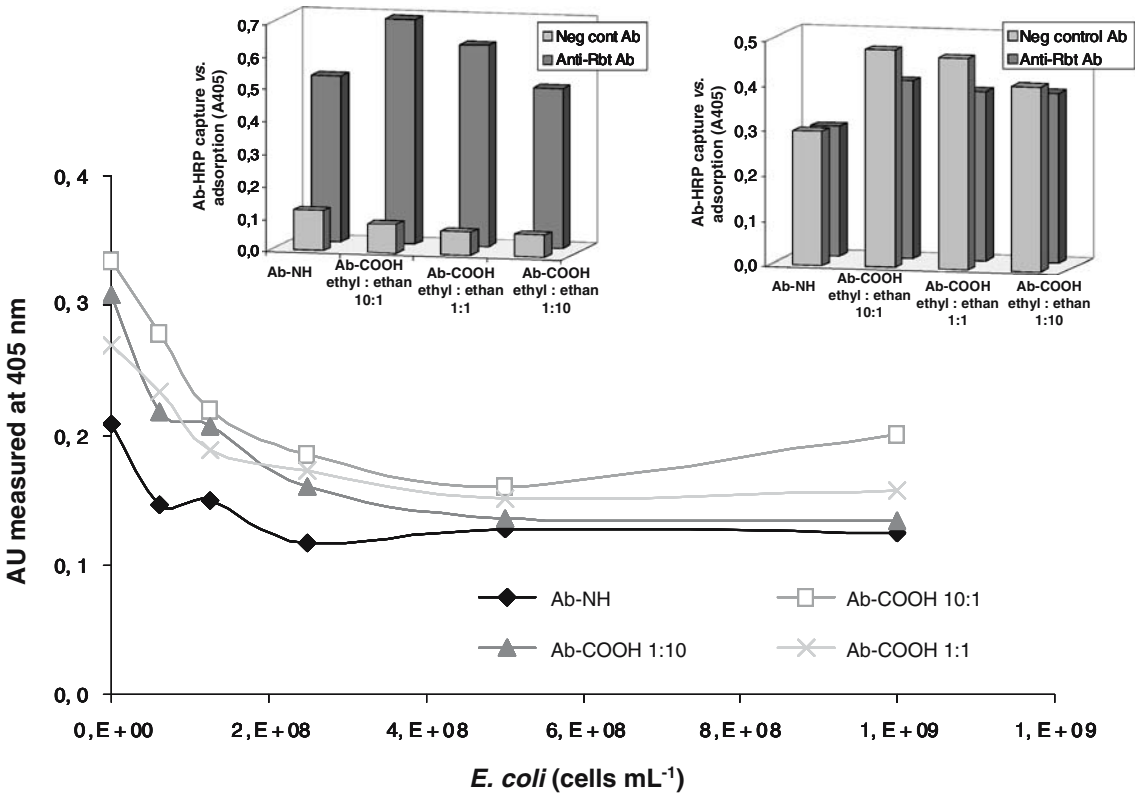

Fig. 1 Gold immunofunctionalisation by Ab conjugation to a dextranised cysteamine SAM. Anti-rabbit and negative control Ab were conjugated in parallel via their –NH groups to EDC/NHS-activated dextranised gold rods. Alternatively, Abs were EDC/NHS-activated and conjugated through their –COOH groups to dextranised surfaces, which had been previously treated with three different molar ratios of ethylenediamine and ethanolamine. *Inset*, *left* comparison of capture efficiency of the target Ab-HRP by the four abovementioned surfaces, modified with either positive or negative control Ab. Target capture appears to be highly specific. *Inset*, *right* the same surfaces, following incubation with a second Ab-HRP which this time recognises the Ab present on surface. The signals recorded indicate that similar amounts of positive/negative control Abs had been immobilised for each surface type. *Main figure* none of the dextranized surfaces bearing anti-*E. coli* Ab recognizes specifically *E. coli,* while nonspecific adsorption of the bacteria is detected in all of them in the shape of negative signals when Ab-HRP is added in excess

