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Research paper

Gold immuno-functionalisation via self-assembled monolayers: Study of critical parameters and comparative performance for protein and bacteria detection

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ABSTRACT

Surface functionalisation is of extreme importance in assay and biosensor development because it ensures the selective capture and detection of the targets of interest. In the present report, we compare the performance of several gold functionalisation strategies/chemistries, based on SAM self-assembly and Ab conjugation, for protein and bacteria detection.
The first part of the work summarises the optimisation of the various protocols considered. Their efficiency was initially evaluated in terms of reduction of biomolecule non-specific adsorption and specific detection competence impairment, using as a model-target an enzyme-labelled protein. With this purpose, the effect of several parameters, such as thiomolecule length and concentration, self-assembly time and temperature, polymer incorporation, or Ab conjugation strategy was determined. The three best performing strategies consisted of antibody (Ab) conjugation to self-assembled monolayers (SAM) containing mercaptoundecanoic acid alone, or conjugated to either long-chain hydrophilic diamines or CM-dextran. In the three cases, results demonstrated that Abs had been successfully incorporated and remained functional for protein detection. Nevertheless, as showed in the second part of the work, we demonstrate for the first time that these chemistries can be inadequate for bacteria detection. The possible reasons and implications will be discussed. Ab physisorption is proposed as a cost-effective gold immuno-functionalisation strategy alternative to SAM-based Ab incorporation for bacteria detection.

1. Introduction

Biosensors are a promising alternative to conventional analytical methods (Patel, 2006; Pejcic et al., 2006; Gonzalez-Martinez et al., 2007; Lazcka et al., 2007; Shankaran et al., 2007; Tudorache and Bala, 2007). They display improved sensitivity, require small sample volumes thanks to their reduced size, and can be incorporated to integrated "user-friendly" devices. This leads to methods that are faster and easier to use by non-technical staff "in the field", away from sophisticated installations. The specificity of the sensing event, however, depends on the incorporation of capture or recognition elements to the transducer surface, which in biosensors are biocomponents (Mohanty and Kougianos, 2006). In traditional methodologies, such as ELISA, surface functionalisation is often attained by non-specific adsorption of the biocomponent onto a relatively big surface (e.g. the well in a microtitter plate), ensuring that the amount of molecules preserving their function is sufficient for the assay to work. As biosensors base their performance on significantly smaller sensing surfaces, oriented/directed anchoring is preferred instead. Many strategies and components have been employed with this aim, such as the immobilisation of protein A and G for immunoglobulin capture (Goding, 1978; Anderson et al., 1997), or (strept)avidin for binding previously biotinylated components (Wilchek and Bayer, 1988; Wilchek et al., 2006; Laitinen et al., 2007). When gold surfaces are considered, however, one of the most exploited strategies consists in the incorporation of antibodies via self-assembled monolayers (SAM) (Wink

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et al., 1997; Chaki and Vijayamohanan, 2002; Mirsky, 2002). In this respect, gold is a very convenient material for bacteria biosensing thanks to its inertness, stability and lack of toxicity.

SAMs form because sulphur containing molecules (thiols, sulfides and disulfides) have a strong affinity for gold and interact with it in a near covalent manner (Love et al., 2005; Vericat et al., 2005). The reaction is spontaneous, and thiol-based SAMs are obtained by simple immersion of the gold surface into a solution of the thiol of choice. The SAM will be ideally composed of tightly packed and well ordered chains, although several factors may lead to the formation of defects and irregularities (Vericat et al., 2005). The chains composing a SAM, on the other hand, can bear reactive groups (amino, carboxyl, maleimide, etc.) or components (biotin) on their free end (see for example Fig. 1). It follows that SAMs can be exploited for the directed conjugation/incorporation of biocomponents and, therefore, surface functionalisation (Chaki and Vijayamohanan, 2002; Schaeferling et al., 2002). Consistently, an important number of works have been published reporting the incorporation of biomolecules (either Ab or Ab-capture protein) to SAM-coated surfaces for the detection of proteins and small molecules. Reports dealing with bacteria detection are less frequent (Oh et al., 2004; Su and Li, 2004; Jyoung et al., 2006; Subramanian et al., 2006; Taylor et al., 2006; Waswa et al., 2006). Alternatively, a number of works report on bacteria detection by means of biocomponents (i) conjugated onto silanised surfaces, (ii) randomly deposited on the sensing surface or (iii) modified with cross-linkers of different chain lengths and self-assembled on gold (Park and Kim, 1998; Ruan et al., 2002; Liju et al., 2004; Radke and Alocilja, 2004; Radke and Alocilja, 2005; Su and Li, 2005). Interestingly, few of the cited works actually provide the due negative controls ensuring that the signal is truly specific and not partly due to the non-specific adsorption of bacteria or other bio-materials on the sensing surface. For example, directed cross-binding of the Ab towards the inter-electrode gaps can hardly avoid a certain level of Ab and bacteria non-specific adsorption on the electrode surface. In other occasions, a certain biosensor may be reported not to recognise unrelated bacteria, but the information regarding their relative concentrations is lacking. Also, providing information about the biosensor response under buffer containing no bacteria gives little information about the system specificity.

