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Electrochemical Biosensors Featuring Oriented Antibody Immobilisation via Electrografted and Self-assembled Hydrazide Chemistry

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ABSTRACT

Appropriate site-directed chemistry is essential to maximise the performance of immunosensors. We present two new functionalisation strategies that preserve proper folding and binding potential of antibodies by forcing their oriented immobilisation. Both strategies are based on the

formation of hydrazone bonds between aldehyde groups on the Fc moieties of periodate-oxidised antibodies and hydrazide groups on functionalised gold electrodes. Those hydrazide groups are introduced by electrografting of diazonium salts or by self-assembly of mono- and dithiolated hydrazide linkers, resulting in films with tailored functional groups and, thus, antibody distribution and spacing. Their barrier properties and permeability towards electroactive species are evaluated. To demonstrate the potential of these new functionalisation strategies, detection of bacteriophage MS2 is performed through either a direct assay using electrochemical impedance spectroscopy (EIS) or through a sandwich assay using differential pulse voltammetry (DPV). Diazonium and monothiolated self-assembled monolayers-modified electrodes enable the detection of less than 1 plaque forming unit (pfu)/mL in a direct EIS assay. However, non-specific adsorption renders measurements in river water samples difficult. In contrast, sandwich-assays on electrodes with electrografted diazonium salts and monothiolated self-assembled monolayers do not show significant matrix effects using river water samples, but the limits of detection are 10^8 times higher than those of the direct assay. Best results are achieved for immunosensors based on mixed monolayers of hydrazide and hydroxyl diothiolated linkers (15 pfu/mL). These new functionalisation techniques are facile to implement. They afford the possibility to tune surface composition and tailor the electrochemical properties of electrochemical sensors. These advantages should translate into broad interest in this type of surface chemistry for biosensor development.

INTRODUCTION

Efficient protocols for the immobilisation of bioreceptors onto electrode materials are of utmost importance for biosensor development.[1] Special emphasis has to be placed on the immobilisation of biological receptors without negatively affecting their binding affinity and selectivity. To that purpose, site-oriented immobilisation procedures are preferred, through reactions that do not involve functional groups that are essential for the bioactivity of the receptor.[2] Much effort has been devoted to immobilise antibodies preserving their oligovalent binding potential. Some studies have employed immobilisation strategies based on the functionalisation of the electrode surface with ligands showing affinity for specific regions of the antibody remote from the antigen-binding sites. Antibodies have been bound through affinity interactions between a histidine-rich sequence located in the C-terminal portion of their Fc region and transition metals, commonly nickel, that had been previously immobilised onto the electrode surface using chelating compounds such as iminodiacetic or nitriloacetic acid.[3] This immobilisation approach however lacks selectivity since other proteins, having cysteine or histidine residues can react as well. Moreover, the stability of the binding is easily affected by changes in pH or ionic strength. The high affinity of proteins A and G towards the Fc region of antibodies has been exploited for oriented antibody immobilisation.[4] Whilst this is a simple approach that does not require any antibody pretreatment, the distribution of the immobilised antibodies is variable and strongly depends on the protocol used to immobilise protein A or G. Yet other studies have been focused on electrode functionalisation strategies addressed to form stable bonds with native thiol groups of antibodies. In some cases, fragments of antibodies, such as half-antibody molecules or Fab fragments obtained after reduction of the disulfide bonds located at the hinge region that hold the heavy chains together, have been attached onto gold surfaces via the generated thiol groups.[5] However, this process may lead to denaturation of the

antibodies due to the proximity of the surface. Alternatively, some authors have taken advantage of the fact that all immunoglobulins are glycoproteins and thus, their sugar moieties are able to specifically react with certain functional groups, ending up in an oriented antibody immobilisation. Some examples of the site-directed immobilisation of antibodies through their sugar moieties include their reaction with boronic acid to form boronate esters[6] or the affinity interaction with the multiple-site sugar-binding protein concanavalin A.[7] The first method is limited by the reversibility of the boronic acid-saccharide binding, while the affinity interaction between sugar moieties and concanavalin A can be affected by changes in pH or temperature.

Advanced bioconjugate chemistry for immunosensors should therefore minimise the loss of biological activity upon antibody immobilisation. Furthermore, immobilisation chemistry should consider the spatial orientation of antibodies required for the formation of antibody-antigen immunocomplexes. And last, but not the least, non-specific adsorption from the matrix on the electrode surface needs to be taken into account and minimised.

With the advantages and disadvantages of the existing immobilisation protocols in mind, we describe here two novel electrode functionalisation strategies to introduce hydrazide groups on gold electrodes, ready to react with the aldehyde groups on the Fc region of oxidised antibodies, enabling their oriented immobilisation through the formation of stable hydrazone bonds. Carbohydrate moieties on the Fc region of antibodies can be readily oxidised with $NaIO_4$ to produce aldehyde groups.[8,9] Hydrazide groups were introduced on sensor electrode surfaces by electrografting of hydrazide-phenyl diazonium cation generated *in situ* from 4-aminobenzoic hydrazide, and by self-assembly of thiolated hydrazide compounds.

