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Time-of-Flight Secondary Ion Mass Spectrometry Investigation of the Orientation of Adsorbed Antibodies on SAMs Correlated to Biorecognition Tests

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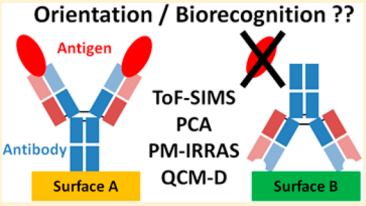
Supporting Information

ABSTRACT: The adsorption of an antiglutamate dehydrogenase (Anti-GDH) antibody on different surfaces was studied to probe its orientation and bioactivity. Three different situations were investigated: physisorption on a −COOH-terminated thiols self-assembled monolayer (SAM) on gold, covalent grafting on the same SAM using NHS-EDC activation, and physisorption on a $-CH_3$ SAM. The orientation of the antibody was investigated combining time-of-flight secondary ion mass spectrometry and principal component analysis. Several orientations are proposed for each case and compared to the results of biorecognition measurements with the antigen (GDH). At each step, protein layers were characterized ex-situ with polarization-modulated infrared reflection absorption spectroscopy and in situ (i.e., in the liquid phase) with quartz crystal microbalance with dissipation monitoring. Biorecognition measurements showed interesting correlations with proposed protein orientations. The role of hydrophobic and/or electrostatic interactions and that of covalent bonding are discussed to underline the influence of the orientation on the bioactivity of adsorbed Anti-GDH.



INTRODUCTION

Development of new biosensors or biomaterials would undoubtly gain from a better understanding and control of the orientation of proteins adsorbed onto surfaces.[1−3] In the case of antibodies, their active sites must be oriented opposite to the surface to ensure a good recognition of antigens.[4,5] Moreover, it is important that physisorption or chemisorption of proteins on surfaces does not induce major changes in their conformation that affect their bioactivity.[6] The orientation will be driven by the distribution of charges and hydrophobic areas on the protein and on the surface. Electrostatic and hydrophobic interactions are known to greatly influence the resulting orientation.[7,8]

Chemical control of metal surfaces has been achieved in the past few decades by developing the self-assembly of organic monolayers (SAMs), especially on gold.[9,10] Thiolate SAMs on gold have been described as a good template for protein adsorption because of the tunability of alkylthiol molecule end-groups (−COOH, $-NH_2$, $-CH_3$, ...) and lengths.[11,12] In parallel, several surface-sensitive techniques allowing the detection of adsorbed proteins were developed. For example, X-ray photoelectron spectroscopy (XPS) in vacuum or polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) in air allow the characterization of protein layers.[13−16] Moreover, quartz crystal microbalance with dissipation monitoring (QCM-D) is able to follow the adsorption of biological molecules[17,18] and especially monitor the antibody−antigen interaction in the liquid phase.[5,19,20] However, these techniques lack the ability to determine the orientation or conformation of a protein in a direct way.

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is also described in the literature as an important tool for analysis of protein-covered surfaces. Because of its high mass resolution (up to 10000 $m/\Delta m$) ToF-SIMS allows the discrimination of amino acid (a.a.) fragments coming from protein films.[21,22] Its low sampling depth (a few nanometers)[23] informs on the outmost layer of the protein and its amino acid composition leading to the identification of various orientations and/or conformations.[24−27] In addition to the direct analysis of a.a. fragments that could be a tedious work because of the large quantity of information contained in one spectra, multivariate analysis techniques such as principal component analysis (PCA) were introduced to analyze ToF-SIMS results. Protein films formed by competitive adsorption of insulin and albumin[28,29] or the "end-on" or "head-on" orientation of antibodies on surfaces were described.[30]

In a previous work,[31] we described a methodology to probe the orientation of β-lactoglobulin (βLG) on different SAMs. ToF-SIMS together with principal component analysis (PCA) allowed us to detect the preferential orientation of the protein on $-NH_2$- or $-CH_3$-terminated SAMs. In that case, the protein was a model globular protein which can reasonably be

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considered as "hard". It was then possible to determine precisely its orientation knowing its a.a. composition by analyzing PCA results. In this work, we investigated a more realistic and complex case; the adsorption of a monoclonal mouse IgG1 antibody directed toward glutamate dehydrogenase (Anti-GDH) on −COOH- or $-CH_3$-terminated SAMs. The studied antibody/antigen (Ab/Ag) couple is used in strip test for the detection of gastroenteritis of bacterial origin developed by Coris BioConcept (Gembloux, Belgium). The orientation of the antibody adsorbed or grafted on the −COOH surfaces and adsorbed onto the $-CH_3$ surfaces have been investigated using the combination of ToF-SIMS and PCA described earlier. This methodology gives crucial information on the preferential orientations resulting from each interaction. In a second stage, PM-IRRAS and QCM-D measurements were used to perform biorecognition tests linked to the orientation in each case.