(expected to be mainly due to specific capture) were always significantly higher than those registered for the negative control Ab (attributable to nonspecific adsorption). In contrast, the results for the detection of the immobilised Ab carried out in parallel (Fig. 1, inset right) indicate that similar concentrations of bicomponent have been incorporated for the two Ab used within each functionalisation procedure. Accordingly, we concluded that the Abs had been successfully incorporated onto the gold surface, that they remained functional and that they showed the expected specificity for the protein target. Ab cross-linking via –COOH groups, expected to induce a lower deleterious effect on the target recognition moieties, generated slightly better results than Ab-NH conjugation, as the former presented slightly lower levels of nonspecific adsorption and higher specific signals were recorded. The reason was thought to be either better orientation of the Ab on surface, or a certain level of Ab cross-binding induced by the Ab EDC/NHS activation, thus incorporating onto the surface "clusters" of Ab rather than individual Ab units.

The abovedescribed immunofunctionalised surfaces were then applied to bacteria detection. Unexpectedly, none of the previously assayed protocols worked as predicted for *E. coli* detection. Figure 1 shows the results for an assay carried out on the dextranised surfaces, bearing Ab conjugated via –NH or via –COOH groups. The increase in Ab-HRP concentration (0.5 to 2 μg mL^{-1}) did not improve the results but, unsurprisingly, increased the level of nonspecific Ab-HRP adsorption. In this context, nonspecific adsorption of *E. coli* was detected as a negative signal. Indeed, the signal due to nonspecifically adsorbed Ab-HRP decreased as the concentration of *E. coli* increased. This is presumably due to the additional blocking effect brought about by nonspecifically bound bacteria. Attempts to decrease adsorption by blocking the surface with 2% (w/v) BSA were unsuccessful on the surface with Ab conjugated via the –NH groups. This could be due to electrostatic repulsions between the negatively charged BSA and dextran molecules which prevented the formation of a stable BSA cover. In the case of surfaces bearing Ab conjugated through –COOH groups, the conjugation of ethylenediamine incorporates positive charges on the modified surface. Under these conditions, BSA seems to attach to the surface, inducing a decrease in both nonspecific Ab-HRP and bacteria adsorption (data not shown). Nevertheless, no specific capture of *E. coli* could be detected for the concentration range assayed (10^1–10^9 cells mL^{-1}) on these immunofucntionalised surfaces.

Results on surfaces modified by conjugation of Ab-NH on plain MUA or MPA SAMs were less promising for protein detection as, in general, lower specific signals were detected (Fig. 2, inset). This was presumably due to a lower level of Ab incorporation onto these surfaces, compared with the dextranised ones. In the case of MPA, higher