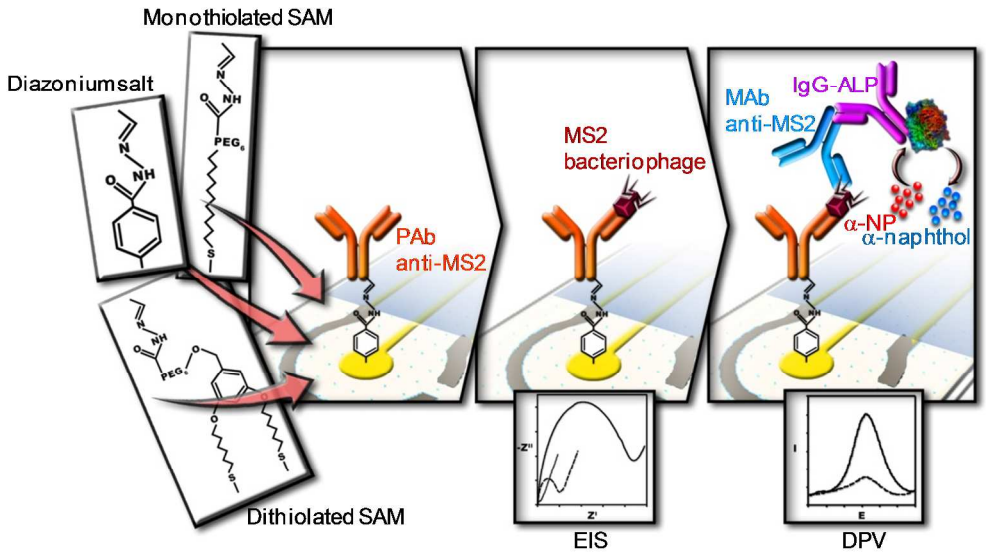
Over the past decade, aryl diazonium salts have been widely used to functionalise electrode surfaces. Apart from their stability, their success is likely due to their easy preparation in one step by reduction of *in situ* generated diazonium cations from aniline derivatives.[10,11] The ever increasing availability of aniline derivatives further enhances the versatility of the aryl diazonium salt chemistry, firstly described by Pinson and coworkers.[12] To the best of our knowledge, this is the first report describing the direct modification of gold electrodes with hydrazide-phenyl groups by electrochemical reduction of *in situ* generated hydrazide-phenyl monodiazonium cations.

Self-assembled monolayers (SAMs) on gold electrode surfaces combine the simplicity and flexibility of surface preparation with the wide-ranging availability of monolayer forming alkanethiols. However, there are issues in relation to lack of thermal stability of the gold-thiol bonds, sub-optimal spatial distribution of the chemical functionalities displayed on the distal of the alkyl chains and non-specific adsorption (fouling).[13,14] Following previous attempts to improve upon those issues, we compared here the use of mono- and di-thiolated molecules,[15] and we directly introduce for the first time hydrazide groups for oriented immobilisation of antibodies.

We demonstrated the potential of these functionalisation protocols by developing immunosensors for the bacteriophage MS2 as an indicator of faecal contamination in water supplies. Bacteriophages are specific for a target bacterial species, more robust than bacterial indicators and can be present in significant numbers. Therefore, they can be used as reliable indicators of microbiological contamination.[16] We have chosen the F-RNA coliphage MS2[17] to develop a proof-of-concept biosensor for faecal contamination that could be further tailored to detect other coliphages[18] or immunoreactive analyte species in general.

We investigated immunosensors for MS2 phage detection based on either a direct assay using EIS, or a sandwich-assay using DPV (Scheme 1). Our results showed that both immobilisation protocols efficiently controlled the orientation of antibodies and the permeability of the electroactive species in solution, resulting in strategies that can be easily tailored to prepare highly sensitive electrochemical immunosensors.

Scheme 1. Immunosensor for the detection of MS2 bacteriophage by EIS and DPV detection.



EXPERIMENTAL SECTION

Reagents. All reagents were used as received. 4-aminobenzoic hydrazide (ABH), 4-aminophenol (APh), *p*-phenylenediamine (PDA), sodium nitrite, sodium periodate, potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), α-naphtyl phosphate (α-NP), mouse IgG antibodies, anti-mouse IgG-alkaline phosphatase (ALP) conjugate, diethanolamine (DEA) and components of buffers were purchased from Sigma-Aldrich. Monothiolalkane-(ethylene glycol)$(EG)_6$-hydrazide, dithiolalkane-aromatic-EG_6-hydrazide and dithiolalkane-

aromatic-EG_3-OH were bought from SensoPath Technologies, Inc. (Bozeman, MT, USA). Monoclonal IgG antibody (MAb, developed in mouse) and polyclonal IgG antibody (PAb, developed in rabbit) against MS2 bacteriophage were obtained from Galahad Sales (Australia). MS2 bacteriophage was kindly supplied by SA Water. All solutions were prepared using Milli-Q water.

Screen-printed gold electrodes were purchased from DropSens (three-electrode system, ref. 250BT, 4 mm diameter, including a platinum counter electrode and a silver reference electrode).

Electrochemical and surface analysis equipment. Electrochemical measurements were performed on an electrochemical analyser (CH Instruments, model 600D series) using a three-electrode electrochemical cell, placed into a Faraday cage. Data acquisition and analysis were accomplished using CH Instruments software (CH Instruments, Inc., Austin, TX, USA). Further characterisation of the surface-modified electrodes was carried out using reflection IR spectroscopy/microscopy (Bruker Hyperion 1000).