MATERIALS AND METHODS

Chemicals and Sample Preparation Protocols. Gold surfaces are obtained by metallization of 3 in. silicon wafers with a 5 nm titanium layer (to enhance gold adhesion on the wafer) and 100 nm of gold on top. Before functionalization, wafers are divided in 1 cm^2 samples, cleaned using UV/O_3 treatment for 15 min, and thoroughly rinsed with absolute ethanol before being dried with N_2.

Gold surfaces were first immersed in 1 mM thiol solutions in absolute ethanol for 24 h, rinsed 2 times with ethanol, and dried under N_2 to form self-assembled monolayers (SAMs).[32,33] Two different alkyl thiols HS-$(CH_2)_{10}$-R with R = COOH (11-mercaptoundecanoic acid, 95%, 450561, Sigma-Aldrich) or CH_3 (1-undecanethiol, 98%, 510467, Sigma-Aldrich) were used. Samples are characterized using PM-IRRAS to ensure formation of SAMs prior to antibodies adsorption.

Two procedures were used for the deposition of antibodies on the −COOH-terminated surface: adsorption and grafting. To perform a chemical attachment (grafting), the acid layer was activated with a mixture of NHS (*N*-hydroxysuccinimide, 98%, 130672, Sigma-Aldrich) and EDC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, E6383, Sigma-Aldrich) in Milli-Q water at 60 and 30 mM, respectively, for 90 min. Samples were then rinsed in Milli-Q water and dried under N_2.

Anti-GDH antibodies and the corresponding antigen GDH (glutamate dehydrogenase) were kindly provided by Coris BioConcept. The monoclonal Anti-GDH antibody (a mouse IgG1) is used in strip test developed by Coris BioConcept. It is directed toward a specific GDH produced in bacteria responsible for certain form of gastro enteritis (*Clostridium difficile*). Both antibodies and antigens were used as received after dilution to 5 $\mu g\,mL^{-1}$ in a carbonate buffer (pH 9.5). Adsorption of the Anti-GDH on the different surfaces was achieved by immersing gold substrates in the protein solution for 90 min then rinsing in carbonate buffer and Milli-Q water. Samples were finally dried under N_2 before ToF-SIMS or PM-IRRAS analysis under UHV or in air, respectively. Commercially available powder milk (Régilait, France) was used to "saturate" the surface and prevent nonspecific adsorption of proteins. To that aim, antibody-covered surfaces were immersed for 60 min in a milk solution at 5 $\mu g\,mL^{-1}$ in carbonate buffer and rinsed in buffer and Milli-Q water before drying under N_2 and analyzing with PM-IRRAS. Finally, for biorecognition tests, samples were immersed in a buffer solution of GDH at 5 $\mu g\,mL^{-1}$ for 90 min, rinsed in buffer and Milli-Q water, and dried in N_2. In the case of QCM-D measurements, the same procedure is applied without Milli-Q water rinsing or N_2 drying.

Analytical Techniques. *ToF-SIMS and PCA.* ToF-SIMS measurements were performed with an IONTOF V spectrometer (IONTOF GmbH, Münster, Germany). The samples were bombarded with pulsed Bi^+ ions (30 keV, 45°) on an area of 500 × 500 μm^2 for 60 s. The primary ion fluence density was lower than 2×10^{11} ions cm^{-2} to remain under the static SIMS limit. The mass resolution $(m)/(\Delta m)$ at m/z 70 was about 8000 for each measured sample. To study proteins with ToF-SIMS, positive mass spectra were analyzed and calibrated using CH_3^+, $C_2H_3^+$, $C_3H_5^+$, and $C_7H_7^+$ (m/z = 15, 27, 41, and 91, respectively).

PCA is used to determine from which peaks the variability between samples is arising after the adsorption of proteins.[34,35] Prior to PCA analysis, a data pretreatment is needed to select the information coming from the proteins on the surface. A selection of 44 peaks corresponding to amino acid fragments in the positive mass spectrum, and providing a signature of adsorbed proteins, was first performed.[21,25,27] The mass spectra were then normalized to the total counts and mean-centered.

PCA calculations were performed using the NESAC/BIO MVA Toolbox (http://mvsa.nb.uw.edu, Dan Graham, University of Washington) for MATLAB (The MathWorks, Inc., Natick, MA). Scores are plotted with the 95% confidence limit; the methodology of the script was described by Wagner et al. in 2001.[25] After the first PCA calculations, the main differentiation between samples was obtained on peaks that could be attributed to SAM surfaces mostly and not only proteins. The peak list was therefore adapted, and several peaks were removed. Details of peak lists used in PCA calculation and removed peaks can be found in the Supporting Information.

