Acta Biomaterialia 9 (2013) 5838–5844

Contents lists available at SciVerse ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Electrochemical functionalization of gold and silicon surfaces by a maleimide group as a biosensor for immunological application

Xin Zhang [a], Aleksei Tretjakov [b], Marc Hovestaedt [c,d], Guoguang Sun [e], Vitali Syritski [b], Jekaterina Reut [b], Rudolf Volkmer [c], Karsten Hinrichs [e], Joerg Rappich [a,*]

[a] Helmholtz-Zentrum Berlin für Materialien und Energie GmbH, Institut für Silizium-Photovoltaik, Kekuléstr. 5, 12489 Berlin, Germany
[b] Department of Materials Science, Tallinn University of Technology, Ehitajate tee 5, Tallinn 19086, Estonia
[c] Charité Universitätsmedizin Berlin, Institut für Medizinische Immunologie, Abteilung Molekulare Bibliotheken, Hessische Str. 3-4, 10115 Berlin, Germany
[d] Charité Universitätsmedizin Berlin, Institute of Biochemistry 18, Reinickendorfer Str. 61, Haus 10, D-13347 Berlin, Germany
[e] Leibniz-Institut für Analytische Wissenschaften – ISAS – e.V., Department Berlin, Albert-Einstein-Str. 9, 12489 Berlin, Germany

ARTICLE INFO

Article history:
Received 2 May 2012
Received in revised form 11 September 2012
Accepted 18 October 2012
Available online 29 October 2012

Keywords:
Biosensor
Au and Si surfaces
Maleimidophenyl
Cys-peptide
Antibody

ABSTRACT

In the present study we investigated the preparation of biofunctionalized surfaces using the direct electrochemical grafting of maleimidophenyl molecules with subsequent covalent immobilization of specific peptide to detect target antibody, thereby extending the application of the biosensing systems towards immunodiagnostics. *Para*-maleimidophenyl (*p*-MP) functional groups were electrochemically grafted on gold and silicon surfaces from solutions of the corresponding diazonium salt. A specially synthesized peptide modified with cysteine (Cys-peptide) was then immobilized on the *p*-MP grafted substrates by cross-linking between the maleimide groups and the sulfhydryl group of the cysteine residues. Accordingly, the Cys-peptide worked as an antigen that was able to bind specifically the target antibody (anti-GST antibody), while it was non-sensitive to a negative contrast antibody (i.e. anti-Flag β). The immobilization of both specific and non-specific antibodies on the Cys-peptide-modified surfaces was monitored by infrared spectroscopic ellipsometry, a quartz crystal microbalance integrated in flow injection analysis system and potentiometric response. The results obtained clearly demonstrated that the direct modification of a surface with maleimidophenyl provides a very simple and reliable way of preparing biofunctionalized surfaces suitable for the construction of immunological biosensors.

1. Introduction

In the materials and life sciences there is a great demand for biofunctionalized surfaces, which offer a variety of innovative biotechnological applications in the field of bioelectronics, biosensors and drug delivery. A great number of surface modification techniques have been developed, providing the possibility of specifically attaching biomolecules. The functionalization of metal and glass surfaces with self-assembled monolayers (SAMs) [1,2] is used extensively for the physical and chemical attachment of proteins [3]. SAMs of alkenethiols are widely employed for the modification of gold metal surfaces [4]. One drawback of this method is the instability of the gold–thiolate bond at reductive potentials, which causes the desorption of the layer [5]. Aryl diazonium salts are a possible alternative to SAMs that has considerable promise due to the high stability of the layers produced and the versatility of the chemistry. Nowadays, electrochemical reduction of organic diazonium salt is a well-known technique to graft organic molecules [6–8], especially aromatic rings, onto metal [9–11], carbon [9,12] and semiconductor surfaces [13–15]. The organic layer formed is covalently attached to a substrate, providing a stable functionalized surface suitable for application in different research areas, such as microelectronics [16] and sensors [10,17]. Different functional groups can be introduced to the surface depending on the substituent on the *para*-position of the aryldiazonium salt. For this, electrochemical grafting of 4-carboxyphenyldiazonium [10], 4-nitrophenyldiazonium [18] and 4-aminophenyldiazonium salts [19] have been used. However, for the immobilization of biomolecules on carboxy-, nitro- and amino-derivatized surfaces, further functionalization is needed, including: the activation of carboxyl function through a carbodiimide route to allow subsequent covalent coupling with an amino group of the biomolecule [20,21]; and chemical coupling reactions with a biomolecule-reactive group, such as *N*-hydroxysuccinimide (NHS) or maleimide. Considering that biomolecular coupling through the amino groups of lysine residues in the case of NHS-modified surfaces leads to additional heterogeneities in the population of molecules, the site-specific immobilization of a biomolecule through thiol groups (i.e. cysteines) on maleimide functionalities is the preferred

* Corresponding author. Tel.: +49 30806241386.
E-mail address: rappich@helmholtz-berlin.de (J. Rappich).

1742-7061/$ - see front matter © 2012 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.actbio.2012.10.022

method [22]. Surface modification with maleimide is usually a multistep process that includes the functionalization of the surface with amino groups followed by the attachment of some heterobifunctional crosslinker containing amine-reactive group on the one end and thiol-reactive maleimide on the other [23,24]. Recently, Harper et al. [9] reported the direct grafting of maleimide on Au and glassy carbon electrodes from *para*-maleimidophenyl diazonium tetrafluoroborate (*p*-MPDT). They showed the possibility of immobilizing biomolecules such as ferrocene and the redox-active protein cytochrome *c* on the maleimidophenyl-modified surface for the development of bioelectronic devices, including biofuel cells, biosensors, and DNA and protein microarrays.