Electrode preparation and electrochemical characterisation. Gold electrodes were electrochemically cleaned in 0.5 M H_2SO_4 by scanning the potential between 0 and 1.6 V at 100 mV s^{-1} for eight cycles.[19] The surface area was calculated from the reduction charge associated to the monolayer of chemisorbed oxygen.[20] All electrochemical data were normalised based on the surface of each electrode. Electrodes were characterised at each modification step using cyclic voltammetry (CV) and EIS. Electrochemical measurements were performed in an unstirred solution of 2 mM ferrocyanide and 2 mM ferricyanide in 100 mM phosphate buffer, pH 7.4. Cyclic voltammograms were obtained by scanning the potential at 0.1 V/s from -0.2 to 0.6 V. EIS measurements were performed under open circuit potential conditions. Frequencies from

10 kHz to 0.1 Hz in logarithmic spacing were applied. The AC amplitude was 10 mV.

Hydrazide-phenyl diazonium salt electrografting: The *in situ* generation of the aryl diazonium was performed by adding 0.5 equivalents of sodium nitrite to an acidic solution (0.5 M aqueous HCl) of ABH. The concentration of the aryl diazonium salt precursor was optimised (1-20 mM). Mixtures at different ratios with another precursor, APh, were also evaluated, to prevent diazo coupling, to improve the spatial distribution and to minimise non-specific adsorptions. These solutions were degassed and left to react for about 30 min in ice, prior to the electrografting process. The electrochemical reductive modification of the gold electrode surface with *in situ* generated diazonium salts was conducted either by cycling the potential between 0.6 V and -0.6 V or by applying a potential of -0.4 V. The number of cycles and the time of applied potential were also optimised. Subsequently, the electrodes were rinsed with copious amounts of Milli-Q water and then subjected to potential scanning between -0.2 V and 0.6 V for 10 cycles at 100 mV/s to remove the physisorbed compounds. ***Self-assembly of thiolated hydrazide compounds:*** Clean gold electrodes were exposed to 20 µL of 2.5 mM of the corresponding monothiolated or dithiolated hydrazide compound in ethanol during 3 h to facilitate the formation of SAMs. After modification, the electrodes were rinsed with ethanol to remove physisorbed molecules.

Oriented immobilisation of capture antibody through hydrazone bonds: Sugar moieties attached to the Fc region of polyclonal antibodies were oxidised to aldehydes, slightly modifying the procedure previously described by Hermanson.[21] Briefly, a solution of 0.3 mg/ml antibody in 100 mM sodium phosphate, pH 7.4, was mixed with 0.2 mM sodium periodate. The mixture was incubated for 30 min protected from light and then diluted 10 times with phosphate buffer to stop the reaction. Residual periodate was removed from the oxidised antibody solution using spin-desalting columns (7K MWCO, Thermo Scientific). 20 µL of the oxidised antibody was

incubated during 30 min at room temperature onto the hydrazide-modified surfaces to form stable hydrazone bonds.

Immunosensing protocol. Mouse IgG antibodies immobilised through hydrazone bonds, were used to directly optimise the protocols of electrode functionalisation based on electrografted diazonium salts and SAMs. To that purpose, 20 µL of a 0.2 µg/mL anti-mouse IgG-ALP solution were incubated during 1 h at room temperature onto each hydrazide-modified electrode to enable the voltammetric detection of the enzyme ALP used as label. Optimum conditions were then used to functionalise electrodes to build a sandwich-based immunosensor for MS2 bacteriophage. In that case, hydrazide groups were reacted with oxidised polyclonal anti-MS2 bacteriophage antibodies as previously described. Afterwards, 1 h incubation of MS2 bacteriophage solutions in a wide range of concentrations (from 1 to 10^{17} pfu/mL) was carried out. Then, as a required step for a sandwich assay, 1 h incubation of 20 µL of a 2 µg/mL MAb anti-MS2 bacteriophage was performed. Finally, 20 µL of a 0.2 µg/mL anti-mouse IgG-ALP solution were incubated during 1 h to introduce the label required for the voltammetric detection. The electrodes were thoroughly rinsed with phosphate buffer between each step. Controls in the absence of each one of the components were systematically performed.

Electrochemical detection protocol. EIS and DPV were used as electrochemical detection techniques. Apart from being an excellent characterisation technique, faradaic EIS was used to directly detect the phage directly in a non-labelled strategy. Alternatively, DPV was used to measure the activity of the enzyme ALP used as label in the sandwich assay.[22] Electrodes were exposed for 20 min to a 2 mM α-NP solution in 0.1 M DEA buffer, pH 9.5. Then, the potential was scanned at 0.05 V/s from 0 to 0.4 V. DPV data was corrected by subtraction of the voltammogram obtained in the absence of α-NP. Electrode assays were performed in triplicate.

Detection of bacteriophage in river water samples. Aliquots of alum-treated water (from Morgan reservoir, Murray river, South Australia) were spiked with MS2 bacteriophage to obtain dilutions from 1 pfu/mL to 10^{17} pfu/mL MS2. To perform electrochemical measurements, samples of 20 µL were incubated onto the electrodes, instead of phage solutions prepared in phosphate buffer.

RESULTS AND DISCUSSION

Hydrazide-phenyl diazonium salt electrografting: The first approach for oriented immobilisation of antibodies involved the electrochemical grafting of aryl groups substituted with hydrazide groups in one step by electrochemical reduction of the corresponding *in situ* generated diazonium cations in aqueous media (Scheme S-1). ABH was used as the amine precursor of the diazonium cations. CV of ABH in the presence of nitrite showed reversible electrochemical behaviour with redox potential at -0.19 V (Fig. S-1(A)).