In this work, we study several surface functionalisation protocols, which are based on Ab incorporation to gold surfaces modified with different SAMs, and their applicability

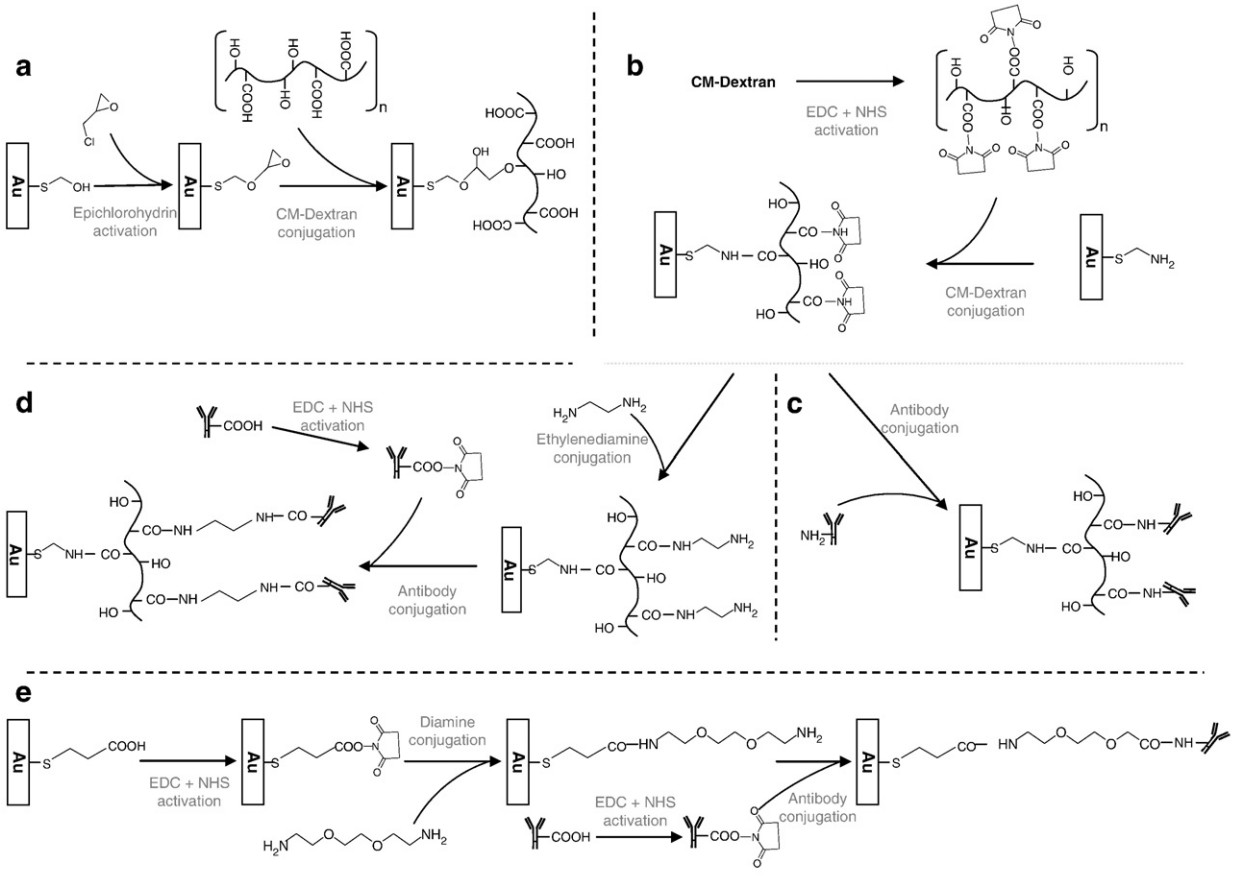

Fig. 1. Reaction mechanism for the different surface functionalisation strategies described in the text. (a) Alkanethiol self-assembly, followed by epichlorohydrin activation and CM-dextran conjugation. (b) CM-dextran activation with EDC/NHS, followed by conjugation on a cysteamine SAM and antibody conjugation through its NH– (c) or COOH-ter (d). (e) Mercaptoacid self-assembly, followed by EDC/NHS activation, long-chain diamine incorporation, and conjugation of EDC/NHS-activated Ab.

to bacteria detection. We will initially compare the performance of SAM components of different length in diminishing the level of biomolecule non-specific adsorption. The selected surfaces are then submitted to functionalisation using different protocols for Ab conjugation, and subsequently assayed for protein detection using an HRP-labelled Ab as the target model. The best performing strategies are finally assayed for bacteria detection with the final goal to develop electrochemical immunosensors. As it will be demonstrated, none of the SAM-based functionalisation strategies tested worked for bacterial detection. On the contrary, bacteria were successfully detected on gold bearing randomly adsorbed Abs. Our results are coherent with the fact that bacteria are bigger and more complex than simpler targets such as proteins, and suggest that careful and extensive studies have to be undertaken in order to obtain sensing surfaces that are really suitable for bacteria detection.

2. Experimental procedures

2.1. Reagents and biocomponents

Unless otherwise stated, reagents and molecules were purchased from Sigma (Barcelona, Spain). The molecules used for surface modification, as well as the abbreviations used for each of them, have been summarised in Table 1. ABTS liquid substrate and ABTS-Enhancer were mixed in a 11/1 proportion and incubated in the dark for 1 min before use. Phosphate Buffer Saline 0.01 M (PBS) was obtained in tablets from Invitrogen, and reconstituted as recommended by the provider. Unlabelled and horseradish peroxidase-labelled anti-*Escherichia coli* polyclonal antibodies (developed in rabbit) were provided by AbCam (Cambridge, UK). An anti-rabbit polyclonal antibody (developed in goat) was provided by Sigma (Barcelona, Spain). During protocol optimisation and in protein detection (results, Sections 2 and 3), 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 antibody used as a protein target (Ab-HRP). For bacteria detection (results, Section 4), the unlabelled and peroxidase-labelled anti-*E. coli* Abs were respectively used immobilised and in solution, as the capture (first) and detector (second) reagents.

Table 1
Non-specific adsorption of the Ab-HRP on the different SAM-modified surfaces described in the text

Reagent	Abbreviation	Ab-HRP adsorption
Bare gold		100
Mercaptoethanol	MEol	–
Mercaptopropanol	MPol	>99
Mercaptopropanoic acid	MPA	
Mercaptobutanol	MBol	88
Mercapto butanoic acid	MBA	
Mercaptohexanol	MHol	80
Mercapto hexanoic acid	MHA	
Mercaptoundecanol	MUol	65
Mercapto undecanoic acid	MUA	53
2,2′ (Ethyelenedioxy)bis(ethylamine)	Diamine	–
MPA+diamine		125
1:1 MPA:MPol+diamine		156
1:9 MPA:MPol+diamine		120
MUA+diamine		52
1:1 MUA:MPol+diamine		116
1:9 MUA:MPol+diamine		99
CM-Dextranomer C-25-120	CM-dextran	–
Mercaptoethanol+epichlor+CM-dextran		83
Mercaptoethanol/Cysteamine+epichlor+CM-dextran		90
Cysteamine+epichlor+CM-dextran		100
Cysteamine+EDC/NHS+CM-dextran		40
Cysteamine/MEol+EDC/NHS+CM-dextran		80

Values normalised as to indicate % of the maximal signal generated onto bare gold.