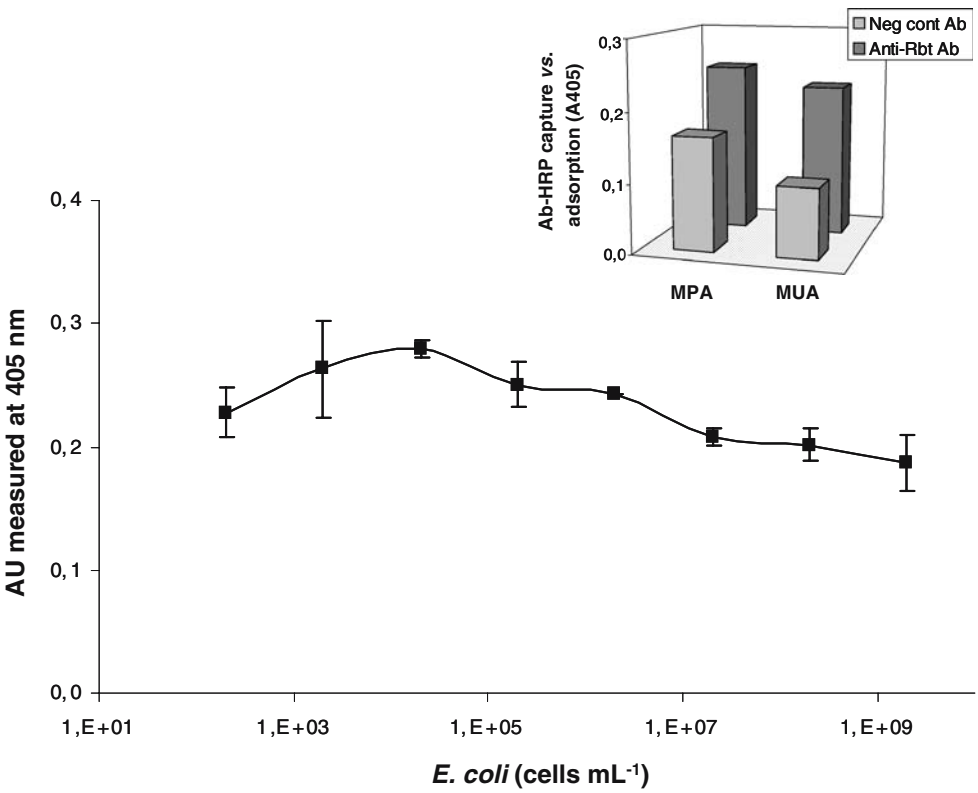

Fig. 2 Gold immunofunctionalisation by Ab conjugation to MPA or MUA SAMs. Anti-rabbit and negative control Ab were conjugated in parallel via their –NH groups to MPA or MUA SAMs, previously activated with EDC/NHS. *Inset* comparison of capture efficiency of the target Ab-HRP by gold surfaces modified with positive control or negative control Ab. Even if target capture is higher for the anti-rabbit Ab, detectability was significantly lower than in the case of the previously described dextranised surfaces. *Main figure* a gold surface coated with a MUA SAM and functionalised with an anti-*E. coli* Ab not showing reactivity towards the target bacteria

signals due to nonspecific Ab-HRP adsorption were also generated. This is consistent with previous reports indicating that longer SAMs induce lower levels of nonspecific biomolecule adsorption [9]. Attempts to detect *E. coli* by means of Ab-NH conjugation to MUA were also unsuccessful (Fig. 2), with no reactivity of the surface for the bacterial target and certain levels of nonspecific *E. coli* adsorption over the SAM components.

In contrast, direct adsorption of anti-*E. coli* Abs on bare gold, followed by BSA blocking, generated trends similar to those obtained on microtiter plates and allowed *E. coli* detection down to 5×10^5 cells mL^{-1} (Fig. 3). Nonspecific *E. coli* adsorption onto a similar surface, modified with only BSA but no Ab, was nearly undetectable for concentrations of up to 10^8 cells mL^{-1}. Surface functionalisation by physisorption is easier, faster and cheaper than any of the SAM-based strategies previously described, as it requires fewer reagents and steps to be carried out. Additionally, Ab deposition could be optimised and improved if capture proteins were physisorbed, followed by Ab capture in a more or less directed manner. These alternative strategies are currently being investigated at our laboratory for bacterial biosensing purposes.

