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Site-directed introduction of disulfide groups on antibodies for highly sensitive immunosensors

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1 **ABSTRACT**

2 The interface between the sample and the transducer surface is critical to the performance of a
3 biosensor. For immunosensor applications the affinity biosensor interface consists of antibodies, which
4 are preferably covalently attached to the transducer surface, and optimal orientation of the immobilized
5 antibody is essential for maximal signal and consequent improved detection limits. In this work we
6 compared different strategies for covalent self-assembly of antibodies onto bare gold substrates by
7 introducing disulfide groups into the immunoglobulin structure and following target binding using
8 surface plasmon resonance (SPR). Disulfide groups acted as anchor molecules able to chemisorb
9 spontaneously onto clean gold surfaces and were chemically introduced to the antibody via the primary
10 amines, carboxylic acids and carbohydrates present in its structure. The site-directed modification via
11 the carbohydrate chains exhibited the best performance in terms of analyte response using a model
12 system for the detection of the stroke marker Neuron Specific Enolase. SPR measurements clearly
13 showed the potential for creating biologically active densely packed self-assembled monolayers (SAMs)
14 in a one step protocol of this particular immobilization technique compared to both mixed SAMs of
15 alkanethiol compounds and commercial immobilization layers. The ability of the carbohydrate strategy
16 to construct an electrochemical immunosensor was thus investigated using electrochemical impedance
17 spectroscopy (EIS) and differential pulse voltammetry (DPV) transduction.

18

19 **KEYWORDS**

20 SPR and electrochemical immunosensors

21 Site-directed protein modification

22 Surface chemistry

23 Self assembled monolayer (SAM)

24 Neuron Specific Enolase (NSE)

1. INTRODUCTION

Immunosensors are analytical devices based on the specific interaction between antibodies and antigens that results from the integration of a biological receptor molecule onto a transducer element [1-3]. They have shown many practical applications to several research fields, from medical diagnostics to environmental analysis, and have great potential for commercialization, but do still face a challenge in achieving a simple, robust and inexpensive surface functionalization method compatible with mass-manufacturing techniques. Generally, functionalization processes require the biochemical modification of either the sensor surface or the antibody or both, and this usually involves several steps that in some cases are time consuming, costly and difficult to implement in large-scale production process.

The immobilization of antibodies on solid support transducers is a critical issue for the sensor performance, as in the majority of cases, the affinity, orientation and stability of the antibodies are affected by the immobilization strategy selected [4]. There is an important variety of surface chemistry methods reported in the literature, which indicates that a universal method is not yet available, however, those introducing orientation and maintaining native antibody conformation are of greatest relevance [5, 6]. Specific orientation should expose free antigen-binding regions of the antibody following surface anchoring, resulting in increased analyte binding and improved sensitivity. . Passive adsorption of biomolecules on solid substrates has been widely explored [7] due to its' simplicity, but this strategy results in a random and non-specific adsorption of the species without control of orientation, low stability and protein unfolding [8, 9]. In order to provide a better control, the use of self-assembled monolayers (SAMs), with two terminal functional groups, with one group enabling binding of molecules to the sensor surface, and the other allows coupling of the bioelement. This technique has been widely used [10–14], due to the high diversity of functionalized SAMs and chemical cross-linkers available, and provides a strong and stable attachment. Nevertheless, it needs a previous functionalization of the surface and the molecules attached to the SAMs can be randomly oriented [15]. To minimize random

1 orientation and position the antibodies in a uniform orientation on the surface, several strategies have
2 been developed. The use of receptors immobilized via Fc binding proteins bound on the surface
3 (proteins A, G, L, anti-Fc) improves sensitivity [4, 16–18], but requires an initial surface modification.
4 Immobilization via antibody fragments through sulfhydryl groups also improves the sensitivity [19–22]
5 but may form a very compact layer which may give rise to significant steric hindrance effects [23] and
6 the potential loss of biological activity of the antibody fragments [24]. A further interesting approach is
7 immobilization via oxidized oligosaccharide moieties of the antibodies, achieved by the chemical or
8 enzymatic oxidation of the oligosaccharide moieties and subsequent coupling to amine or hydrazine
9 modified solid supports, providing great sensitivity [25–27], but requiring a surface pre-
10 functionalization step. Alternatively, sulfur-containing molecules can be introduced into the bioelement
11 structure prior to its chemisorption onto gold [1, 26], reducing surface biofunctionalization to a single
12 incubation step (Scheme 1) and suitable anchoring groups that carry sulfur atoms can be introduced into
13 the bioelement using different approaches. Antibody molecules possess a number of functional groups
14 suitable for modification including lysine ϵ -amine, N-terminal α -amine groups, and C-terminal aspartic
15 acid and glutamic acid residues. Chemical conjugation reactions with antibody molecules are generally
16 more successful at preserving activity if the functional groups utilized are present in limiting quantities
17 and only at discrete sites on the molecule. Such “site-directed conjugation” schemes exploit cross-
18 linking reagents that can specifically react with residues that are only in certain positions on the
19 immunoglobulin surface, usually chosen to be well removed from the antigen binding sites. In one
20 approach, the disulfide in the hinge region of the antibody that holds the heavy chains together can be
21 selectively cleaved with a reducing agent such as 2-mercaptoethylamine to create two half-antibody
22 molecules, each containing an antigen binding site [28]. An alternate method is based on the
23 modification of antibodies via their carbohydrates, which are located in the C_H^2 domain within the Fc
24 region. Mild oxidation of the polysaccharide sugar residues with sodium periodate generates aldehyde
25 groups, which can then be used for coupling to another molecule [28]. By proper selection of the

1 conjugation reaction and knowledge of antibody structure, the immunoglobulin molecules can be
2 oriented so that its bivalent binding potential for antigen remains available.