2.2. Bacteria

E. coli K12 (ATCC 10536) from the American Type Cells Collection, was cultured overnight at 37 °C in AB minimal liquid medium. The culture was spectrophotometrically quantified (550 nm), serially diluted and agar plated as 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 were discarded and the pellets were stored at −20 °C. Previous to reconstitution, each *E. coli* pellet was taken to room temperature for 1 min, completely resuspended in 50 µl PBS, and dissolved in PBS to the desired concentration.

2.3. Gold pre-treatment

Gold wire 0.5 mm diameter (Sigma; Barcelona, Spain) was cut into 4 mm long regular rods. The gold rods were immersed for 3 min in a hot piranha solution (3/1 v/v H_2SO_4/H_2O_2), rinsed with distilled water, sonicated for 3 min in isopropanol, rinsed in ethanol and thoroughly rinsed in deionised water. (Warning: piranha solution is highly reactive and may explode on coming into contact with organic solvents. Extreme precautions must be taken at all times). The rods were at this point transferred to Eppendorf tubes. A maximum of three rods per tube was used to prevent them from touching each other and scratching their surfaces, and thus facilitate surface coverage.

2.4. SAM formation

Fig. 1 shows the different functionalisation strategies tested. Unless otherwise stated, all thiols were dissolved to 10 mM in ethanol and incubations carried out at room temperature (RT) for 1 h. This was followed by two washes with ethanol and other two with PBS. During protocol optimisation, thiol concentrations ranging from 1 to 10 mM and incubation times of some few min to 24 h were assayed. CM-dextran conjugation was attempted via two alternative chemistries (each incubation step was followed by two washes with PBS containing 0.05% v/v Tween 20, PBST, and other two with PBS). Mercaptoethanol was self-assembled as described above, followed by activation of the OH– groups with 10% (w/v) epichlorohydrin in 0.4 M NaOH for 1 h. CM-dextran, 8 mg/ml in PBS, was added and allowed to react overnight (Fig. 1a). Alternatively, cysteamine hydrochloride was self-assembled for 1 h. CM-dextran, 8 mg/ml in PBS, was added and incubated in the presence of 100 mM EDC and NHS for 0–60 min (Fig. 1b).

2.5. Antibody conjugation

Two different conjugation routes, illustrated in Fig. 1, were tested. The first one involved conjugation of the antibody to the cysteamine-dextran surfaces, following dextran activation with EDC/NHS (Fig. 1c). The Abs were then dissolved to 1.25–10 μg/ml in PBS, and incubated on the modified gold for 1 h at either 37 °C or RT. The gold rods were then washed and incubated with ethanolamine to block any remaining activated groups on the surface. In case the Ab conjugation via the –NH groups affected their functionality, Abs were alternatively conjugated through their –COOH terminal groups using also the EDC/NHS chemistry (Fig. 1d). 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 4 washes with PBST. In parallel, the Abs were pre-activated for 15 min at RT in 2-(*N*-morpholino)ethanesulfonic acid buffer pH 5.3 (MES buffer) 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. The later protocol can lead to Ab cross-linking and/or polymerisation, yet the formation and precipitation of insoluble products were never noticed during the course of the experiments.

2.6. Protein capture versus non-specific adsorption

The gold rods, whether bare or SAM-modified, and/or containing positive or negative control Ab, were incubated for 1 h at 37 °C in PBS containing 0.5 μg/ml of Ab-HRP. The rods were then washed 4 times with PBST and individually transferred to the wells of a microtitter plate. To each well, 100 μl of enzyme substrate was added and incubated for 120 min at RT in the dark. The reaction was stopped with 50 μl/well of 1% (w/v) SDS, and colour development recorded at A405 nm using a ThermoElectron Multiskan plate reader. The concentration of Ab-HRP had been previously optimised by titration and was selected as the concentration generating 80% of the maximum detectable signal. Unmodified bare gold rods incubated with Ab-HRP served as control for the level of non-specific adsorption named 100%. Each experiment was repeated up to 6 times. The values shown correspond to the average from at least 3 replicates.

2.7. E. coli detection on functionalised gold

Gold rods functionalised with unlabelled anti-*E. coli* Ab or BSA, were incubated with different concentrations of *E. coli* for 1 h at 37 °C, washed 3 times with PBS, incubated with HRP-Ab for 1 h at 37 °C and washed four times more with PBST. The rods were developed with enzyme substrate as described above.

3. Results and discussion

3.1. SAM self-assembly optimisation: effect on biomolecule non-specific adsorption

It has been reported that SAMs contribute to decreasing biomolecule non-specific adsorption compared to bare gold. The incorporation of SAMs in biosensor development may thus play two roles: (i) to aid incorporate the biomolecule receptor in an oriented manner and (ii) to favour specific capture of the target by decreasing non-specific adsorption of unrelated compounds. Our work started with the evaluation of the performance of different thiolated molecules, following their self-assembly on gold, in terms of subsequent level of non-specific adsorption of a peroxidase-labelled Ab (Ab-HRP) (Fig. 2a). The signals recorded were compared to those obtained following Ab-HRP non-specific adsorption on bare gold. Three different modification strategies have been evaluated, including alkanethiol self-assembly, conjugation of a long-chain hydrophilic diamine onto alkanethiol SAMs, and conjugation of CM-dextran on cysteamine SAMs. Our results have been summarised in Table 1.