In the present study we investigated the possibility of preparing biofunctionalized surfaces using the direct electrochemical grafting of maleimidophenyl molecules with subsequent covalent immobilization of specific peptide to detect a target antibody, thereby extending the application of the biosensing systems towards immunodiagnostics. Fig. 1 presents the method used to construct an immunological biosensor.

The first step is the modification of the electrode surfaces with *p*-MP by the electrochemical reduction of *p*-MPDT (step 1 in Fig. 1). *p*-MP is a very favourable reactive group in the cross-linking process and reacts specifically with sulfhydryl (–HS) groups in aqueous solution at a pH value between 6.5 and 7.5 to form a stable and non-reversible thioether linkage [25]. An HS group could be introduced into a target peptide (antigen) by chemical modification at its N-terminus with cysteine, so that the obtained Cys-peptide could be covalently immobilized on a maleimide-modified surface by cross-linking (as sketched for *p*-MP at step 2 in Fig. 1). We prepared biofunctionalized electrodes by the covalent immobilization of Cys-peptide on *p*-MP modified Au and/or Si substrates and investigated their specificity to interact with the target antibody (anti-GST antibody) (step 3 in Fig. 1), identifying both the specific infrared vibrational signature of amide bonds by infrared spectroscopic ellipsometry (IRSE) [26] and the mass change upon the antibody binding by the quartz crystal microbalance integrated in flow injection analysis (QCM-FIA) technique [27]. IRSE was used to characterize all the steps of the electrode modification process, including the grafting of the maleimide group, Cys-peptide and target antibodies immobilization by their specific vibrational signature. Additionally, the interaction between Cys-peptide and the antibody was monitored by zero current potential (U_Z) measurements between the biofunctionalized electrode (as a working electrode) and the reference electrode during the anti-GST antibody binding. Thus, the biosensing process was displayed in real time via a simple electrochemical response.

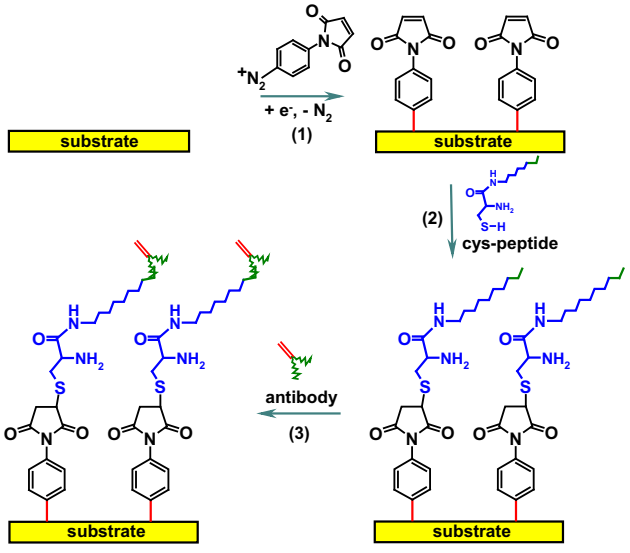

Fig. 1. Scheme of an antibody immobilization onto *p*-MP modified substrate: (1) electrochemical modification by *p*-MP functional groups; (2) coupling of Cys-peptide; and (3) target antibody immobilization.

2. Experimental section

2.1. Substrates

A 200 nm polycrystalline Au film deposited on the glass with a 3 nm Ti coadhesive layer and p-Si(1 1 1) substrates were used as electrodes for the electrochemical grafting process. The Au surfaces were cleaned using fresh piranha solution (97% H_2SO_4:30% H_2O_2 = 1:1) for 20 s, then rinsed copiously with deionized water. p-Si(1 1 1) wafers (0.5–2.5 Ω cm), covered by a 100 nm thick oxide layer and polished on one side, were purchased from the Leibniz Institute for Crystal Growth (IKZ). Before any modification, the SiO_2/Si samples were treated ultrasonically in 2-propanol for 10 min before being washed with deionized water. Then hydrogenated p-Si(1 1 1) surfaces were prepared in three steps. First, the Si samples were immersed in 5% HF for 10 min to remove the oxide layer. Second, the samples were reoxidized for 10 min in freshly prepared piranha solution. Next, the Si samples were rinsed by water and immersed in 40% NH_4F for 6–8 min to obtain a flat and hydrogen-terminated Si surface [15]. Finally, the samples were washed with deionized water and dried by nitrogen. The HF, NH_4F, H_2SO_4 and H_2O_2 solutions used were supplied by Aldrich® with grade Selectipure® (VLSI grade). The water used was purified by a PURELAB® Ultra water system (⩽18 MΩ cm at 25 °C).

2.2. Chemicals and materials

The *p*-MPDT used in this work was synthesized from *N*-(4-aminophenyl)maleimide (TCI Europa) and identified by nuclear magnetic resonance spectroscopy in CD_3-CN (8.55 dblt, 2H; 8.12 dblt, 2H; 7.07 singlet, 2H). The reagents and solvents employed were used as received, and were as follows: anhydrous acetonitrile (ACN, 99.8%, Sigma–Aldrich) and electrochemical grade tetrabutylammonium tetrafluoroborate (Bu_4NBF_4, ⩾99.0%, Fluka). Phosphate-buffered saline powder (PBS, pH 7.4), and Tris-buffered saline powder (TBS, pH 8.0) were bought from Sigma and dissolved in deionized water to obtained 0.05 M buffer solution.