ABH was electrografted onto a gold electrode at -0.4 V. The barrier properties of the grafted layers, their permeability towards electroactive species and the analytical performance of the resulting immunosensor, were evaluated. After electrografting, the electrochemical response for the redox probe ferrocyanide/ferricyanide and the potential peak separation between the anodic and cathodic peaks was investigated using CV. Electrografting resulted in decreasing current and increasing peak separation (Fig. 1(A)). EIS was also used to confirm the electrografting. The recorded impedance spectra were fitted to a Randles circuit, corresponding to the basic equivalent circuit depicted in Fig. 1(B). The Randles circuit includes the following parameters: the solution resistance (R_s), the charge transfer resistance (R_{ct}), the Warburg impedance (W) and the double-layer capacitance (C_{dl}). Due to the effect of the surface roughness that leads to the

dependence of the interfacial capacitance on the potential modulation frequency, a constant phase element (CPE) was used instead of an ideal C_{dl}. The R_{ct} obtained from the diameter of the semicircle of the corresponding Nyquist plots (physical barrier properties) in Fig. 1(B) show a significant increase of R_{ct} after electrografting, from 100 Ω for a bare electrode to 25500 Ω for a diazonium salt-modified electrode electrografted from a 10 mM ABH solution.

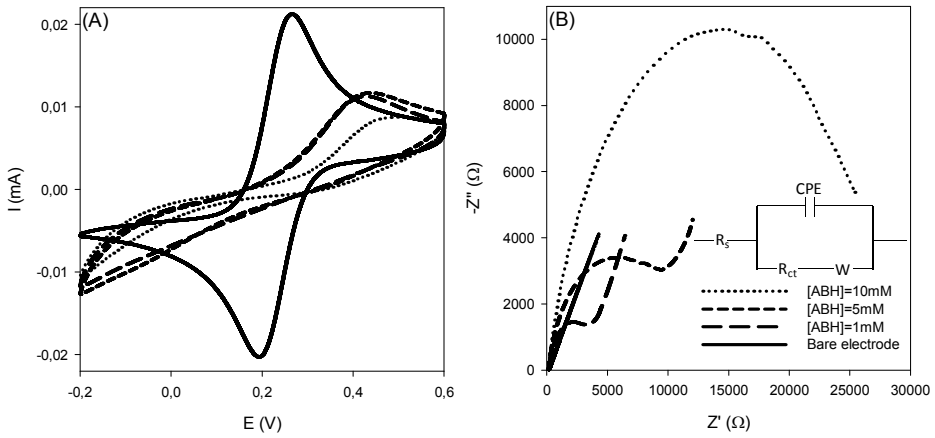


Figure 1. (A) CVs and (B) Nyquist impedance plots recorded at electrodes electrografted using different concentrations of ABH precursor. Measurements were performed in a 2 mM ferrocyanide/ferricyanide solution in phosphate buffer. Inset in (B): Equivalent circuit used to fit experimental data.

IR spectroscopy provided further proof for successful electrografting (Fig. S-2). The peak at 3437 cm^{-1} corresponds to the absorption of N-H stretching vibration; the absorption peaks at 2957 cm^{-1}, 2837 cm^{-1} and 728 cm^{-1} were attributed to CH_2 symmetrical stretching vibrations, asymmetrical stretching vibrations and rocking vibrations, respectively. The absorption band at 1730 cm^{-1} stems from the C=O vibration of benzoic hydrazide. The absorption bands at 1501 cm^{-1}, and 1481 cm^{-1} are related to the benzene vibrations. The presence of C-N groups on the gold

surface is confirmed by the band at 1530 cm^{-1}. The band at 1620 cm^{-1} was attributed to the angular deformation of N-H in NH_2 and to the C=C bond stretch in aromatic rings (this band could also indicate the presence of phenyl multilayers).

The diazonium grafting efficiency of aryl groups depends on the nature, position on the aromatic ring and chain length of the alkyl substituent. The electrochemical grafting of aminophenyl groups at the surface of gold electrodes in one step by reduction of *in situ* generated aminophenyl monodiazonium cations, using *p*-phenylenediamine (PDA) as the amine precursor has been described.[11] Other aromatic-aliphatic diamines were studied, concluding that in contrast to aliphatic diazonium cations, aromatic diazonium cations are relatively stable at low temperature because the positive charge is delocalised and, thus, resonance-stabilised by the aromatic system.[23] The amine group on the phenyl ring is known to be selectively oxidised by nitrosonium ions in the presence of other amines on the molecule.[24] Therefore, we assume that, in the case of ABH, the introduction of positive charge after diazonium formation from the aromatic amine reduces the reactivity of the hydrazide. However, high ratios of sodium nitrite to ABH may lead to bisdiazonium formation.[25] In our case, best results were achieved when using 0.5 equivalents of sodium nitrite.