3.1.1. Alkanethiol SAM

The efficiency of mercaptothiols of different length (MUol, MHol, MBol and MPol), as well as the equivalent mercapto-acids, in "blocking" the non-specific adsorption of the Ab-HRP has been evaluated and compared to the level of adsorption observed on bare gold. As expected, the level of Ab-HRP adsorption registered was inversely related to the length of the molecule composing the SAM (Table 1 and Fig. 2b), the best results having been obtained for the longest chain assayed, MUol (40–50% decrease compared to bare gold). Conversely, the SAM formed by MPol showed in all the cases non-specific signals similar or higher to those obtained using bare gold (up to 160% in some experiments). This is in agreement with other works (Knoll et al., 2000) and suggests that using longer chain SAMs is better for biosensor performance. In spite of this, the possible limitations that such a cover may introduce in signal transduction, for instance to electron transfer in electrochemical detection, have to be taken into account. For example, the performance of an amperometric biosensor is expected to get worse the longer the chain length of the thiol used. This is because for a well-packed monolayer, the distance over which electrons need to jump, from the solution to the electrode, increases with thiol chain length.

Interestingly, the SAM formed by MUA generated lower non-specific signals than the SAM formed by MUol. This was consistent with the improved performance of mixed SAMs we observed when combinations of 10 mM mercaptothiol plus 1 mM of a mercapto-acid of similar length were assayed.

The potential effect of incubation temperature was also studied, in case self-assembly could be accelerated at a higher temperature and assuming that more ordered SAMs would generate lower levels of biomolecule non-specific adsorption. SAMs were thus formed in parallel both at RT and at 37 °C (Fig. 2c). The rest of the experiment was carried out as previously described. Our results indicated that, in most cases, little effect could be attributed to the incubation temperature. Nevertheless, a slight increase in Ab-HRP non-specific adsorption was detected on MUol SAMs self-assembled at 37 °C compared to RT. On the other hand, the formation of MPol SAMs appeared to be favoured to some extent at higher temperatures. This is presumably related to the different interactions at play between nearby chains during SAM formation/stabilisation in each case. Most authors defend that SAMs form very fast (within a few minutes), but that they require longer times to arrange themselves properly. This is especially true for long chains, for which inter-molecular interactions contribute to SAM

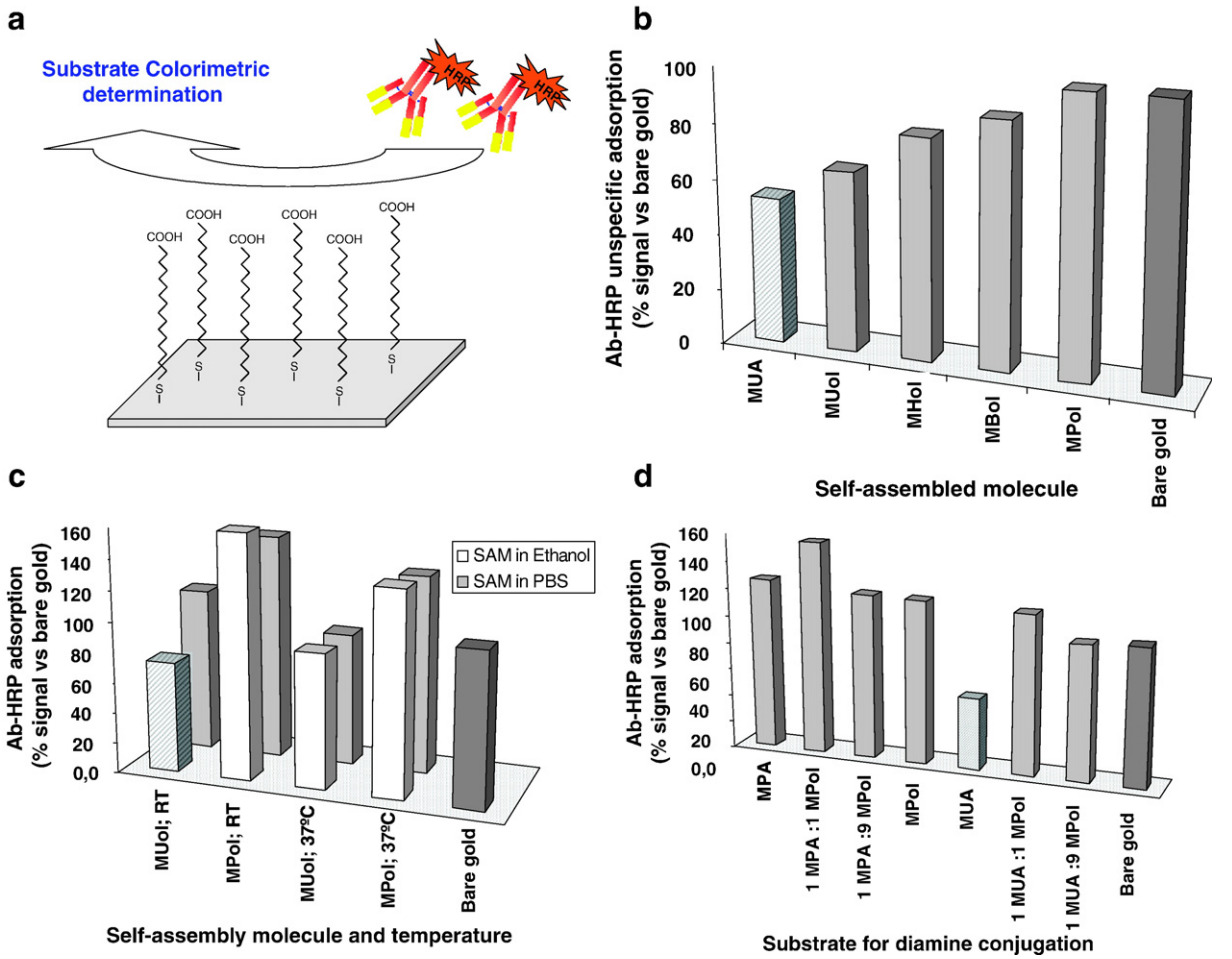

Fig. 2. (a) Study of non-specific adsorption of an HRP-labelled Ab (Ab-HRP) onto SAMs. Different SAMs were self-assembled as described in the text. Bare and modified gold surfaces were then incubated in parallel in the presence of the Ab-HRP. The level of Ab-HRP non-specific adsorption was assessed colorimetrically by adding the appropriate enzymatic substrate. (b) Non-specific adsorption of the Ab-HRP on gold with self-assembled thiols of different lengths. (c) Non-specific adsorption of the Ab-HRP onto different SAMs, conjugated via EDC/NHS chemistry to a long-chain hydrophilic diamine. Values are expressed in all cases as % of the level of Ab-HRP non-specific adsorption, registered on bare gold surfaces.