PM-IRRAS. Gold samples were placed in the external beam of the FT-IR instrument (Nicolet Nexus 5700 FT-IR spectrometer), and the reflected light was focused on a nitrogen-cooled Mercury−Cadmium−Telluride (MCT) wide band detector. Infrared spectra were recorded at 8 cm^{-1} resolution by coaddition of 128 scans. A ZnSe grid polarizer and a ZnSe photoelastic modulator were placed prior to the sample to modulate the incident beam between p and s polarizations (HINDS Instruments, PEM90; modulation frequency, 36 kHz). The detector output is sent to a two-channel electronic device that generates the sum and difference interferograms. Those are processed and undergo Fourier transformation to produce the PM-IRRAS differential signal $(\Delta R/R_0) = (R_p - R_s)/(R_p + R_s)$. Using a modulation of polarization enabled us to perform rapid analyses of the sample after treatment in various solutions without purging the atmosphere or requiring a reference spectrum. At each step (thiol SAMs, Anti-GDH adsorption, milk saturation, and GDH recognition), a minimum of three samples were analyzed using PM-IRRAS.

QCM-D. The adsorption of proteins on SAMs was monitored in situ (i.e., in the liquid phase) by quartz crystal microbalance with dissipation monitoring (QCM-D). Measurements were performed with a Q-Sense E4 System (Gothenborg, Sweden) at 20.0 ± 0.1 °C. Oscillations of the crystal at the resonant frequency (5 MHz) or at one of its overtones (15, 25, 35, 45, 55, and 65 MHz) were obtained when applying AC voltage. The variations of the resonance frequency (Δf) and of the dissipation (ΔD) were monitored during adsorption of proteins. Solutions were injected into the measurement cell using a peristaltic pump (Ismatec IPC-N 4) at a flow rate of 50

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μL min^{-1}. Before protein adsorption, a carbonate buffer solution was injected to establish the baseline. The protein solution was then flowed into the measurement cell. Subsequently, rinsing was performed using carbonate buffer solution. The presented data correspond to the fifth overtone. Mass uptakes Δm were calculated with the Sauerbrey equation[36] assuming the deposited films behave as a rigid layer.

$$\Delta f = -N\frac{\Delta m}{C_f} \quad (1)$$

where C_f (= 17.7 ng cm^{-2} Hz^{-1}) is the mass sensitivity factor at f = 5 MHz and N (= 3, 5, 7, 9, ...) is the overtone number. For both −COOH-terminated surfaces, two experiments were performed, each with two different functionalized QCM crystals, giving a total of four samples. In the case of the −CH_3-terminated SAMs, two crystals were analyzed the first time and only one the second time, giving a total of three samples. Calculations presented in this work take into account the delta in frequency for all four (or three) samples in each case in addition to the results taken on the third, fifth, seventh, and ninth overtones. These results are available in the Supporting Information.

RESULTS

Anti-GDH Adsorption on SAMs. The formation of −COOH- and −CH_3-terminated SAMs was checked using PM-IRRAS (spectra not shown). The characteristic features of SAM formation have been described in detail in a previous work[31] and were evidenced here. Activation of the carboxylic acid terminated SAMs was followed in PM-IRRAS with the band at 1745 cm^{-1} characteristic of C═O stretch in ester functions[14] and in ToF-SIMS following peaks previously identified as characteristic of the NHS group.[37] Details about the characterization of the activated layer can be found in Supporting Information. Then, protein adsorption was investigated at each step by PM-IRRAS. The amide I and II bands at 1660 and 1550 cm^{-1} evidence the presence of proteins. Moreover, the integrated areas of these bands provide quantitative information on the amounts of adsorbed proteins.[38] Integrated amide I and II band areas are presented in Figure 1. Results in Figure 1 show that Anti-GDH is adsorbed onto all surfaces. In the case of adsorption on −COOH-terminated SAMs prior or after NHS-EDC activation, the quantity of adsorbed antibodies is similar (2.1 and 2.2 au for the amide I and II band area). Conversely, a larger quantity is observed in the case of physisorption on the −CH_3-terminated surface with an area of 3.0 au. This result is in agreement with our previous work showing that proteins tend to adsorb preferentially on this surface because of strong hydrophobic interactions.[31]

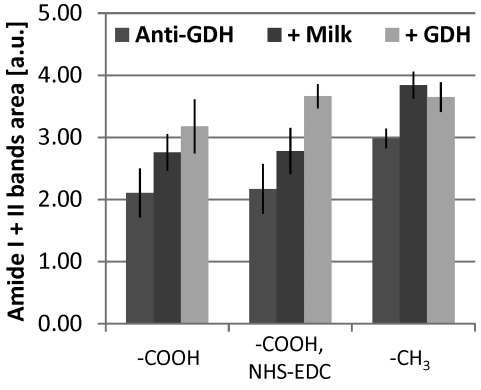

Figure 1. PM-IRRAS analysis. Amide I and II band area variation at each step on −COOH SAMs, on −COOH SAMs after activation, and on −CH_3 SAMs.