The results above showed that electrografting was successful and the IR results indicated that the hydrazide group was still available after electrografting. However, it is important to demonstrate that these hydrazide groups on the gold electrode can be used to immobilise a periodate-oxidised antibody under mild conditions forming a stable hydrazone bond.[26] We first incubated the ABH-electrografted surface mouse antibody for immobilisation and then incubated with an anti-mouse IgG-ALP conjugate to enable voltammetric detection of enzymatic activity in the presence of α-naphtyl phosphate (α-NP). As controls, we used surfaces electrografted with

aminophenol (APh) and PDA, the former lacking any group that could react with the antibody and the latter displaying amine groups which can react with amine groups on the antibody in a non-oriented fashion. DPV results of the enzymatic activity of ALP for immunosensors prepared by ABH electrografting showed that ABH electrografting clearly facilitated the immobilisation of antibodies (Fig. 2). In contrast, on the APh-modified electrodes, there was no ALP activity. When comparing the peak current of the DPV on ABH- and PDA-electrografted surfaces, we observed a 3-fold higher signal for the former electrode. The lower signal obtained with PDA-electrografted electrode was explained by the contribution of antibody binding via formation of an unstable Schiff base that results from the interaction between amine and the antibody's aldehyde groups.

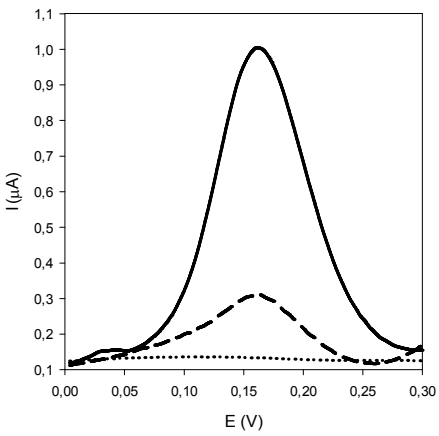


Figure 2. DPV responses of the ALP activity after adding α-NP on immunosensors prepared by electrografting of APh (dotted line), PDA (dashed line) and ABH (solid line), incubation with mouse IgG and further incubation with anti-mouse IgG-ALP antibody.

Impedance spectra showed a significant increase in R_{ct}, from 624 Ω in the absence of antibody to 19750 Ω after incubation with the oxidised antibody. Upon incubation with non-oxidised

antibody the R_{ct} increased to 4599 Ω, presumably due to non-specific adsorption of the antibody (Fig. S-3). Therefore, we can conclude that ABH electrografting onto gold electrodes allowed immobilisation of antibodies via their oxidised Fc regions.

We next optimised electrografting conditions as a compromise between fractional electrode coverage (θ) (calculated as $\theta = 1-(R_{ct}^0/R_{ct}^f)$, where R_{ct}^0 and R_{ct}^f are the R_{ct} obtained prior and after diazonium salt electrografting, respectively)[27] and intensity current from DPV, obtained after mouse antibody immobilisation on the electrografted surface and labelling with anti-mouse ALP labelled antibody (Table S-1). Best results were achieved after 20 cycles of potential between 0.6 and -0.6 V in a 20 mM ABH solution.

We also investigated whether mixed monolayers generated from mixtures of ABH and APh (not reactive with antibody) gave further improvements in DPV currents as a measure of ALP activity. Mixed diazonium salts were grafted using different ratios of ABH and APh. θ decreases as APh concentration in the mixture increases. This may be the result of the formation of less dense mixed layers compared to pure ABH. ALP activity showed a maximum for equal concentrations of ABH and APh (both 10 mM). Electrodes prepared with mixed diazonium salts gave 2-fold higher DPV peak currents than those obtained using pure ABH while non-specific adsorption (measured using non-oxidised antibody) remained equal, therefore, greatly improving the signal-to-noise ratio.

In order to study the efficiency of the electrografted electrode surface towards electrochemical detection of α-naphthol, the product of ALP conversion, electrodes prepared with 10 mM ABH and 10 mM APh were used and ALP was added in solution to catalyse the dephosphorylation of α-NP. Cyclic voltammograms of α-NP in the presence of ALP in solution show a plateau of low

intensity current that starts at 0.25 V at a bare electrode, whereas a well-defined oxidation peak was obtained at 0.19 V at the electrografted electrode (Fig. 3). At the same time, the anodic peak current increased from 0.2 µA to 1.6 µA. These results (reduced overpotential and increased peak current) indicate that the mixed monolayers catalyse the oxidation of α-naphtol.

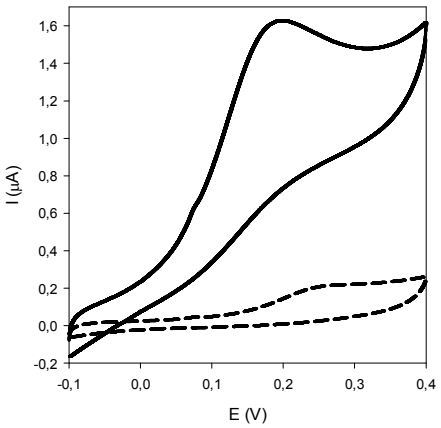


Figure 3. Cyclic voltammograms at a bare electrode (dashed line) and at a mixed diazonium salt electrografted electrode (solid line), after adding 2 mM α-NP to an ALP solution.

Furthermore, peak current (I) for these electrodes was directly proportional to the square root of scan rate ($\nu^{1/2}$) (r^2= 0.9987), indicating a diffusion controlled electrode reaction. $I_p/\nu^{1/2}$ *vs.* ν showed a characteristic shape typical of an EC_{cat} process[28] where the grafted diazonium salt chemically reacts with the α-naphtol diffused toward the electrode surface, while the simultaneous regeneration of the grafted diazonium salt increases the anodic current (Fig. 4). We hypothesise that the hydroxyl group on the grafted diazonium salt can play an important role in the electron transfer between the grafted layer and the α-naphthol in solution (Scheme 2).