3
4 In this work, we developed a simple one-step surface functionalization method based on the covalent
5 coupling of antibodies. We studied different antibody immobilization strategies on bare gold substrates
6 via a direct bio-SAM approach. This approach was achieved by chemical introduction of sulfur-
7 derivative molecules into the antibody structure prior to its adsorption onto gold via the primary amines,
8 carboxylic acids and carbohydrates present in its structure. Additionally, the classic SAM approach was
9 also evaluated using unmodified antibodies attached to a long-chain of alkanethiols previously self-
10 assembled onto a bare gold substrate. The model antibody-antigen system applied to carry out this work
11 consisted of two monoclonal antibodies, a capture anti-NSE21 antibody and a reporter anti-NSE17
12 antibody, against neuron specific enolase (NSE), a dimeric isoenzyme of the glycolytic enzyme enolase
13 and derives from neuronal cytoplasm and neuroendocrine cells [29]. Several studies have demonstrated
14 significantly lower NSE concentrations in serum in healthy subjects, with levels lower than 12.5 ng/ml,
15 than in patients with acute ischemic stroke [30], and has thus been identified as a possible biological
16 marker for the diagnosis of ischemic stroke [31]. A Biacore® 3000 surface plasmon resonance (SPR)
17 system was employed to characterize the different immobilization techniques and monitor the antibody-
18 antigen interactions, and the optimal antibody modification applied to its electrochemical detection
19 using an immunosensor exploiting differential pulse voltammetry with a clinically relevant detection
20 limit of 4.6 ng/ml.

22 **2. EXPERIMENTAL**

23 **2.1. Chemicals and materials.** Neuron specific enolase (NSE), anti-NSE21 monoclonal antibody
24 (MAb) and anti-PSA66 (Prostate Specific Antigen) MAb were kindly supplied by Fuijirebio Diagnostics
25 (Gothenburg, Sweden). Human CEA (Carcino Embryonic Antigen) was purchased from SCIPAC

1 (Sittingbourne, UK). Both anti-PSA66 MAb and human CEA were employed for non-specific binding
2 studies as non-specific ligand and as non-specific marker, respectively. Bare gold substrates (SIA-kit),
3 gold substrates mounted in chip (Au chip) dextran-coated gold substrates (CM5 chip) and HEPES
4 buffered saline (HBS) (10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, 150 mM NaCl, 3.4
5 mM ethylenediaminetetraacetate, and 0.005% Tween 20 (pH 7.4)) were purchased from Biacore (GE
6 Healthcare, Barcelona). Thiolated polyethyleneglycol 1-(mercaptoundec-11-yl)-tetra(ethylene glycol)
7 (PEG) was supplied by SensoPath Technologies (Bozeman, USA). Cystamine dihydrochloride, 1-Ethyl-
8 3-(3-dimethylamino-propyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), sulfo-NHS,
9 dithiopropionic acid succinimidyl ester (DTPS), 16-mercapto-1-hexadecanoic acid (16-MHA), 11-
10 mercapto-1-undecanol (11-MUOH), and carbonate-bicarbonate capsules for preparation of carbonate
11 buffer (0.05 M, pH 9.6) were purchased from Sigma (Barcelona, Spain). Ethanol, acetone, dimethyl
12 sulfoxide (DMSO), sodium di-hydrogen phosphate (NaH_2PO_4) and di-sodium hydrogen phosphate
13 (Na_2HPO_4) were obtained from Panreac Química (Barcelona, Spain). Sodium chloride (NaCl), sodium
14 hydroxide (NaOH), potassium chloride (KCl), sodium acetate, acetic acid and sodium periodate were
15 supplied by Scharlau (Barcelona, Spain). Centrifugal filter membranes of 100 molecular weight cut off
16 (MWCO) and 0.2 μm membrane filters were supplied by Whatman GmbH (Dassel, Germany).
17 Deionised water was produced using a Milli-Q RG system (Millipore Ibérica, Madrid, Spain). The
18 concentration of the chemically modified antibodies was determined using a Cary 100 UV-Vis
19 spectrophotometer supplied by Varian (Barcelona, Spain). To evaluate the different immobilization
20 techniques a Biacore® 3000 Surface Plasmon Resonance (SPR) system was used.

21

22 **2.2. Chemical modification of biocomponents.** Disulfide groups were covalently introduced into the
23 structure of both anti-NSE21 and anti-PSA66 via its primary amines, carboxylic acids and
24 carbohydrates, whereas the anti-NSE17 was used in its' unmodified state as second primary antibody for
25 sandwich assay experiments.