The specific peptide with the amino acid sequence LAKDLIVPRR was specially synthesized for immobilization of the target antibody – a murine anti-GST IgG (Sigma–Aldrich). The selectivity and sensitivity of the synthesized peptides was verified by complete substitution analysis and affinity determinations with surface plasmon resonance spectroscopy. These binding of these peptides to other antibodies was also tested, including anti-p24 (HIV) CB 4-1 IgG, anti-TGFα-IgG, anti-Flag β antibody and anti-mouse IgG. None of the antibodies showed any interaction with the peptide used. Thus, the LAKDLIVPRR peptide and anti-GST IgG interaction is very specific, and is very well suited as a model reaction for the biosensor study. Anti-Flag ß antibody was taken as the negative contrast. A variant of peptide LAKDLIVPRR was produced then with an N-terminal cysteine (Cys)-linker βAla in milligram amounts by solid-phase synthesis.

2.3. Electrochemical grafting

The *p*-MP layers were electrochemically grafted onto either Au or Si surfaces via the appropriate procedures described below. For the Au surface grafting process, an electrochemical cell accommodating three electrodes, i.e. a gold-coated glass slide as the working

electrode, a gold wire counter electrode and an Ag/AgCl (3 M KCl) reference electrode, was used. All the electrodes were connected to a potentiostat/galvanostat (Ivium Technologies, CompactStat™) and the deposition of the *p*-MP layers was carried out from ACN solution containing 2 mM p-MPDT and 0.1 M Bu_4NBF_4 by cyclic voltammetry, ramping the potential between +0.75 V and −0.6 V at a rate of 50 mV s^{-1} several times. After the deposition, the *p*-MP-functionalized Au surface was cleaned ultrasonically in ACN for 2 min and dried in N_2 stream.

For electrochemical grafting of the *p*-MP layer onto the Si surface, chronoamperometry in combination with the injection technique was used [15]. The hydrogenated p-Si(1 1 1) surfaces were immersed in 4 units of volume of the acetonitrile solution with 0.1 M tetrabutylammonium salt. The solution was stirred using a magnet bar and a potential of −2 V was applied to prevent the Si surface from oxidizing and to ensure the reduction of the diazonium ions. After a short time period (about 20 s), 1 unit of volume of 10 mM *p*-MPDT solution was injected into the electrochemical cell. The total concentration of the diazonium ions in the cell was quickly changed to 2 mM under stirring.

2.4. Biofunctionalization of the p-MP modified surfaces

The specific synthesized Cys-peptide was immobilized on the maleimide-modified surface at room temperature by a cross-linking reaction performed in PBS solution containing 0.5 mg ml^{-1} of Cys-peptide for several hours. The Cys-peptide-modified surfaces were then tested for biological recognition ability by immersing them in the TBS solution containing 0.08–50 μg ml^{-1} of either specific (anti-GST) or non-specific (anti-Flag ß) antibodies. The biosensing process was monitored by IRSE, QCM-FIA and potentiometric response, as described below.

2.5. IRSE measurements

The IR ellipsometric measurements were performed with a photometric ellipsometer attached to a Bruker 55 or Vertex 70 Fourier transform spectrometer as described in detail elsewhere [26,28]. The Kramers–Kronig-related ellipsometric parameters $\tan\psi$ and Δ, which are defined through $\tan\psi e^{i\Delta} = r_p/r_s$, where r_p and r_s are the complex reflection coefficients polarized in parallel and perpendicularly, respectively, with respect to the plane of incidence. The spectra were obtained at an angle of incidence of 65° with a resolution of 4 cm^{-1} using a mercury cadmium telluride detector (KV104-1, Kolmar Technologies, Newburyport, MA, USA). All samples were purged for 0.5 h in a dry air environment before starting the measurements to eliminate the influence of H_2O and CO_2. IRSE was established recently for the characterization of electrochemically prepared organic films on Au and Si [29,30].

2.6. Electrochemical quartz crystal microbalance (EQCM)

The EQCM measurements were performed using the QCM100 system (Stanford Research Systems, Inc., Sunnyvale, CA, USA) connected to a potentiostat/galvanostat (Reference 600™, Gamry Instruments, Inc, Warminster, PA, USA) and a counter (PM6680B, Fluke Corporation, Everett, WA, USA), as described in Ref. [27]. A custom-made 8 ml Teflon electrochemical cell accommodated three electrodes, where the liquid face of a one inch diameter, polished, gold-coated 5 MHz crystal (Maxtek, Inc.) served as the working electrode, a rectangular shaped platinum plate (4 × 1.5 cm^2) was used as the counter electrode and Ag/AgCl (3 M KCl) was used as the reference electrode, respectively.

2.7. QCM-FIA measurements

The antibody binding to the Cys-peptide functionalized Au surface was studied in a QCM-FIA system comprising two programmable precision syringe pumps (Cavro® XLP 6000, Tecan Nordic AB, Mölndal, Sweden), a motorized six-way port injection valve (C22-3186EH, VICI® Valco Instruments Company Inc., USA) controlled by a microelectric actuator, and a small volume (150 μl) axial flow cell attached to the QCM sensor holder (Stanford Research Systems, Inc.) [27]. All elements of the system were connected to a PC and controlled by software written in Labview.