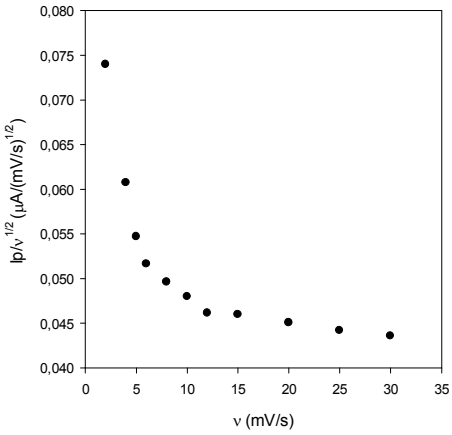
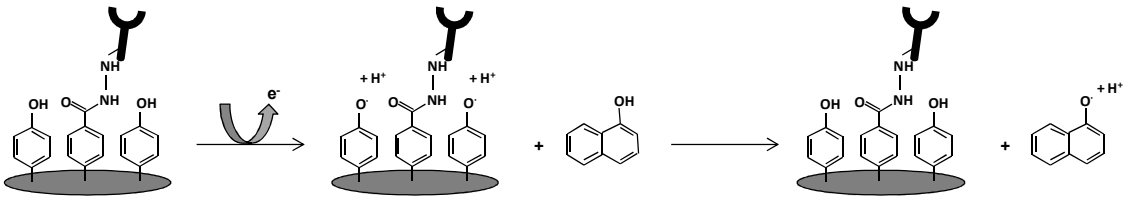


Figure 4. Electrochemical characterisation of mixed diazonium salt-electrografted electrodes in the presence of α-naphthol in solution. Plot of variation of the peak current normalised to the $\nu^{1/2}$ ($I_p/\nu^{1/2}$) *vs.* ν.

Scheme 2. Possible mechanism involved in the electron transfer between the ABH-electrografted layer and the α-naphthol in solution.



Self-assembly of thiolated hydrazide compounds: The second strategy for oriented antibody immobilisation via hydrazides involved formation with thiolated hydrazides that contain oligoethylene glycol units in order to reduce non-specific adsorption.[29] Successful SAM formation of monothiolated and dithiolated hydrazide compounds (shown in Fig. 5(A)) was confirmed by CV and EIS. The CV currents of the ferrocyanide/ferricyanide redox couple observed at a bare gold electrode were almost completely suppressed after SAM formation due to the blocking effect of a well-packed monolayer (Fig. 5(B)). For both thiolated compounds, we

observed a significant decrease in peak current. The residual current on the SAM of the dithiolated compound is 46% lower than that obtained with the monothiolated compound, suggesting that the former one exerts a more pronounced barrier effect. These results are consistent with those found using EIS (data not shown), showing higher values of R_{ct} for SAM-modified electrodes than those obtained for a bare electrode, and also being higher for electrodes prepared with the dithiolated compound than with the monothiolated one.

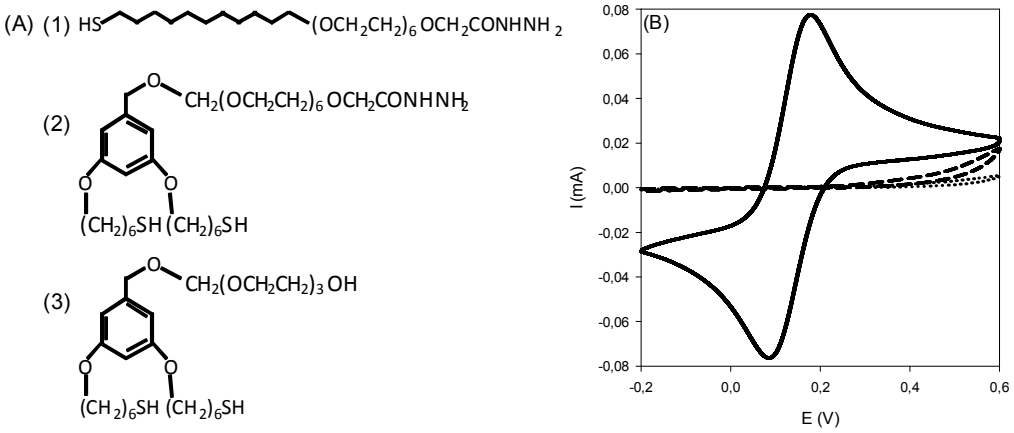


Figure 5. (A) Chemical structure of (1) monothiolalkane-EG_6-hydrazide, (2) dithiolalkane-aromatic-EG_6-hydrazide and (3) dithiolalkane-aromatic-EG_3-OH. (B) CVs recorded at a bare electrode (solid line), and SAM-modified electrodes of monothiolated (dashed line) or dithiolated (dotted line) compounds, respectively. Measurements were performed in a 2 mM ferrocyanide/ferricyanide solution in phosphate buffer.

The θ values obtained after 3 h incubation of each thiolated compound were close to 0.9998, indicating close-packed monolayers for both mono- and dithiolated compounds. The observed differences in electron transfer permeability were hence not due to different fractional coverages.

Instead, an explanation could be a difference in the degree of hydrophobicity of both thiolated compounds, being higher for the dithiolated-aromatic compound.