26

2.2.1. Chemical introduction of disulfides in anti-NSE21 antibody using -NH₂ residues. Primary

amine groups are known to have a high nucleophilic behaviour at basic pH. In the presence of an active ester, the free electron pair of the amine group can easily attack the electrophile carboxylic carbon of the ester. Introduction of disulfides by this synthetic route was performed by reacting terminal primary amines and lysine residues of IgG with a disulfide-containing active ester, giving rise to a covalent attachment between disulfide groups and IgG. Therefore, 0.5 mg of anti-NSE21 (3.6×10^{-9} mol) diluted in 0.5 mL of 0.01 M carbonate buffer pH 9.5 were mixed with 0.07 mg of dithiopropionic acid succinimidyl ester (DTPS) (1.8×10^{-7} mol) prepared in 0.05 ml of DMSO. The mixture was allowed to react in dark conditions for 5 hours at room temperature under vigorous stirring. The excess of DTPS was removed by ultrafiltration (100 kDa Molecular Weight Cut Off (MWCO) membranes) and the modified antibody was recollected in PBS buffer pH 7.4. The concentration of the modified antibody was determined by UV-Vis spectrophotometry at 280 nm using an extinction coefficient of 1.38 ml/mg•cm for 1 mg/ml IgG solutions in a 1-cm path length [32].

2.2.2. Chemical introduction of disulfides in anti-NSE21 antibody using carbohydrates. The

introduction of disulfide groups into IgG structures was achieved by oxidizing the polysaccharide residues to aldehydes that react with primary amine groups of cystamine via the formation of Schiff bases. Alcohol groups of carbohydrates can be partially oxidized in aldehydes under the oxidative power of periodate (IO_4^-), in an acidic medium. These aldehydes then easily react with primary amines and form Schiff bases. The unstable imines formed have to be reduced under the action of borohydride, to stable secondary amines. Initially, 0.5 mg of anti-NSE21 were diluted in 100 μL of 0.01M acetate buffer pH 5.0, and then a 5 mM solution of sodium periodate was added to the antibody solution. The mixture was left to react in the dark and under stirring conditions for 1 h at room temperature. The oxidized antibody solution was slowly added to 900 μL of a 0.1 M cystamine solution diluted in 0.05 M carbonate buffer (pH 9.5) and left to react for 3 hours at room temperature. In the next step, unstable imines were reduced to amine bonds by dropping 10 mM cyanoborohydride into the solution. Finally,

the antibody solution was purified from the large excess of cystamine by filtration using 100 kDa MWCO membranes and, the modified antibody subsequently recollected in PBS buffer pH 7.4.

2.2.3. Chemical introduction of disulfides in anti-NSE21 antibody using –COOH residues.

Disulfides were covalently attached to the IgG structure through the terminal carboxylic acids and the glutamic acid residues. At acidic pH, carboxylic acids can react with carbodiimide derivates in the presence of N-hydroxysuccinimide and form an active ester. At basic pH, a primary amine can easily attack the electrophile carbon of the ester. Firstly, 0.5 mg of anti-NSE21 antibody (3.6×10^{-9} mol), 7.8 mg of sulfo-NHS (3.6×10^{-5} mol) and 6.9 mg of EDC (3.6×10^{-5} mol) were mixed and allowed to react for 10 min under stirring conditions at room temperature in 100 μ L of 10 mM acetate buffer pH 4.5. Subsequently, this mixture solution was added to 900 μ L of 50 mM HEPES buffer pH 8.5 containing 20 mg of cystamine (9×10^{-5} mol). Nucleophilic substitution took place during 2 hours at room temperature under vigorous stirring conditions. Excess reagents and by-products were removed by filtration (100 kDa MWCO membranes) and the antibodies were dissolved in PBS buffer pH 7.4. The concentration of the modified antibody was determined by using UV-Vis spectrophotometry at 280 nm.

2.3. Biological functionalization of bare gold substrates. Different biological functionalization strategies of bare gold substrates were characterized using a Biacore® 3000 SPR system. The immobilization degree of proteins in a Biacore SPR system is given in refractive units (RU), which corresponds to a shift in resonance angle of approximately 0.1 millidegree [33]. Surface functionalization was exploited using two different approaches: (i) Direct bio-SAM using the chemically modified antibodies containing disulfide groups and (ii) Classic SAM using unmodified antibodies attached to a long-chain of alkanethiols previously self-assembled onto a bare gold substrate (Scheme 1). Additionally, the efficiency of these two approaches was compared with the performance of both a commercial carboxymethylated dextran surface (CM5 chip) and the immobilization of the unmodified antibody directly on gold substrates.