stabilisation to a higher extent. Our results indicate that self-assembly is accelerated at higher temperatures, but the establishment of inter-chain interactions and SAM stabilisation requires more time and seems more efficient at low temperatures. Consistently, a long-chain SAM will attain a more ordered structure when self-assembled at lower temperatures, while higher temperatures will induce higher energy states, thus higher molecule vibration levels and less ordered SAMs. In this respect, we observed that incubating at 37 °C only improved self-assembly of long-chain alkanethiols when carried out in 80/20% (v/v) ethanol/PBS mixtures instead of ethanol alone. This is, however, most likely due to a slight increase in the molecule water solubility at higher temperatures. Consistently, self-assembly of short chains, which are soluble in water, generated similar results when dissolved in PBS or ethanol.

3.1.2. Alkanethiol SAM conjugation to a long-chain hydrophilic diamine

2,2'(Ethyelenedioxy)bis(ethylamine) is a long-chain diamine, which incorporation has been reported to reduce biomolecule adsorption over the treated surface. In an attempt to additionally block the modified surface, this diamine was chemically conjugated to the previously self-assembled SAMs via EDC/NHS chemistry (Fig. 1e). SAMs consisting of either MPA or MUA only, and 1/1 or 1/9 molar mixtures of MPA or MUA with MPol were assayed. According to our data, all SAMs using MPA in combination with the hydrophilic diamine generate higher or similar levels of non-specific binding as bare gold (Table 1 and Fig. 2d). In contrast, the conjugation of the long-chain diamine on a MUA SAM reduced non-specific binding by approximately 50% compared to bare gold.

3.1.3. Alkanethiol SAM conjugation to CM-dextran

Dextrans are natural and inexpensive polymers, often reported to repel biomolecule non-specific adsorption thanks to their abundant hydroxyl groups. The carboxymethylated variants were described as a tool to incorporate biomolecules to surfaces in the early 1990s (Johnsson et al., 1991) and are one of the key components of the widely used CM5 chips for SPR detection using Biacore instrumentation (O'Shannessy

et al., 1992). We assayed the conjugation of CM-dextran to previously self-assembled SAMs via two alternative chemistries in parallel. In the first case, mercaptoethanol SAMs were activated with 10% epichlorohydrin for 1 h at RT. The epoxy groups generated were then reacted overnight towards the hydroxyl groups present in the dextran (Fig. 1a). Alternatively, the CM-dextran was conjugated onto cysteamine SAMs for 1 h at RT via EDC/NHS chemistry, followed by a 15-minute chemical blocking step with 1 M ethanolamine at pH=8.5 (Fig. 1b).

The best results, in terms of lower non-specific signal, were obtained for the SAM produced with cysteamine-EDC/NHS-dextran (60% reduction compared to bare gold) (Table 1). The use of a mixed cysteamine/mercaptoethanol SAM, despite the incorporation of hydrophilic groups, generated higher background signals, presumably due to a decreased rate of CM-dextran conjugation. This is consistent with the results obtained while evaluating the efficiency of mercaptothiol SAMs of different chain lengths, indicating that short chain thiols are less capable of preventing non-specific adsorption.

3.2. SAM functionalisation by Ab conjugation

The best performing surfaces, in terms of reduced levels of non-specific adsorption, were functionalised in parallel as to incorporate two different Abs: (i) a goat anti-rabbit IgG as a positive control, and (ii) an unrelated negative control Ab (unlabelled anti-*E. coli* Ab). Following exposure to the Ab-HRP, signals generated on (i) are expected to be mainly due to specific capture, while signals on (ii) should be attributed to non-specific adsorption (Fig. 3a).

We show the results obtained on the dextranised surface described above, to which Abs were initially conjugated through their NH– groups (Fig. 1b and c). As expected, the signals recorded on surfaces functionalised with the positive control Ab were always significantly higher than those registered for the negative control Ab (Fig. 3b). This indicated that the Abs had been successfully crosslinked to the modified gold surface, and that they produced higher levels of target specific capture than an analogous but unrelated surface. Also unsurprisingly, signals due to non-specific adsorption on the negative control Ab were significantly lower than the ones recorded on a similar surface lacking Abs. This confirms that incorporating Abs further contributes to the physical blocking of the surface. Moreover, the signals measured when incubating the Ab-HRP on unmodified bare gold were always lower than the signals obtained on the conjugated positive control Ab, but higher than signals due to non-specific adsorption on the negative control Ab. This is consistent with the partial unfolding/denaturation of the Ab-HRP following adsorption on bare gold, presumably affecting the enzyme activity. As anticipated, its indirect capture through the conjugated anti-rabbit Ab leads to a higher and/or more organised number of functional Ab-HRP molecules on surface.