Application of electrografted surfaces and SAMs in impedimetric and voltammetric immunosensors is discussed in the next section. We focused on the contribution of each monolayer to obtain a proper spatial distribution of the immobilised antibodies as well as on minimising non-specific adsorptions.

MS2 bacteriophage detection: Scheme 1 illustrates the stepwise procedure for the preparation and working protocol of the immunosensor for the detection of MS2 bacteriophage. We used EIS as a sensitive, non-destructive, and label-free electrochemical sensing method for MS2 bacteriophage on electrodes functionalised by oriented immobilisation of polyclonal anti-MS2 antibody via hydrazone bonds. EIS is especially useful as a highly sensitive electrochemical technique when large species are involved in the biological sensing interface. Therefore, apart from using it as a surface characterisation technique, we evaluated its suitability as a transducer technique. As mentioned before, the parameters derived from fitting the Nyquist plots of the impedance spectra with a Randles circuit include the Warburg impedance and the R_s that are related to the bulk properties of the electrolyte solution and diffusion features of the redox probe in solution and, thus, are not affected by changes in the biological interface.[30] The other two parameters involved in the fitting are the CPE and the R_{ct}, corresponding to the dielectric and insulating features at the electrode/electrolyte interface. These two parameters are influenced by changes on the electrode surface. R_{ct} changes are more pronounced than CPE changes and therefore they can afford a higher sensitive detection of the interaction of the MS2 bacteriophage with the immobilised antibody. Therefore, R_{ct} was used as the impedance signal responsive to the presence of bacteriophage. To allow comparison among immunosensors, all R_{ct} values were

normalised as follows: $(R_{ct}^{f}-R_{ct}^{0}))/R_{ct}^{0}$, where R_{ct}^{0} and R_{ct}^{f} are R_{ct} values prior and after bacteriophage interaction. After bacteriophage interaction with the immobilised antibodies, R_{ct} increased due to the reduced access of the ferrocyanide/ferricyanide couple towards the modified surface. It is interesting to note that the highest sensitivity, taken as the slope of the linear part of the calibration curves, was achieved with those immunosensors prepared by electrografting of diazonium salts (Table 1). This result can be explained by the difference in the thickness of the films: electrodes modified with diazonium salts placed the interaction phage-immobilised antibody closer to the electrode surface than the electrodes prepared with SAMs. As a result, access of the redox probe to the electrode surface was facilitated and changes onto the electrode surface were sensitively detected. Regarding SAMs prepared with monothiolated or dithiolated compounds, the latter gave immunosensors showing higher sensitivity. In this case, the amount and distribution of antibodies seems to play a role: dithiolated compounds introduced less hydrazide groups on the electrode surface than monothiolated compounds per unit area, and thus more intermolecular empty space among antibodies, facilitating the proper orientation of their binding sites towards bacteriophage present in solution.[31]

Table 1. Curve parameters derived from the linear regression fitting of the plots of normalised R_{ct} *vs.* MS2 bacteriophage concentration.

Functionalisation strategy	**Sensitivity**[a]	**LOD (**pfu/mL)[b]	**SD**[c]	***R***
Electrografted diazonium salt	0.31	0.84	0.23	0.9978
Monothiolated SAM	0.13	0.24	0.08	0.9981

Dithiolated SAM	0.21	2.20	0.72	0.9989

[a] Sensitivity taken as the slope of the linear part of the calibration curves $\left[\frac{\left(R_{ct}^{f}-R_{ct}^{0}\right)}{R_{ct}^{0}}\right]$ *vs*. MS2 concentration.

[b] LOD calculated as $y_b+3*\sigma_b$,[32] where y_b is the value for the blank (R_{ct} of the phosphate buffer solution without bacteriophage) and σ_b is the standard deviation.

[c] SD values refer to the maximum SD values found in the calibration curve.

Even though EIS proved to be a sensitive detection technique, showing low limits of detection (Table 1), high non-specific adsorption was observed when measurements of bacteriophage-spiked water samples were conducted (Fig. S-4). Non-specific adsorption was minimised by using either mixed diazonium salts or mixed SAMs that introduce functional groups known to have an anti-fouling effect. However, EIS was still too sensitive towards interferences from the matrix.

We also investigated the performance of sandwich immunosensors using electrodes modified by either diazonium salt electrografting or SAMs (Scheme 1). Electrochemical measurements of the ALP activity used as label were performed using DPV. Fig. 6 shows the non-linear fitting of plots obtained from the DPV measurements of immunosensors prepared by electrografting or SAMs followed by PAb MS2 antibody immobilisation, incubation with buffer and river water samples spiked with MS2 bacteriophage, interaction with MAb MS2 antibody and labeling with anti-mouse IgG-ALP. Percentages of slope deviation between calibration curves in buffer and in water samples were lower than 7% for immunosensors prepared with diazonium salts and monothiolated compounds, showing their ability to detect MS2 in spiked river water samples, without significant matrix effects. The percentage of slope deviation increased up to 65% for immunosensors functionalised with dithiolated compounds, possibly due to the previously