1

2 **2.3.1. Direct bio-SAM approach.** Bare gold substrates were employed for the construction of the
3 bio-SAM structure. Cleaning of these substrates involved rinsing with acetone and incubation for 15
4 min in an UV/O₃ chamber provided with an ozone producing Mercury Grid Lamp to remove all organic
5 contaminants on gold substrates [34]. Covalent immobilization of chemically modified antibodies
6 containing disulfide groups was achieved by injecting 100 µl of the modified antibody solution (100
7 µg/ml in PBS buffer pH 7.4), followed by an injection of 35 µl of a 1 mM PEG solution in order to
8 block remaining free sites on the surface. A continuous flow of HEPES buffered saline (HBS) (10 mM
9 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, 150 mM NaCl, 3.4 mM
10 ethylenediaminetetraacetate, and 0.005% v/v Tween 20 (pH 7.4)) at 5 µl/min was maintained during the
11 immobilization step. To test the non-specific adsorption of NSE on the PEG blocking layer, a control
12 experiment was performed by injecting 100 µl of the non-specific PSA66 antibody (100 µg/ml in PBS
13 buffer pH 7.4) previously modified with disulfides via its primary amines, carboxylic acids and
14 carbohydrates as described above. Subsequently, the surface was backfilled by an injection of 35 µl of a
15 1 mM PEG solution. NSE detection experiments were performed by injecting serial dilutions of NSE
16 (3.1 – 200 ng/ml) prepared in HBS buffer over the antibody-immobilized surface at a flow rate of 20
17 µl/min. After 6 min of association, the sample solution was replaced by a HBS buffer flow for 7 min,
18 allowing the complex to dissociate. Regeneration of the surface was performed by injecting two pulses
19 of 10 mM glycine (pH 2.2) between each analyte injection. The recognition experiments were carried
20 out at 25 °C. Specificity of the recognition layer for NSE was tested by injecting two concentrations of
21 human CEA (1 and 10 µg/ml) as a non-specific marker.

22

23 In addition to the NSE recognition experiments, sandwich assays were also performed to test the
24 specificity of the NSE binding. These assays consisted of a NSE recognition step by injecting both 100
25 ng/ml and 200 ng/ml of NSE for 6 min, followed by a 10 µg/ml solution of unmodified NSE17 for a

further 6 min. Subsequent to association, the sample solution was replaced by a HBS buffer flow for 7 min at 20 µl/min.

2.3.2. Classic SAM approach. Bare gold substrates were also used for SAM deposition. These substrates were cleaned as previously described. Immediately after cleaning, the gold substrates were immersed in a mixture of thiol solutions. Mixed SAM solutions of thiolated molecules were obtained by mixing 5 % of a 1mM 16-mercapto-1-hexadecanoic acid (16-MHA) solution in a matrix of 95 % a 1mM 11-mercapto-1-undecanol (11-MUOH) solution, both in ethanol. Mixed SAMs were prepared in glass recipients cleaned with 2M NaOH for at least 1 h. After 3 h of SAM deposition, the substrates were thoroughly rinsed with ethanol and dried under a stream of nitrogen, producing stable and fully covered SAMs on gold [35]. The mixed SAM used here is considered as optimal for obtaining a high degree of antibody immobilization with maximal elimination of non-specific adsorption due to the high content of thiols with OH end groups [36].

Covalent immobilization of unmodified anti-NSE21 monoclonal antibody was accomplished via coupling to their primary amines. A continuous flow of HBS at 5 µl/min was maintained during the immobilization step. The carboxylic groups of the mixed SAM were activated by injection of 50 µl of a 1:1 mixture of EDC (400 mM in deionized water) and NHS (100 mM in deionized water). Subsequently, 100 µl of the antibody solution (100 µg/ml in 10 mM acetate buffer pH 5.0) was injected, followed by injection of 35 µl of ethanolamine (1.0 M in deionized water, pH 8.5) in order to block remaining NHS ester active groups and by two 10 µl injections of 10 mM glycine (pH 2.2) in order to remove non-specifically bound molecules from the surface. To test the non-specific binding of NSE, a control experiment was performed by injecting 100 µl of unmodified anti-PSA66 solution (100 µg/ml in 10 mM acetate buffer pH 5.0) as control antibody. The experiments were carried out at 25 °C.

2.3.3. Bio-functionalization of CM5. A commercial carboxymethylated dextran surface (CM5 chip) was used to immobilize the unmodified anti-NSE21. Immobilization was achieved via coupling to their primary amines to the previously activated carboxylic groups of the dextran matrix. The immobilization was performed in the same manner as described above for the classic SAM approach.

2.3.4. Direct immobilization on gold substrates of unmodified anti-NSE21. Immobilization of unmodified anti-NSE21 was carried out by injecting 100 μ l of the antibody solution (100 μ g/ml in PBS buffer pH 7.4), followed by an injection of 35 μ l of a 1 mM thiolated PEG solution.

2.4. Electrochemical instrumentation. Electrochemical measurements were performed on a PC controlled PGSTAT12 Autolab potentiostat (EcoChemie, The Netherlands) with an in-built frequency response analyzer FRA2 module. Electrochemical impedance measurements were performed using a standard three-electrode configuration (reference electrode: Ag/AgCl(sat), counter electrode Pt wire) in 1 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl. The electrochemical impedance spectra (EIS) were recorded over the frequency range of 10 kHz – 0.1 Hz at a bias potential of +0.22 V and AC amplitude of 5 mV. The impedance data was represented in the complex domain (Nyquist plot) and the electrochemical parameters were obtained from simulation using Autolab FRA software of the equivalent circuit $R_s(R_{ct}C_{dl})$, where R_s is the solution resistance, R_{ct} the charge transfer resistance and C_{dl} the double layer capacitance (Scheme 2). Sputtered gold working electrodes were fabricated as reported elsewhere [37]. The electrode surface was electrocleaned by applying 20 potential cycles in 0.5 M H_2SO_4 in the range 0-1.6 V at 0.5 V/s.