In order to improve surface functionality, several parameters in the functionalisation protocol were at this point reworked. For example, either too high or too low an amount of NH– groups on the modified surface was expected to induce either too low or too high yields of conjugated dextran, leading to lower Ab conjugation efficiency. Thus cysteamine self-assembly times of 1 min to overnight and concentrations

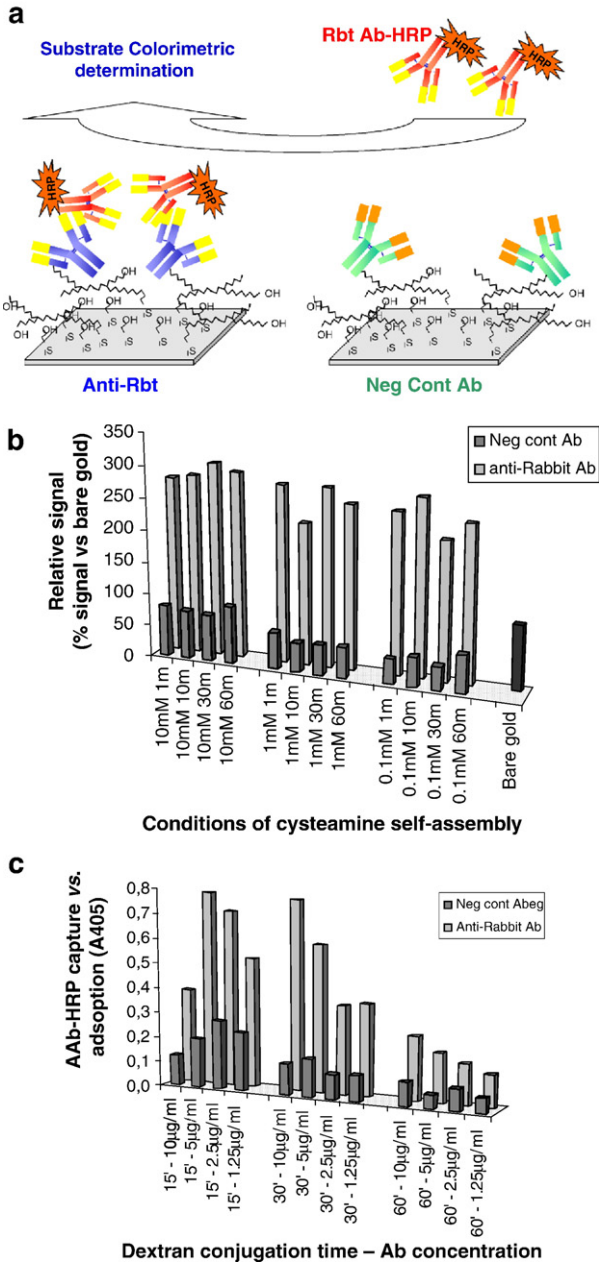

Fig. 3. (a) Non-specific binding versus specific capture of the Ab-HRP on Ab-modified surfaces. Two different Abs are immobilised in parallel, behaving respectively as a positive and a negative control. The two surfaces are then exposed to the HRP-labelled target, followed by incubation with the appropriate enzymatic substrate. Any signal registered on the positive control Ab is expected to be mainly due to specific capture. On the other hand, signals on the negative control Ab are related to non-specific adsorption. (b) Effect of cysteamine concentration and self-assembly time in the generation of dextranised immuno-functionalised surfaces. In all cases, the specific capture of the Ab-HRP by the immobilised positive control anti-rabbit Ab is significantly higher than non-specific adsorption on an unrelated negative control Ab. Values are expressed as % of the level of Ab-HRP non-specific adsorption, registered on bare gold surfaces. (c) Effect of EDC/NHS-dextran activation time and concentration of Ab, conjugated in the generation of dextranised immuno-functionalised surfaces. Values indicate absorbance units measured at 405 nm.

between 0.1 and 10 mM were studied (Fig. 3b), and cysteamine 1 mM self-assembly for 30 min was accordingly chosen for subsequent experiments. In the same way, CM-dextran activation time with EDC/NHS and concentration of Ab conjugated were optimised (results summarised in Fig. 3c). In our experiments, the shortest incubations with EDC/NHS generated the highest levels of non-specific binding, suggesting incomplete dextran coverage, while 60-minute incubations correlated with decreased non-specific but also low specific signals. This could be due to (i) a high level of CM-dextran cross-binding to the cysteamine SAM, and thus lower availability of functional groups for Ab conjugation, but also to (ii) Ab over-crowding on surface, and thus steric hindrance and decreased target recognition/binding efficiency. At the same time, enhancement in target binding efficiency was observed at higher Ab concentrations (Fig. 3c). Under these conditions, surface saturation does not seem to be reached, except for poorly dextranised surfaces, which also generate particularly increased levels of non-specific binding (e.g. dextran ECD/NHS activated for only 15 min). The best results, in terms of highest ratio specific/non-specific signal, were obtained when incubating the activated CM-dextran on the SAM for 30 min, followed by conjugation of 5–10 μg/ml Ab for 1 h at 37 °C. Ab conjugation at room temperature generated lower performance (data not shown).

The Ab target recognition moieties rely on their NH-terminal groups. It was thus feared that conjugation through the Ab –NH groups might partly compromise functionality and target capture efficiency. For this reason, an alternative protocol was assayed, including conjugation of ethylenediamine to the dextran, followed by cross-binding of the Ab –COOH groups, previously activated by EDC/NHS chemistry (Fig. 1b and d). As shown in Fig. 4, all the conditions tested generated better results than Ab–NH conjugation, meaning that lower levels of non-specific adsorption and higher specific signals were recorded. This is presumably due to a better orientation of the Ab on the surface, compared to conjugation via the NH-ter. Nonetheless, as this protocol potentially induce a certain level of Ab cross-binding, groups of Ab rather than Ab individual units may have been incorporated to the surface, contributing to increase immuno-functionality.

3.3. Protein detection on functionalised gold

Gold surfaces, immuno-functionalised as above mentioned with positive or negative control Ab, were also applied to protein detection. In parallel we assayed surfaces functionalised by simple Ab physisorption. The target Ab-HRP was serially diluted in PBS containing 0.1% Tween and 0.5% BSA, incubated on the different surfaces for 1 h at 37 °C and the surfaces processed as previously described. As shown in Fig. 5, little differences in performance were observed between surfaces for this specific target, which showed in all case limits of detection around 2 nM Ab-HRP.