For the QCM-FIA measurements, the Cys-peptide functionalized QCM sensor was placed into the flow cell and the working buffer (TBS, pH 8.0) was pumped at 62.5 μl min^{-1} by the syringe pump until a constant baseline of the QCM sensor resonant frequency was reached. Subsequently, various concentrations of either anti-GST IgG antibody (specific) or anti-Flag ß antibody (non-specific) (8×10^{-5}–5×10^{-2} mg ml^{-1}) prepared in the same buffer were injected into the flow stream via an injection loop (250 μl). The amount of the antibodies adsorbed was calculated from the recorded resonant frequency change using the Sauerbrey equation.

2.8. Immunoelectrode potentiometric response measurement

The change in the potential difference between the immunoelectrode and a reference electrode, U_Z, was determined during galvanostatic control so that no net current flow (I = 0) was observed, thereby avoiding any electrochemical reduction/oxidation of the solvent or surface molecules. Any change in potential should then be due to the change in the surface potential caused by the adsorbed and/or bonded surfaces species (i.e. antibodies). Thus, the behaviour of U_Z should follow the trend observed for the QCM-FIA measurement.

3. Results and discussion

3.1. Electrochemical grafting of the p-MP layer

Fig. 2a shows the cyclic voltammograms of an Au surface in contact with the electrolyte solution containing 2 mM *p*-MPDT and 0.1 M Bu_4NBF_4 in ACN. The current peak, which occurred at about 200 mV during the first potential scan, is attributed to the reduction of the diazonium ions. The diazonium group of the *p*-MPDT is reduced by an electron transfer from the Au electrode followed by the separation of an N_2 molecule from the aryl ring (reaction 1). The aryl radical thus originated reacts with the Au surface to form a stable covalent bond [19], and a *p*-MP layer is formed.

$$p - \mathrm{MP} - \mathrm{N_2^+} + \mathrm{e^-} \rightarrow p - \mathrm{MP}\bullet + \mathrm{N_2} \uparrow \quad (1)$$

In the second potential scan, the current response is strongly reduced, indicating that the surface is already passivated by *p*-MP groups. Therefore, additional scans show a further weak reduction in current since the *p*-MP layer continues to grow and consequently blocks the electron transfer at the solution/electrode interface. Also, the mass response, recorded by the EQCM, reflects relevant behaviour coincident with the effect of the growing MP layers. The mass increases significantly in the first scan, by about 0.28 μg cm^{-2}; thereafter, the mass increase is reduced by every additional potential scan, from 0.06 down to about 0.03 μg cm^{-2}, respectively (Fig. 2a, top). Thus, the first scan generates a *p*-MP layer with the thickness of about one monolayer, assuming the surface area of maleimidobenzene to be 10.9 $Å^2$ and close-packed *p*-MP layer growth. After five potential scans, the *p*-MP layer reaches a nominal thickness of about two monolayers.

The cyclic voltammetry method is not applicable for deposition of *p*-MP layer on Si surfaces, due to the oxidation of the hydrogenated Si(1 1 1) surface at potentials that are more anodic than the reduction of the diazonium ion. To avoid the oxidation of Si–H surfaces, another method – the injection technique (chronoamperometry plus the addition of the diazonium salt at a certain time) – was used. The chronoamperometric curves for the electrochemical grafting of *p*-MP molecules onto the hydrogenated p-Si(1 1 1) surfaces are shown in Fig. 2b. The cathodic current shows a rapid increase when the diazonium compound is added to the solution, followed by a strong decrease in current flow up to about 60 s and a levelling out of the current at longer times. This sharp current peak shows that the first monolayer of grafted *p*-MP molecules is completed within 60 s, as confirmed by quantitative calculation of the transferred electrons according to reaction (1). The following current decrease after that turning point (t = 60 s) is much slower because of the steric hindrance for the further reaction of MP radicals by the organic layer. The thickness of the *p*-MP layer on the Si surface is about two monolayers after 250 s deposition, as calculated from the charge consumed during the deposition considering a two-electron transfer reaction [14]. This very thin layer is one reason for the steric hindrance of *p*-MP in the *ortho* position [31]. Therefore, the typical polymer formation via phenyl radical binding to this *ortho* position [7,8,32] is strongly inhibited for maleimidobenzene due to the size of the maleimido group. This layer thickness is in general agreement with the one found for other thin layers, as obtained for binding of poly(n-butyl methacrylate) via a $-C_6H_4-CH(CH_3)-Br$ aryl layer [33], 3,5-bis-*tert*-butyl benzene [31] and nitrobiphenyl [34].

The steric effect of the *p*-MP group is a crucial point for the whole deposition process, leading to its low efficiency (~20%), as obtained from the in situ EQCM experiment correlating the mass and charge responses.

3.2. IR spectroscopy of the p-MP layer

The presence of the *p*-MP layer on the Au and Si surfaces was confirmed by its characteristic molecular vibrational signature as measured by the IRSE technique (Fig. 3). The curves represent the tanΨ spectra of the maleimidophenyl-modified Au and Si surfaces, respectively. The strong peak at 1728 cm^{-1} and the weak peak at 1785 cm^{-1} are assigned to the symmetric and antisymmetric stretching vibrations of two C=O groups from the maleimide ring, respectively [35,36]. The peaks at 1418 and 1383 cm^{-1} are assigned to the symmetric vibration of C–N–C from maleimide and the vibration of the aryl C–N bond, respectively [35,36]. The peak