2.4.1. Electrochemical characterization of SAM formation of anti-NSE21-CHO modified electrodes. For the impedimetric study of SAM formation, clean gold electrodes were modified by immersion in a freshly prepared 1 μ g/ml solution of NSE-CHO in PBS for fixed times followed by rinsing with copious amounts of PBS-Tween. After each modification and washing, Faradaic EIS were

recorded using 1 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl as an electroactive probe; the electrodes were then washed with Milli-Q water, argon dried and immersed again in the NSE-CHO solution.

2.4.2. Electrochemical immunosensor construction and optimization. The incubation time of the specific recognition of NSE was optimized by immersion of a NSE-CHO modified electrode in a 100 ng/mL solution of NSE in PBS at different incubation times in the range 0-60 min and recording the change in R_{ct} of the faradaic response 1 mM $\text{Fe}(\text{CN})_6^{3-/4-}$ in 0.1 M KCl before and after incubation with NSE.

2.4.3. Electrochemical Detection of NSE. A SAM of NSE-CHO was formed by immersion of a clean electrode on a 1 $\mu\text{g}/\text{ml}$ solution of NSE-CHO in PBS for three hours followed by blocking with 1 mM 1-(mercaptoundec-11-yl)-tetra(ethyleneglycol) in PBS for 30 minutes. The electrodes were then exposed to different concentrations of NSE antigen in PBS for 30 min, rinsed with PBS-Tween and further incubated with 10 $\mu\text{g}/\text{ml}$ of anti-NSE17-HRP conjugate in PBS for 10 at room temperature. The differential pulse voltammetry (DPV) response was recorded in triplicate in the potential range 0.2 to -0.4 V versus Ag/AgCl using a modulation amplitude of 25 mV, a step potential of 5 mV, and a scan rate of 50 $\text{mV}\cdot\text{s}^{-1}$) after 5 min of addition of a mixture of hydroquinone (1 mM) and hydrogen peroxide (1 mM) in PBS pH 6.

3. RESULTS AND DISCUSSION

3.1. Immobilization efficiency of modified and unmodified antibodies. Immobilization efficiency of the different functionalization strategies was evaluated by monitoring the coupling level of the antibodies on the gold substrates. For all immobilization strategies, alcohol groups-containing species, PEG or ethanolamine, depending on the surface, were used to backfill the remaining free sites on the surface and to cap (i.e. block) the activated groups respectively. Coupling levels for each

1 immobilization strategy are depicted in Figure 1. Immobilization levels were measured after the addition
2 of the backfiller or capping agent depending on the experiment. Antibodies modified via their
3 carbohydrates exhibited the highest coupling level. Unmodified antibodies coupled on the CM5 chip
4 showed higher immobilization levels, probably due to stronger electrostatic attractive forces between
5 the receptor molecules and these functionalized surfaces that lead to an accumulation of receptors close
6 to the surface as this favors the receptor pre-concentration effect and enhances its subsequent
7 immobilization. For the pre-concentration effect to occur, the pH of the acetate coupling solution was
8 chosen to be 5. At this pH, the carboxylic groups of both the mixed SAM and CM5 are negatively
9 charged ($\text{pK}_{\text{a Surface}} \approx 3.5$ [38]), while the anti-NSE21 is positively charged ($\text{pI} \approx 5.9 - 7.1$ [39]), so that
10 the surface and the ligand carry opposite net charges, which increases the electrostatic attractive forces.
11 In addition, electrostatic interactions involved in pre-concentration are favored by low ionic strength in
12 the coupling buffer, and therefore a 10 mM acetate buffer, pH 5 solution was used. Although this
13 receptor pre-concentration effect also occurred in the mixed SAM functionalized surface, the low
14 amount of carboxylic groups present on that surface (original mixture of thiols only contained a 5 %
15 (v/v) of 16-MHA) limited the immobilization of the IgG, yielding low coupling levels. The highest
16 immobilization levels were observed for unmodified antibodies coupled on a commercial CM5 chip due
17 to the high density of carboxylic groups present in this surface. Antibodies immobilized on both the bare
18 gold substrates and the mixed SAM form an ordered two-dimensional (2D) monolayer, while on a CM5
19 chip the antibodies are immobilized within a three-dimensional (3D) carboxylated dextran matrix.
20 Compared to the 2D chips, the 3D CM5 chip allowed for a slower immobilization rate of unmodified
21 IgGs due to diffusion limitations within the dextran matrix, but with higher amounts of the immobilized
22 antibodies due to the greater number of reactive sites. The lowest coupling levels were exhibited by the
23 unmodified IgG immobilized without any coupling chemistry. The coupling of unmodified antibodies is
24 mainly based on weak interaction forces, basically hydrophobic and electrostatic, and at a minor level
25 on strong interaction forces, due to the covalent link between the thiol group of the cysteine moieties
26 and the gold surface.