3.4. E. coli detection on functionalised gold

Following the above results, the best performing SAM-based functionalisation protocols were readapted for *E. coli* detection. Thus, either unlabelled anti-*E. coli* Ab or BSA as a

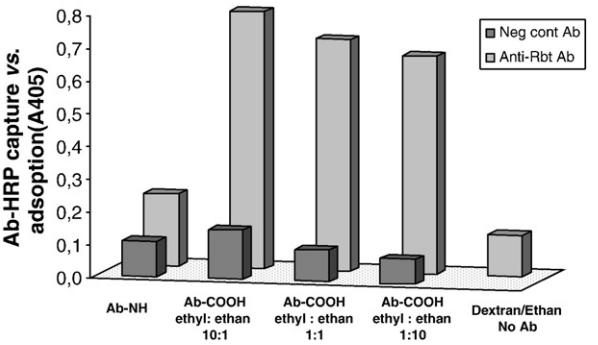

Fig. 4. Ab conjugation through COOH-ter compared to NH-ter. Ab, previously activated with EDC/NHS, were conjugated on gold surfaces with different molar ratios of self-assembled ethylenediamine (ethyl) and ethanolamine (ethan). Our results indicate that this protocol may generate slightly better specific versus non-specific signal ratios than the immobilisation via the Ab NH-ter groups.

negative control was immobilised onto the modified gold rods. The rods were then washed, incubated with different concentrations of *E. coli* or no bacteria at all, washed again, incubated with anti-*E. coli* Ab-HRP and developed with the appropriate enzyme substrate. Unexpectedly, none of the previously assayed protocols worked as predicted for *E. coli* detection.

Fig. 6a shows an example of the results obtained for the dextranised gold surface, with anti-*E. coli* Abs conjugated through their NH-ter. The increase in Ab-HRP concentration (0.5 to 2 μg/ml) did not improve the results but only generated extremely high levels of Ab-HRP non-specific adsorption. In this context, non-specific adsorption of *E. coli* was detected as negative signals, with the signal due to non-specifically adsorbed Ab-HRP decreasing as the concentration of *E. coli* increases. This is presumably due to the additional blocking effect exerted by non-specifically bound bacteria. Interestingly, the results were similar for gold rods bearing conjugated anti-*E. coli* Ab, BSA or no protein at all, indicating that bacteria adsorption was promoted by the surface itself. Attempts to decrease adsorption by blocking the surface with 2% (w/v) BSA were unsuccessful. We propose that this can be due to electrostatic repulsion between the negatively charged BSA molecules and the unreacted –COOH groups on the dextran, which prevented the formation of a stable BSA cover over the dextranised gold. In accordance to our results, dextran had been previously reported to non-specifically bind proteins present at high concentrations (Masson et al., 2006), and to be used by some bacteria as a carbon source (Khalikova et al., 2005).

The alternative conjugation of either Ab or BSA through their –COOH was performed as previously described in the text (Fig. 1b and d). In this case, ethylenediamine is first conjugated to dextran, incorporating positive charges to the modified surface. Under these conditions, BSA efficiently attaches to the surface as suggested by the important drop registered in both Ab-HRP and bacteria non-specific adsorption (Fig. 6b). However, no specific capture of *E. coli* is detected for the concentration range assayed (10^1 to 10^9 cells/ml). Attempts to detect *E. coli* by means of the previously optimised SAMs bearing the long-chain hydrophilic diamine and on MUA SAMs with directly conjugated

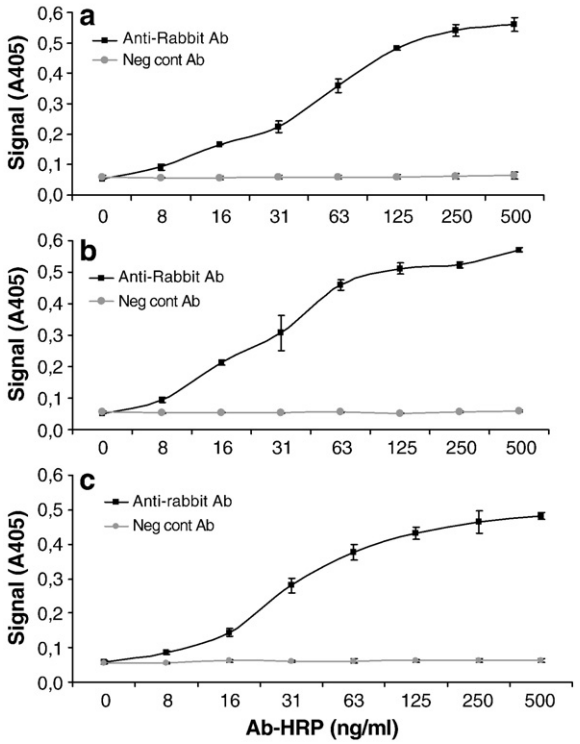

Fig. 5. Capture of different concentrations of the target Ab-HRP on gold surfaces functionalised with positive or negative control Ab. (a) Dextranised surface: CM-dextran was conjugated on a cysteamine SAM via EDC/NHS chemistry, followed by Ab conjugation through NH-ter as described in the Experimental section. (b) MUA SAM: MUA was self-assembled for 1 h, activated with EDC/NHS for 15 min and conjugated to NH-Ab. (c) Ab random physisorption. As to guarantee very low levels of non-specific adsorption, the target Ab-HRP was serially diluted in PBS containing 0.1% Tween and 0.5% BSA. Under these conditions, the performances of the three surfaces compared were very similar for this specific protein target and the negative control Ab generated in all cases very low levels of non-specific adsorption.

Ab or BSA were also unsuccessful. In contrast, direct physisorption of anti-*E. coli* Abs on bare gold, followed by BSA blocking, generated trends similar to those obtained on microtitter plates and allowed *E. coli* detection down to 5×10^5 cell/ml (Fig. 6c). These results differ from those obtained for protein detection. It is unclear whether SAM-based immuno-functionalisation generates Ab poorly exposed on surfaces (for example, Ab partly embedded within the SAM or buried under the BSA cape). In this case, the SAM-conjugated Ab could easily bind small molecules/proteins present in solution, but hardly access epitopes less exposed on a rigid bacterial wall. In this respect, the Ab used had been produced against whole *E. coli* cell preparations, and thus recognise a variety of epitopes.