mentioned intermolecular empty space in between antibodies which might allow electroactive interfering compounds present in water samples better access to the electrode surface. For the immunosensors prepared with diazonium salts, the LODs were $2.3 \cdot 10^8$ and $8.2 \cdot 10^7$ pfu/mL for buffer and river water samples, respectively. For the immunosensor prepared with monothiolated compounds, the LOD values were $1.1 \cdot 10^8$ and $7.7 \cdot 10^8$ pfu/mL for buffer and river water samples, respectively. The immunosensor prepared with SAMs of dithiolated compounds showed a greatly reduced LOD down to $3.6 \cdot 10^5$ and $1.5 \cdot 10^6$ pfu/mL in buffer and in river water samples, respectively, (see Table S-2). However, as mentioned before, there was a significant matrix effect when spiked-water samples were used that strongly affected the accuracy of the results. To minimise this effect, mixed SAMs, combining the hydrazide dithiolated compound with a hydroxyl dithiolated compound (Fig. 5(A3)), were evaluated. Nonspecific adsorption was eliminated, as shown by the percentages of slope deviation between calibration curves in buffer and in water samples that was lower than 4% (Fig. 6). Moreover, LOD values decreased down to 15 pfu/mL in buffer and 214 pfu/mL in river water.

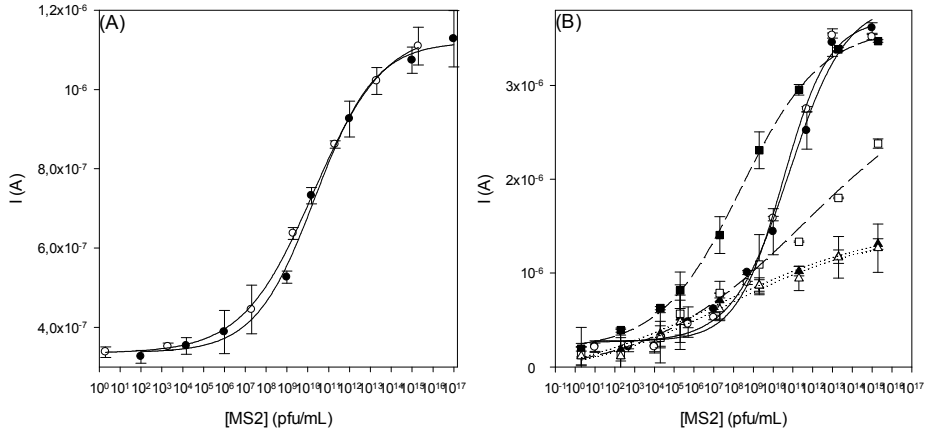


Figure 6. Calibration curves of peak current (DPV) *vs.* [MS2] obtained for MS2 bacteriophage in phosphate buffer (black symbols) and in river water samples (white symbols) using sandwich-

based electrochemical immunosensors prepared by diazonium salt electrografting (A) and self-assembling of monothiolated (circle), dithiolated (square) and mixed hydrazide/hydroxyl dithiolated (triangle) compounds (B).

CONCLUSIONS

In conclusion, two electrode functionalisation strategies were developed to enable oriented antibody immobilisation. Both strategies involved hydrazides able to form hydrazone bonds with the Fc moieties of oxidised polyclonal antibodies. The first strategy relied on hydrazide-phenyl diazonium salts that were electrografted onto the gold electrode surface. The second strategy involved the use of mono- and dithiolated SAMs carrying hydrazide functional groups. To prove the potential of these new funtionalisation strategies, an electrochemical immunosensor able to detect MS2 bacteriophage in buffer and river water at low concentrations was demonstrated. Direct detection of MS2 binding using EIS gave low LODs values below 2.2 pfu/mL for all developed immunosensors. However, adsorption of compounds present in spiked water samples limited the application of this impedimetric sensor. As an alternative, a sandwich assay using DPV detection was evaluated. Diazonium salt and monothiolated SAM-based immunosensors behaved similarly, showing no matrix effects for spiked river water samples, but gave significantly higher limits of detection ($2.3 \cdot 10^8$ and $1.1 \cdot 10^8$ pfu/mL, respectively) than those found using direct impedimetric detection. On the contrary, a better LOD ($3.6 \cdot 10^5$ pfu/mL) was observed for dithiolated-based immunosensors, likely due to the presence of more intermolecular empty space in between antibodies, facilitating the proper orientation of their binding sites towards bacteriophage present in solution. However, the downside of this additional space was that we observed non-specific adsorption events. The use of mixed SAMs proved to minimise this problem, offering immunosensors able to detect 214 pfu/mL in spiked river samples.

ASSOCIATED CONTENT

Supporting Information. Experimental results of diazonium salt-modified electrodes: CV of the electrografting process, characterisation by reflection IR spectroscopy, EIS results proving the immobilisation of antibodies via their oxidised Fc regions, optimisation of electrografting conditions by EIS and DPV and EIS measurements showing the non-specific adsorption observed in the direct detection of MS2 bacteriophage. A table summarising the curve parameters of the plots obtained for MS2 bacteriophage using sandwich-based electrochemical immunosensors. This material is available free of charge via the Internet at http://pubs.acs.org."

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ABBREVIATIONS

EIS, electrochemical impedance spectroscopy; DPV, differential pulse voltammetry; CV, cyclic voltammetry; SAM, self-assembled monolayer; ABH, 4-aminobenzoic hydrazide; APh, 4-aminophenol; PDA, *p*-phenylenediamine; α-NP, α-naphtyl phosphate; IgG-ALP, IgG-alkaline phosphatase; R_s, solution resistance; R_{ct}, charge transfer resistance; W, Warburg impedance; C_{dl}, double-layer capacitance; CPE, constant phase element; θ, fractional electrode coverage; I, current intensity; ν, scan rate.

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