Based on the linear regression of the response and the amount of protein coated on the sensor surface, the theoretical surface concentrations of the antibodies were determined assuming that for matrix surfaces, like those of CM5 chips, an SPR signal of 1000 RU corresponds to a 1 ng/mm² of protein [40]. For planar surfaces, however, this assumption needs to be corrected by a factor 3, so that 3000 RU equals 1 ng/mm² [41, 42]. Additionally, each sensor's maximum antigen binding capacity (R_{max}) was also evaluated for each immobilization strategy by using the following relation (1):

$$R_{\max} = (Mw_{\text{Analyte}}/Mw_{\text{Ligand}}) \cdot R_{\text{Ligand}} \cdot V_{\text{Ligand}} \quad (1)$$

where Mw_{Analyte} and Mw_{Ligand} are the molecular weights of the analyte and ligand respectively, R_{Ligand} is the SPR response due to the ligand immobilization, and V_{Ligand} is the valency of the ligand (amount of binding sites). The valency for IgGs is considered to be equal to 2 (Table 1).

3.2. Binding efficiency of the different functionalization strategies. NSE detection. The number and accessibility of functional binding sites is essential for optimal analyte detection. Functional receptors should be well oriented, i.e. with their analyte binding site fully exposed to the solution, and should not sterically hinder each other for analyte interaction. The NSE recognition efficiency of the different immobilization strategies was evaluated by capture assay by injecting serial dilutions of NSE (3.1 – 200 ng/ml) prepared in HBS buffer over the antibody-immobilized surfaces for an association time of 6 min. A zero analyte concentration was also included to obtain measurements for system related bias. SPR signals for NSE recognition levels of gold substrates functionalized with both disulfide-containing and unmodified IgGs are depicted in figures 2 and 3, respectively. The degree of binding was calculated by measuring the response signal at the end of the dissociation phase in three replicate experiments subtracted by the signal from the control surface (non-specific PSA66 MAb). Regarding the disulfide-containing antibodies (Figure 2), only for the antibodies modified via their carbohydrates moiety, the absorbance and the target concentration exhibited a typical sigmoidal response. Very low responses to different NSE concentrations were obtained for the IgGs modified via their amine or carboxylate groups. The carbohydrate chains are attached to the CH₂ domain within the

Fc region of the IgGs. This site-directed conjugation orientates the attached molecule away from the antigen binding regions, preventing blockage of these sites and thus preserving activity [28]. Regarding the MAbs modified via their amine or carboxylate groups, it should be considered that the distribution of both amine and carboxylate groups within the three-dimensional structure of an antibody is nearly uniform throughout the surface topology [28]. For this reason, conjugation procedures that utilize these groups randomly cross-link to many parts of the antibody molecule. This in turn leads to a random orientation of the antibody within the conjugate structure, often blocking the antigen binding sites, resulting in a decrease in antigen binding activity. The level of non-specific adsorption of CEA on these surfaces containing the modified IgGs was lower than 9 RU and therefore insignificant.

Figure 3 illustrates the target response levels for the unmodified anti-NSE21 immobilized on a CM5 chip, a mixed SAM and on a bare gold substrate. NSE was only detected using the CM5 and mixed SAM functionalized surfaces. Target binding levels on these two immobilization strategies are very similar up a concentration 100 ng/ml; at higher antigen concentrations the mixed SAM surface starts to level off, suggesting that most of the antibodies have been associated with the target. On the other hand, the response on the CM5 keeps increasing linearly. This indicates the capacity of the CM5 chip to accommodate more antigen molecules, as was expected taking into account the high immobilization level obtained for this surface. The response of the Au surface containing unmodified IgG randomly immobilized showed no dependency on the target concentration, indicating that this is not a recommendable immobilization strategy. The level of non-specific adsorption of CEA on these surfaces originally prepared with unmodified IgGs was lower than 5 RU for the both the CM5 and the surface containing unmodified IgG, whereas for the mixed SAM the levels were lower than 11 RU. The performance of the NSE detection of the carbohydrates modification, CM5 and mixed SAM functionalization strategies were also evaluated by measuring assay critical parameters such as the limit of detection (LOD), the sensitivity, EC50 (concentration of target needed to obtain a 50 % of the maximum signal) and dynamic range. The sensitivity was determined by the slope of the calibration

curve as the absorbance units (RU) per concentration in ng/ml (RU/(ng/ml)). The sensitivity, EC50 and dynamic range was not determined for the CM5 chip because the top plateau was not defined by the experimental data and thus, these parameters would lack on accuracy. The LOD was taken as the mean concentration value obtained for three blanks plus three times the standard deviation of the blank. The goodness of fit of each calibration curve was determined by the linear regression coefficient (R^2). The low LOD (6.8 ± 2.1 ng/ml) obtained for the -CHO modification compared well with the other two strategies, classic SAM (1.8 ± 0.3 ng/ml) and commercial layer (3.3 ± 1.3 ng/ml), and also meet the requirements of the clinical assays.