Physisorption is accepted to negatively affect protein integrity and function (Heitz and Van Mau, 2002), in some extreme cases inducing inactivation of up to 90% of the Ab (Butler, 2000). Nevertheless, physisorption has been used by other authors in immuno-sensor development, due to its simplicity and cost-effectiveness (Kobayashi et al., 2005; Pemberton et al., 2005; Sharma et al., 2006; Taylor et al., 2006; Volpe et al., 2006), and is the only immuno-functionalisation strategy we assayed having generated acceptable results for bacteria detection on gold with reasonably low levels of non-specific adsorption. In the present case, the size of the target bacteria exceeds that of the Ab. This implies that target capture presumably induces a "shadowing" effect on the immuno-functionalised surface, eclipsing a number of unoccupied Ab. In this context, the loss of function of a small fraction of Ab due to physisorption may affect detectability in a less deleterious manner than in the case of detection of smaller targets. We thus propose physisorption as a suitable alternative to SAM-based gold functionalisation for bacteria detection. In this respect, immuno-functionalisation will be presumably improved if the Abs are indirectly captured by a physisorbed capture protein.

The results presented are consistent with the complexity of bacteria walls, and suggest that surface functionalisation for microorganism specific detection demands functionalisation strategies of their own. At this point, we should stress the importance of carrying out appropriate series of negative controls during assay and biosensor development in order not

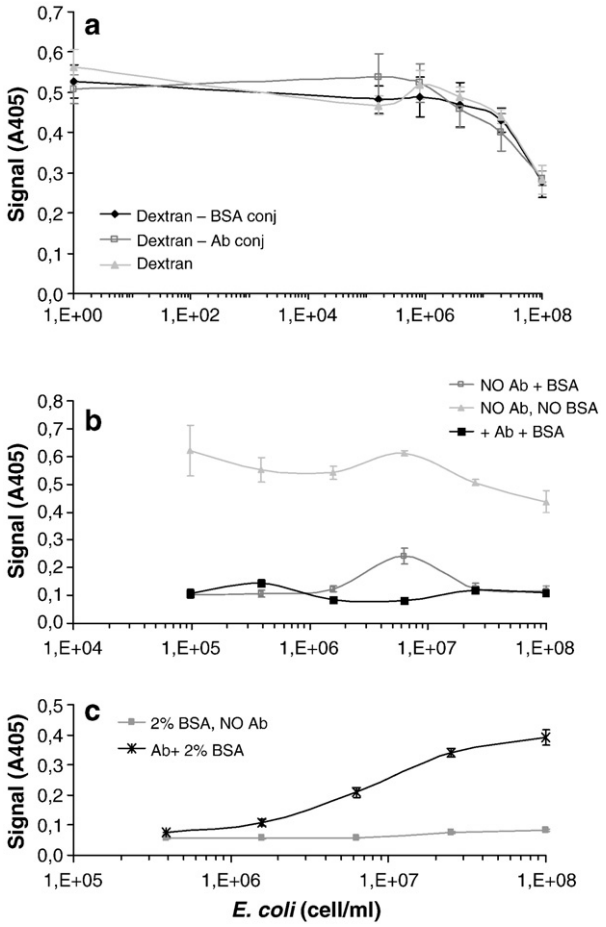

Fig. 6. *E. coli* detection on different modified gold surfaces. (a) Dextranised gold with Ab or BSA conjugated through their NH– groups. (b) Dextranised gold with/without Ab conjugated through their COOH– groups, and blocked or not with 2% BSA. (c) Gold surface with/without Ab non-specifically adsorbed, followed by blocking with 2% BSA.

to misinterpret bacteria non-specific adsorption on Ab-modified surface as specific capture.

4. Conclusions

In this work, we have assayed and optimised various strategies for gold functionalisation via SAM self-assembly and Ab conjugation. In this context, we have studied the effect of several parameters in both immuno-functionalisation efficiency and decrease on biomolecule non-specific adsorption, including thiomolecule length and concentration, self-assembly time and temperature, or polymer incorporation, as well as Ab conjugation strategy. Three of the SAM-based protocols, consisting on self-assembly of mercaptoundecanoic acid alone or conjugated to either a long-chain hydrophilic diamine or CM-dextran, contributed to reduce the level of Ab-HRP non-specific adsorption by up to 60% compared to bare gold. Ab conjugation onto those surfaces contributed to its physical blocking against non-specific adsorption and allowed the specific detection of an HRP-labelled protein target. These results demonstrated that Abs had been successfully incorporated and remained functional. As anticipated, Ab incorporated via COOH– groups performed better that those conjugated via their NH-ter.

On the other hand, the SAM-based optimised surfaces failed to detect *E. coli*, which was successfully detected when using Ab random physisorption instead. These results were completely unexpected. We could effectively detect both bacteria at a gold surface containing physisorbed antibodies as well as proteins on a SAM functionalised surface. This suggests that both functionalisation strategies work on gold and that they can be confidently transferred to produce biosensors. Our results imply that the interferences observed are specific to bacteria detection on SAM-based surfaces. Considering the significant differences between protein and bacterial targets, it is likely that both the bacterial size and its complexity (mainly due to the complex wall composition) are playing a role in this case. Our results indicate that immuno-functionalisation protocols having shown good performance for the detection of relatively simpler targets, such as proteins, may not work equally well for bacteria recognition. Consistently, surface functionalisation should be accompanied in these cases by an accurate evaluation, comprising the performance of the appropriate series of controls in order to differentiate specific capture from non-specific adsorption. This is of special importance in the field of biosensors, where reagentless assay formats are preferentially exploited. We finally propose Ab physisorption as a cost-effective gold immuno-functionalisation strategy, alternative to SAM-based Ab incorporation for bacteria detection.

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