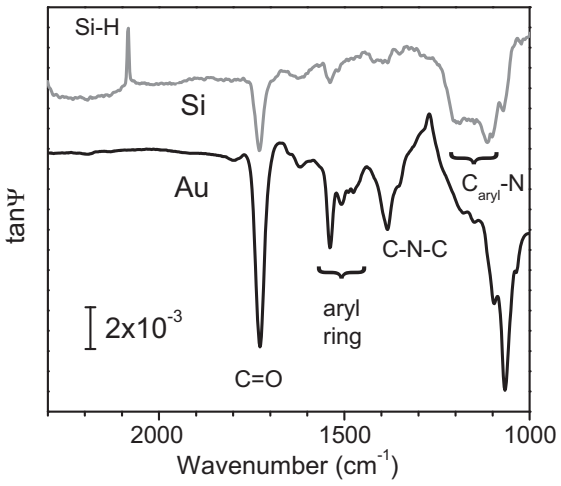

Fig. 3. IRSE spectra of *p*-MP covered Au and Si surfaces. The loss of Si–H (at 2083 cm^{-1}) is clearly seen after the grafting of *p*-MP by the pronounced C=O and N–H bond vibration structure.

at 2083 cm^{-1} for the Si sample is a result of the loss of Si–H bonds by the grafting of the maleimidophenyl group.

3.3. IR spectroscopy for the Cys-peptide and antibody-modified p-MP layer

Biofunctionalization of *p*-MP modified surfaces by the covalent attachment of the specially synthesized Cys-peptide and the subsequent affinity binding of the specific (Fig. 4a) or non-specific (Fig. 4b) antibody were also investigated by the IRSE technique. To highlight the changes during every recognition process, the IRSE spectra after modification by Cys-peptide were normalized to the spectra of the surfaces modified with *p*-MP. After coupling with antibodies, the IRSE spectra were normalized to the spectra of the surfaces modified with Cys-peptide. Because all of the peptide and antibodies are identified by the amide I and II bands [37], only the amide bands and C=O vibration are discussed to show the modification process.

The curves 1 in Fig. 4a and b show the *p*-MP modified Si surfaces as identified by C=O vibration at 1728 cm^{-1} and other small bands (discussed above). The successful modification with the Cys-peptide was confirmed by the strong amide I and II peaks at 1676 and 1538 cm^{-1} in curves 2, respectively. The peak upwards at about

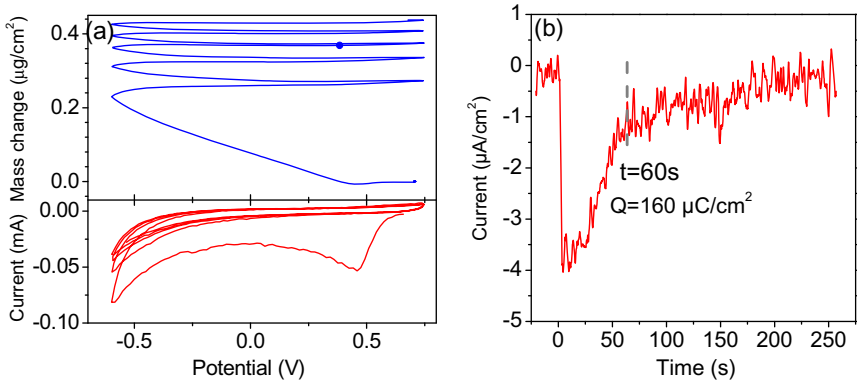

Fig. 2. (a) Cyclic voltammogram (bottom) and respective mass response (top) measured on Au-coated QCM in 2 mM *p*-MPDT with 0.1 M Bu4NBF4 in ACN at 50 mV s^{-1}; (b) chronoamperometry during the electrochemical grafting of *p*-MP molecules on hydrogenated p-Si(1 1 1) surface from 2 mM *p*-MPDT in ACN solution. The diazonium salt was injected at time position 0.

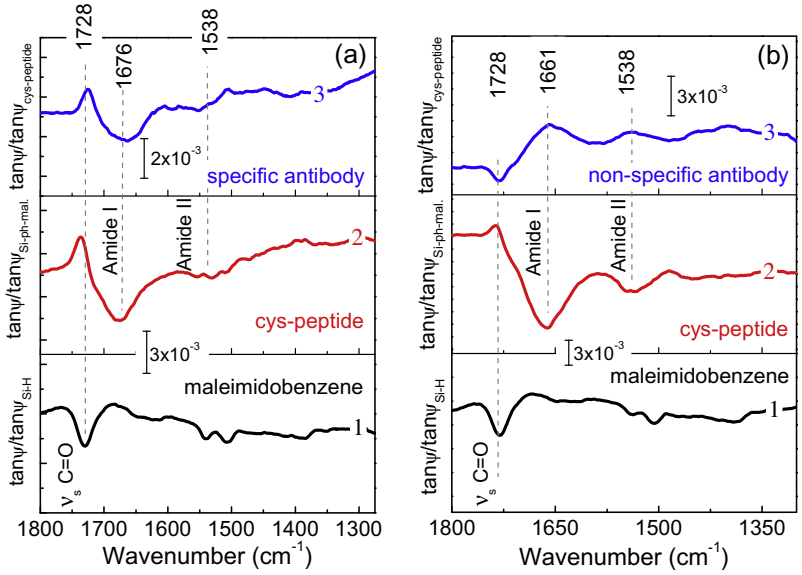

Fig. 4. TanΨ spectra of Si surfaces terminated by *p*-MP normalized to bare surface (black line, 1), to a Cys-peptide-modified *p*-MP layer normalized to the *p*-MP terminated surface (red line, 2), and (blue line, 3) after coupling with specific antibody (left, a) and with non-specific antibody (right, b) normalized to the Cys-peptide-modified surface.