SPR was also used to determine the strength of the antibody-antigen binding by calculating the dissociation constant (K_D) using the BIAevaluation® software from Biacore. Experimental kinetic data was fitted to the 1:1 binding model and the goodness of fit was assessed by calculating the chi-square (χ^2). The K_D for the -CHO, CM5 and mixed SAM strategies was of $4.4 \cdot 10^{-11}$ M ($\chi^2=2.3$), $4.8 \cdot 10^{-12}$ M ($\chi^2=2.2$) and $1.9 \cdot 10^{-9}$ M ($\chi^2=5.1$) respectively. Besides the low chi-square values obtained that suggested the good fitting to the ideal 1:1 binding model, differences in the K_D of 1 and 2 orders of magnitude were exhibited among the different strategies. These results clearly indicate that binding kinetics were affected by the different immobilization approaches, mass-transfer limitations and surface heterogeneity due to the covalent immobilization procedures. The 3D surface of the CM5 chip is prone to mass transport limitations and therefore the kinetic parameter is underestimated, probably due to (i) the slower transport step of the antigens to the immobilized anti-NSE21 because of the three-dimensional dextran structure and (ii) the high binding capacity of ligands of this chip. Immobilization procedures on both the CM5 and mixed SAM are achieved via coupling primary amines of the antibodies, and this can modify the antigen binding sites, resulting in a decrease in affinity. On the other hand, the -CHO strategy offers a 2D surface, less subjected to mass transfer limitations, where the antibody binding sites have been unaffected by chemical reactions and thus, this surface offers a higher reliability on binding kinetic calculations.

NSE recognition levels did not reach the theoretical maximum antigen binding capacity (R_{max}) for any of the immobilization techniques. This revealed that all immobilized ligand molecules were not fully accessible or functional, or that the NSE concentration assayed was not high enough to interact with all ligand molecules. The stoichiometry of the binding of the NSE to antibody was calculated using the molecular mass values for the anti-NSE MAb (150 kDa) and NSE (78 kDa) and their immobilization degree and response level. To this end, experimental stoichiometries of anti-NSE21 were 0.11, 0.20 and 0.03 for carbohydrate modification, mixed SAM and CM5 chip, respectively.

For the recognition experiments, the flow rate was increased to 20 μ l/min to minimize rebinding and to reduce mass transport limitations, allowing quick diffusion of the analyte from the bulk solution to the surface. Sensor chips were regenerated by selective dissociation of the analyte from the covalently immobilized ligand. Conditions were chosen to achieve complete dissociation of the analyte without affecting the binding characteristics of the ligand. Regeneration of the surfaces was achieved by injecting two pulses of 10 mM glycine (pH 2.2) between each analyte injection [43]. Regeneration efficiencies were higher than the 90 % for all experiments. Sandwich assays were performed to test the specificity of the NSE binding. These assays consisted in a NSE recognition step by injecting both 100 ng/ml and 200 ng/ml of NSE, followed by the injection of 10 μ g/ml of unmodified NSE17 for each detection step during 6 min. A zero analyte concentration was also tested to obtain measurements for system related bias. SPR signals for the anti-NSE17 binding are displayed in Figure 4. Binding responses obtained for the addition of the second primary antibody demonstrated the higher presence of analyte molecules bound on the surface prepared using the –CHO modification, mixed SAM and CM5 chip, confirming the results obtained for direct capture of NSE. Both carbohydrate modification and mixed SAM displayed the highest responses for 100 ng/ml of NSE, while for an antigen concentration of 200 ng/ml the best surface was the –CHO modified. These results revealed that this surface performs well also for sandwich assays, obtaining high binding efficiencies of the second antibody. Taking into

account the high binding levels obtained for the direct capture of NSE in a CM5 chip, low binding levels of NSE17 were observed in this surface, which can be attributed to a too dense surface to accommodate the subsequent binding of the second antibody.

3.3. Electrochemical detection of NSE. Since modification through the CHO groups was found to give the highest sensitivity for NSE detection, the immobilization of CHO-modified NSE on gold electrodes was studied with the aim to construct an electrochemical immunosensor. The duration of SAM formation of NSE21-CHO on gold electrodes was first optimized by monitoring the impedance changes at different times of exposure of a gold electrode to a solution of NSE-CHO. Charge transfer resistance (R_{ct}) values, which are indicative of the opposition of the interface to the passage of electrical current from a electroactive probe present in solution, were obtained from simulation of the equivalent circuit shown in Scheme 2, where C_{dl} is the double layer capacitance and R_s is the solution resistance of the circuit. The R_{ct} values increased steadily with time (Figure 5), reaching saturation after 3 hours. This impedance increase does not account for multilayer formation since each point represents the constant impedance value obtained after repeated washings to remove physically adsorbed molecules. The maximum deposition time for NSE-CHO is considerably lower than that observed for the formation of SAMs of alkanethiols, which usually require an overnight exposure of the electrodes to the modifying solution in order to form a compact monolayer, highlighting the advantage of the direct attachment of modified antibodies on surfaces in immunosensor construction. The same procedure was employed in order to optimize the time required for the specific recognition of NSE (Figure 5, inset). The impedance response increased up to saturation after 30 minutes of interaction and this time was thus used in the detection experiments. Electrochemical determination of NSE was carried out using anti-NSE17-HRP conjugate as reporter antibody with differential pulse voltammetric detection. Figure 6a shows the DPV responses of the immunosensor with increasing concentrations of NSE in the potential range 0.1 to -0.4 V. As can be seen from Figure 6b the peak height increased with NSE concentration and showed a

linear relationship with the logarithm of the NSE concentration. The limit of detection (4.6 ng/ml) was calculated with a linear relationship between 0-25 ng/ml.