Physisorption is accepted to negatively affect protein integrity and function [17], with examples of inactivation of up to 90% of the Ab following physisorption having been reported [18]. Nevertheless, physisorption has been used by other authors in immunosensor development owing to its simplicity and cost-effectiveness [16, 19–22]. In our case, Ab physisorption allowed bacteria detection on gold with low levels of nonspecific adsorption, while none of the SAM-based strategies produced acceptable results. In contrast, the same surfaces had previously been shown to be efficient for protein detection. In this respect, the differences between both target systems should be stressed. Besides considering the complexity of the bacterial wall, which makes the production of truly antifouling surfaces difficult, the size of the target bacteria is another important factor to consider. As the dimensions of bacteria (up to several microns in length) exceed those of the immobilised Ab ($10\times15\times5$ nm), target capture presumably induces a "shadowing" effect on the immunofunctionalised surface, eclipsing a number of otherwise available Ab. Consequently, loss of function on part of the physisorbed Ab may affect bacterial detection to a lesser extent than it may protein detection. On the other hand, it is unclear whether SAM-based immunofunctionalisation favours the right Ab exposition on surface or if, on the contrary, Ab can appear partly embedded within the SAM components. This could be especially true for the dextranised surfaces. Despite the structural complexity of antibodies and other proteins, these are relatively small molecules and can reach the nooks and crannies of the SAM-covered surface where the antibodies have been attached. Bacteria, on the other hand, are much more cumbersome structures, and thus epitopes present on the surface of the complex and rigid bacterial wall would be less accessible to the immobilised Ab.

Conclusions

Biosensor development for bacteria determination often takes advantage of strategies and formats previously optimized for small-target detection. This is the case of SAM-based surface immunofunctionalisation. Nevertheless, our results indicate that this strategy, although efficient

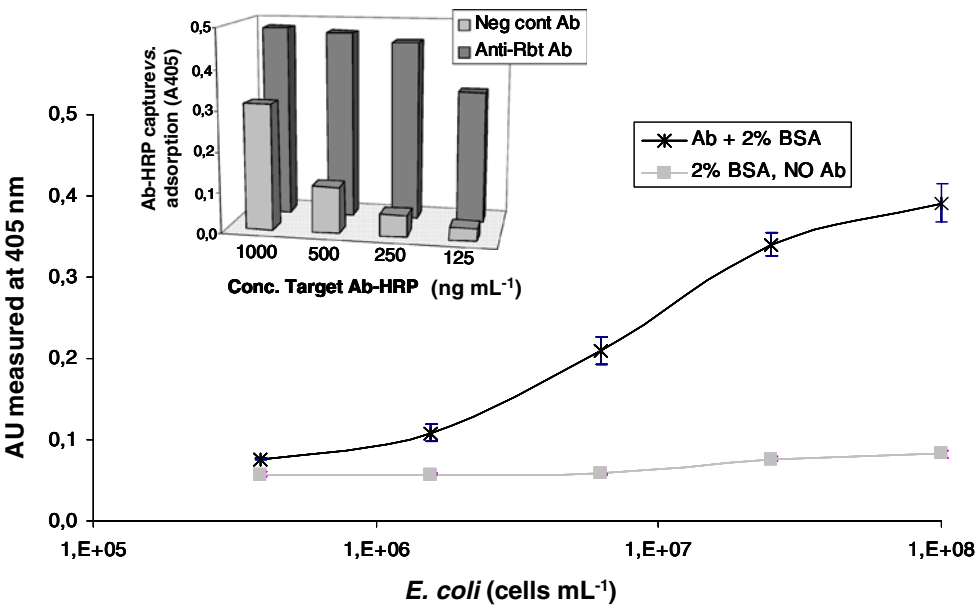

Fig. 3 Gold immunofunctionalisation by Ab physisorption. *Inset* comparative capture of the target Ab-HRP by gold surfaces modified with physisorbed positive control or negative control Ab. The target is preferentially captured by the surface functionalised with the positive control Ab. *Main figure* reactivity of gold surfaces covered with either physisorbed Ab or BSA towards the target bacteria. Ab physisorption was the only strategy among the approaches tested in this work which allowed *E. coli* detection

for small-target detection, might not be appropriate for bacteria. These results are consistent with the size of bacteria and the complexity of their outer membrane and wall, and suggest that surface functionalisation for the specific detection of whole microorganism demands functionalisation strategies of their own, which should be carefully evaluated. In the meantime, we propose Ab physisorption as a suitable alternative to SAM-based gold functionalisation for bacteria detection.

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