1728 cm^{-1} reflects the decoupling of the C=O vibration in the maleimide group by losing the C=C double bond through Cys-peptide binding. The amide peaks in curve 3 of Fig. 4a show the successful binding of specific antibody onto the surface since there is still a strong increase in the amide I and II peaks after the deduction of the peptide signal. The small peaks at 1661 and 1538 cm^{-1} in curve 3 of Fig. 4b indicate that non-specific antibody was not adsorbed on the Cys-peptide surface and a small amount of physically adsorbed peptide was removed. This difference shows the selectivity of the biosensing process. This measurement could also be considered as an ex situ biosensing technique using Si- and Au-based biosensors.

3.4. Application for biological sensors

Because the Cys-peptide could work as a binding site for an antigen, the above-mentioned Cys-peptide-modified surfaces were investigated for their biosensing capabilities with respect to their selective interaction (step 3 in Fig. 1) with the target antibody (anti-GST). We used a QCM-FIA technique to demonstrate the potential analytical efficiency of such Cys-peptide-modified surfaces for the development of biochemical sensors feasible for label-free detection in antigen antibody immunoassays.

Fig. 5a shows the massogram obtained with the Cys-peptide-modified QCM sensors upon consecutive additions of antibody solutions with concentration increasing from 0.08 to 50 μg ml^{-1}. In particular, curves 1 and 2 represent cases for solutions containing the specific (anti-GST) and non-specific (anti-flag β) antibodies, respectively. As expected, the Cys-peptide-modified surface clearly demonstrates its significantly higher affinity for anti-GST than for anti-Flag β. Remarkably, the QCM sensor reveals an increase binding activity at anti-GST concentrations exceeding 10 μg ml^{-1}, with the greatest mass change occurring at 50 μg ml^{-1}. However, in anti-Flag β solutions the sensor reaches the binding saturation value at 10 μg ml^{-1}, with no further increase occurring at higher concentrations. Obviously, some non-specific binding sites still exist on the *p*-MP/Cys-peptide-modified Au surface.

Since the interaction between an antigen and an antibody is a pure biochemical reaction that occurs without any electron transfer, the charge of the Cys-peptide-modified electrode will strongly depend on the amount of antibody specifically bound to its surface and thus will shift the Fermi level of the electrode with respect to the solution. Consequently, potentiometry could be used as a method for detecting antibody [38–40] directly, without the need of labels or indicators. The surface charge caused by immobilized

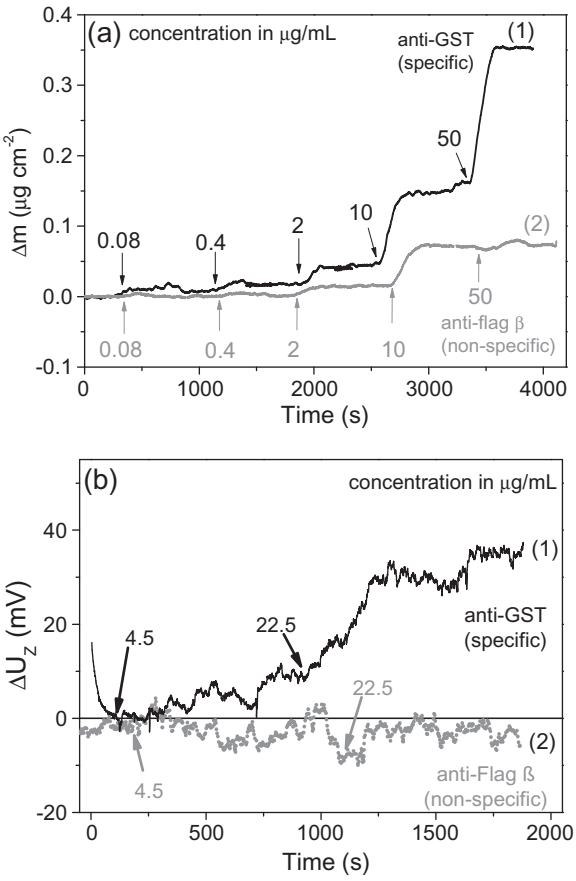

Fig. 5. (a) Frequency response of the Cys-peptide-modified Au electrodes (QCM) as a function of time after the addition of different concentrations (from 0.08 to 50 μg ml^{-1}) of solutions of specifically binding anti-GST antibody (1) and non-specific anti-Flag ß antibody (2); (b) electrical response of a Cys-peptide-modified Si electrode after the addition of anti-GST antibody (1) and anti-Flag ß antibody (2).

biological species will be changed after coupling with corresponding species in the sample. Therefore, we measured the changes in the potential at zero current condition (U_Z) of the Cys-peptide-modified Si electrode (the so-called immunoelectrode sensor) upon exposing it to the buffer solution with different concentrations of the antibodies. The plots in Fig. 5b show the difference in $\Delta U_Z = U_Z - U_Z(t = 150\ s)$ after the addition of a solution containing either the specific or the non-specific antibody (curves 1 and 2, respectively). In the first 120 s, before the injection of 100 μl of anti-GST antibody, the potential drops down and stabilizes at about $U_Z(t = 150\ s) = -0.4$ V. After injection of the specific antibody (to give a concentration in the cell of 4.5 μg ml^{-1}), ΔU_Z increases slowly and becomes slightly noisy.