4. CONCLUSIONS

Different immobilization strategies have been developed for antibody immobilization, based on the chemical modification of their functional groups with disulfide “anchors” able to spontaneously chemisorb onto gold, with no need for surface pre-functionalization. Among the three chemical routes investigated, the site-directed conjugation of antibodies via their carbohydrate chains exhibited a good analyte response in both capture and sandwich assays using SPR. Surfaces prepared with this approach also compared well with both the classic two step SAM scenario and the 3D-CM5 chip in terms of analyte response, LOD and sensitivity, suggesting that the immobilization of carbohydrate-modified antibodies driven by chemisorption of their disulfide moieties represents a successful approach for creating biologically active dense monolayers on gold devices due to an optimisation of orientation of the capture (primary) antibody bioelement. The chemical specificity of the reaction toward carbohydrate residues opens up an attractive option for oriented antibody immobilization since their sugar moieties are specifically located on the constant region of the immunoglobulins. Finally, in terms of simplicity, required time, and minimal use of reagents, the use of modified carbohydrate residues presents an extremely effective approach for antibody immobilization with application in electrochemical, optical, and gravimetric immunosensors.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Scheme 1. Functionalization strategies of bare gold substrates via direct bio-SAM using disulfide-containing antibody chemically modified via their primary amines (A), carbohydrates (B) and carboxylic acids (C) and via classic SAM (D) using unmodified antibodies attached on a long-chain of alkanethiols previously self-assembled onto a bare gold substrate.

Scheme 2. Equivalent circuit used to model the impedance data (R_s : solution resistance, R_{ct} : resistance to charge transfer, C_{dl} : double layer capacitance).

Figure 1. SPR signals for immobilization of unmodified NSE21 antibody on bare gold substrate, disulfide-containing antibody chemically modified via their primary amines (-NH₂), carbohydrates (-CHO) and carboxylic acids (-COOH), unmodified antibody via mixed SAM and unmodified antibody on a commercial layer (CM5 chip).

Figure 2. SPR signals for NSE binding levels (Capture Assay) of gold substrates functionalized with disulfide-containing anti-NSE21 antibodies modified via their primary amines, carbohydrates and carboxylic acids and immobilized on a bare gold substrate. Inset: NSE binding levels obtained with the anti-NSE21 modified via their primary amines and carboxylic acids. Error bars represent $n = 3$.

Figure 3. SPR signals for NSE binding levels (Capture Assay) of gold substrates functionalized with unmodified anti-NSE21. The unmodified antibodies were immobilized on a CM5 chip, a mixed SAM and on a bare gold substrate. Error bars represent $n = 3$.

Figure 4. SPR signals for anti-NSE17 binding levels (Sandwich Assay) for a gold substrate functionalized with anti-NSE21. Unmodified antibodies were immobilized on a CM5 chip, a mixed SAM and on a bare gold substrate. Disulfide-containing antibodies were modified via their primary amines, carbohydrates and carboxylic acids immobilized on a bare gold substrate. Error bars represent $n = 3$.

Figure 5. Complex impedance plot (in 1 mM $K_3Fe(CN)_6$ solution in PBS pH 7.4) for the formation of a SAM of NSE21-CHO at gold electrodes at different exposure times. Inset: Impedance variations for the specific interaction of NSE with NSE21-CHO modified surface.

Table 1. SPR response due to the ligand immobilization (R_{Ligand}), surface concentrations of the immobilized species and sensor's maximum antigen binding capacity (R_{max}) for each immobilization strategy

Immobilization Strategy	R_{Ligand} (RU)	Surface conc. (ng/mm ²)	R_{max} (RU)
Unmodified (Bare Au)	1100	0.4	1144
-NH ₂	2994	1.0	3114
-CHO	4492	1.5	4672
-COOH	3514	1.2	3655
Mixed SAM	2380	0.8	2475
CM5 chip	31443	31.4	32700

1 **Table 2.** Assay performance parameters for the detection of NSE using (i) disulfide-containing NSE21
2 modified via their carbohydrates (-CHO) immobilized on a bare gold substrate and (ii) unmodified
3 NSE21 immobilized on a mixed SAM.

Immobilization Strategy	R ²	EC ₅₀ (ng/ml)	Sensitivity (RU/(ng/ml))	Dynamic range (ng/ml)	LOD (ng/ml)
-CHO	0.999	171 ± 4	1.32 ± 0.20	6.8 – 603	6.8 ± 2.1
Mixed SAM	0.999	59 ± 2	1.28 ± 0.05	1.8 – 1170	1.8 ± 0.3