The increase in ΔU_Z is more pronounced after injection of additional 300 μl of anti-GST antibody (concentration in the cell = 22.5 μg ml^{-1}) after about 1000 s. The potential change is caused by the coupling of the antibody to the Cys-peptide and amounts to about 40 mV, as shown by curve 1 in Fig. 5b. As a negative contrast, a second Si sample modified with Cys-peptide was fixed in the same environment ($I = 0$, in 10 ml of TBS buffer). In the first 100 s, no antibody was injected into the cell (curve 2 in Fig. 5b) and the potentiometric response was noisy but did not change as much as observed for the specific antibody in the cell. After injection of 100 and 300 μl of non-specific antibody (concentrations in the cell of 4.5 and 22.5 μg ml^{-1}, respectively), the potential changed only slightly. The noise of the electrical response is possibly influenced by the light in the laboratory, which induced a charge carrier in the Si substrate.

4. Conclusions

Biofunctionalization of Au or Si surfaces with the specific Cys-peptide based on the direct electrochemical grafting of maleimide group was demonstrated. The electrochemical deposition of maleimidobenzene led to an ultra thin layer with a thickness of about two monolayers after five scans in the case of the Au electrode and after 250 s of deposition on the Si electrode. IRSE spectra confirmed the multilayer formation on the electrode surface.

Each stage of the surface modification was proved qualitatively by IRSE spectra. The biosensing capabilities of the obtained Cys-peptide functionalized surfaces was clearly demonstrated ex situ by an increase in the absorption due to amide I and II bands in the IRSE spectra, as well as in situ by the identification of target antibody specific binding by the QCM-FIA technique and by potentiometric measurements. It was found that the electrical sensing process at zero current (chronopotentiometry) was very sensitive to the coupling of the target antibody on the modified Si surface, and a change in potential of about 40 mV was observed at a concentration of 22.5 μg ml^{-1} whereas no distinct change in potential was visible for the non-specific antibody. QCM-FIA measurements showed the selectivity of the functionalized Au surface towards the target antibody, while a slight affinity to the non-specific antibody was also observed. More tests are required for this type of sensing system. Nevertheless, the results obtained clearly demonstrate that the direct modification of a surface with maleimidophenyl provides a very simple and reliable way to prepare biofunctionalized surfaces suitable for the construction of immunological biosensors. Furthermore, other kinds of biomolecules (DNA, protein, etc.) could be connected to such an electrochemically modified surface to provide a way to construct biosensing devices.

Acknowledgements

The financial support of the European Union through the EFRE Program (ProFIT Grant, Contract No. 10136530/1/2) is gratefully acknowledged. V.S. and J.R. thank the Estonian Ministry of Education and Research Grants (SF0140033s12 and ETF8249) for financial support. We thank Ilona Fischer for technical assistance.

Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1, 2 and 4, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.10.022.

References

[1] Ulman A. Formation and structure of self-assembled monolayers. Chem Rev 1996;96:1533.
[2] Eckermann AL, Feld DJ, Shaw JA, Meade TJ. Electrochemistry of redox-active self-assembled monolayers. Coord Chem Rev 2010;254:1769.
[3] Ferretti S, Paynter S, Russell DA, Sapsford KE, Richardson DJ. Self-assembled monolayers: a versatile tool for the formulation of bio-surfaces. TrAC Trends Anal Chem 2000;19:530.
[4] Nuzzo RG, Zegarski BR, Dubois LH. Fundamental studies of the chemisorption of organosulfur compounds on gold(1 1 1). Implications for molecular self-assembly on gold surfaces. J Am Chem Soc 1987;109:733.
[5] Walczak MM, Popenoe DD, Deinhammer RS, Lamp BD, Chung C, Porter MD. Reductive desorption of alkanethiolate monolayers at gold: a measure of surface coverage. Langmuir 1991;7:2687.
[6] Bélanger D, Pinson J. Electrografting: a powerful method for surface modification. Chem Soc Rev 2011;40:3995.
[7] Chehimi MM, editor. Aryl diazonium salts: new coupling agents in polymer and surface science. Weinheim: Wiley-VCH Verlag; 2012.
[8] Pinson J, Podvorica F. Attachment of organic layers to conductive or semiconductive surfaces by reduction of diazonium salts. Chem Soc Rev 2005;34:429.
[9] Harper JC, Polsky R, Wheeler DR, Brozik SM. Maleimide-activated aryl diazonium salts for electrode surface functionalization with biological and redox-active molecules. Langmuir 2008;24:2206.
[10] Liu GZ, Bocking T, Gooding JJ. Diazonium salts: Sstable monolayers on gold electrodes for sensing applications. J Electroanal Chem 2007;600:335.
[11] Zhang X, Sun G, Hinrichs K, Janietz S, Rappich J. Infrared spectroscopic study of the amidation reaction of aminophenyl modified Au surfaces and p-nitrobenzoic acid as model system. Phys Chem Chem Phys 2010;12:12427.
[12] Delamar M, Hitmi R, Pinson J, Saveant JM. Covalent modification of carbon surfaces by grafting of functionalized aryl radicals produced from electrochemical reduction of diazonium salts. J Am Chem Soc 1992;114:5883.
[13] Buriak JM. Organometallic chemistry on silicon and germanium surfaces. Chem Rev 2002;102:1271.
[14] de Villeneuve CH, Pinson J, Bernard MC, Allongue P. Electrochemical formation of close-packed phenyl layers on Si(1 1 1). J Phys Chem B 1997;101:2415.
[15] Hartig P, Dittrich T, Rappich J. Surface dipole formation and non-radiative recombination at p-Si(1 1 1) surfaces during electrochemical deposition of organic layers. J Electroanal Chem 2002;524:120.
[16] Joyeux X, Mangiagalli P, Pinson J. Localized attachment of carbon nanotubes in microelectronic structures. Adv Mater 2009;21:4404.
[17] Gooding JJ. Advances in interfacial design sensors: aryl diazonium salts for electrochemical biosensors and for modifying carbon and metal electrodes. Electroanalysis 2008;20:573.
[18] Harper JC, Polsky R, Wheeler DR, Lopez DM, Arango DC, Brozik SM. A multifunctional thin film au electrode surface formed by consecutive electrochemical reduction of aryl diazonium salts. Langmuir 2009;25:3282.
[19] Lyskawa J, Bélanger D. Direct modification of a gold electrode with aminophenyl groups by electrochemical reduction of in situ generated aminophenyl monodiazonium cations. Chem Mater 2006;18:4755.
[20] Mandon CA, Blum LJ, Marquette CA. Aryl diazonium for biomolecules immobilization onto SPRi chips. Chemphyschem 2009;10:3273.
[21] Mahouche-Chergui S, Gam-Derouich S, Mangeney C, Chehimi MM. Aryl diazonium salts: a new class of coupling agents for bonding polymers, biomacromolecules and nanoparticles to surfaces. Chem Soc Rev 2011;40:4143.
[22] Zimmermann JL, Nicolaus T, Neuert G, Blank K. Thiol-based, site-specific and covalent immobilization of biomolecules for single-molecule experiments. Nat Protoc 2010;5:975.
[23] Lee CS, Baker SE, Marcus MS, Yang WS, Eriksson MA, Hamers RJ. Electrically addressable biomolecular functionalization of carbon nanotube and carbon nanofiber electrodes. Nano Lett 2004;4:1713.
[24] Zhang X, Sun GG, Hovestadt M, Syritski V, Esser N, Volkmer R, et al. A new strategy for the preparation of maleimide-functionalised gold surfaces. Electrochem Commun 2010;12:1403.
[25] Hermanson GT. Bioconjugate techniques. Elsevier; 2008.
[26] Hinrichs K, Gensch M, Esser N. Analysis of organic films and interfacial layers by infrared spectroscopic ellipsometry. Appl Spectrosc 2005;59:272a.
[27] Syritski V, Reut J, Menaker A, Gyurcsányi RE, Öpik A. Electrosynthesized molecularly imprinted polypyrrole films for enantioselective recognition of L-aspartic acid. Electrochim Acta 2008;53:2729.

[28] Mikhaylova Y, Ionov L, Rappich J, Gensch M, Esser N, Minko S, et al. In situ infrared ellipsometric study of stimuli-responsive mixed polyelectrolyte brushes. Anal Chem 2007;79:7676.
[29] Roodenko K, Mikhaylova Y, Ionov L, Gensch M, Stamm M, Minko S, et al. Ultrathin responsive polyelectrolyte brushes studied by infrared synchrotron mapping ellipsometry. Appl Phys Lett 2008;92:103102.
[30] Rappich J, Hinrichs K. In situ study of nitrobenzene grafting on Si(1 1 1)–H surfaces by infrared spectroscopic ellipsometry. Electrochem Commun 2009;11:2316.
[31] Combellas C, Kanoufi F, Pinson J, Podvorica FI. Sterically hindered diazonium salts for the grafting of a monolayer on metals. J Am Chem Soc 2008;130:8576.
[32] Bernard M-C, Chaussé A, Cabet-Deliry E, Chehimi MM, Pinson J, Podvorica F, et al. Organic layers bonded to industrial, coinage, and noble metals through electrochemical reduction of aryldiazonium salts. Chem Mater 2003;15:3450.
[33] Matrab T, Save M, Charleux B, Pinson J, Cabet-deliry E, Adenier A, et al. Grafting densely-packed poly(n-butyl methacrylate) chains from an iron substrate by aryl diazonium surface-initiated ATRP: XPS monitoring. Surf Sci 2007;601:2357.
[34] Anariba F, DuVall SH, McCreery RL. Mono- and multilayer formation by diazonium reduction on carbon surfaces monitored with atomic force microscopy "scratching". Anal Chem 2003;75:3837.
[35] Hinrichs K, Gensch M, Esser N, Schade U, Rappich J, Kroning S, et al. Analysis of biosensors by chemically specific optical techniques. Chemiluminescence-imaging and infrared spectroscopic mapping ellipsometry. Anal Bioanal Chem 2007;387:1823.
[36] Parker SF, Mason SM, Williams KPJ. Fourier transform Raman and infrared spectroscopy of N-phenylmaleimide and methylene dianiline bismaleimide. Spectrochim Acta A Mol Spectrosc 1990;46:315.
[37] Onodera K, Hirano-Iwata A, Miyamoto KI, Kimura Y, Kataoka M, Shinohara Y, et al. Label-free detection of protein-protein interactions at the GaAs/water interface through surface infrared spectroscopy: discrimination between specific and nonspecific interactions by using secondary structure analysis. Langmuir 2007;23:12287.
[38] Janata J. Immunoelectrode. J Am Chem Soc 1975;97:2914.
[39] Liang RP, Peng HZ, Qiu JD. Fabrication, characterization, and application of potentiometric immunosensor based on biocompatible and controllable three-dimensional porous chitosan membranes. J Colloid Interface Sci 2008;320:125.
[40] Yuan R, Tang DP, Chai YQ, Zhong X, Liu Y, Dai JY. Ultrasensitive potentiometric immunosensor based on SA and OCA techniques for immobilization of HBsAb with colloidal Au and polyvinyl butyral as matrixes. Langmuir 2004;20:7240.