Anti-GDH adsorption on all three surfaces was also monitored in situ (i.e., in the liquid phase) using QCM-D. In Figure 2, frequency shifts at the fifth overtone recorded upon successive protein injections on the three surfaces are represented together with dissipation measurements. Because dissipation remains lower than 3 × 10^{-6}, the observed shifts in frequency can be correlated to the quantity of adsorbed proteins on the surface (Sauerbrey model). Upon rinsing with the buffer, there was no desorption of Anti-GDH, showing that the antibodies stick to the surface. The quantity of Anti-GDH deposited on the −COOH (before or after activation by NHS-EDC) surfaces is lower than that on −CH_3-terminated SAMs, in agreement with the PM-IRRAS data. The frequency shifts observed after buffer rinsing are 12.9 ± 3.5, 12.2 ± 2.8, and 26.3 ± 1.9 Hz on −COOH-, activated −COOH-, and −CH_3-terminated surfaces, respectively. Measured frequency shifts for each surface (3 or 4 samples) and the third, fifth, seventh, and ninth overtones are presented in Supporting Information; they were used to calculate the variability for each type of sample (presented values represent the mean and standard deviation for each type of surface).

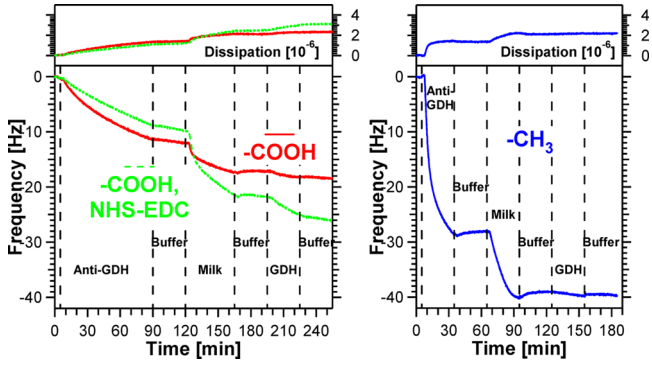

Figure 2. QCM-D results showing all adsorption steps (Anti-GDH, milk, and GDH) on −COOH SAMs (red), −COOH SAMs after activation (green), and −CH_3 SAMs (blue). The fifth overtone for one experiment is presented here.

The same trend is observed for both techniques, with larger adsorption on −CH_3 surfaces and Anti-GDH adsorption stable upon rinsing in each case. The relative difference measured in QCM-D between the −COOH-terminated surfaces (before or after activation) and the −CH_3-terminated surface is larger than that measured by PM-IRRAS. The −COOH to −CH_3 Anti-GDH quantity ratios are 0.7 in PM-IRRAS and 0.4 in QCM-D. Rinsing and drying of the samples prior to analysis for PM-IRRAS could lead to the removal of adsorbed antibodies thus explaining those differences. Influence on GDH recognition will be presented after the characterization of the adsorbed antibody layers on −COOH-, activated −COOH-, or −CH_3-terminated SAMs using ToF-SIMS and PCA calculations.

Exploring Anti-GDH Adsorption Using ToF-SIMS and PCA. PM-IRRAS and QCM-D showed differences in the amounts of adsorbed Anti-GDH but no information about the orientation could be determined at this point. PCA calculations

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were then performed on ToF-SIMS measurements to obtain a direct probe of the preferential orientation, if any, on the surfaces. In Figure 3 are presented the scores for PC1 and PC2; from PC1 (90% of variance), a clear separation of the $-CH_3$-terminated surfaces is observed with large positive scores. Surfaces bearing −COOH-terminated SAMs give negative scores on PC1 when Anti-GDH is adsorbed without prior activation of the acid functions. When Anti-GDH is grafted, the PC1 scores are still negative but with a shift toward positive values (closer to zero). An additional separation of these two surfaces arises from PC2 (only 9% of variance) where activated surfaces exhibit positive scores and nonactivated surfaces negative scores (together with $-CH_3$-terminated surfaces).

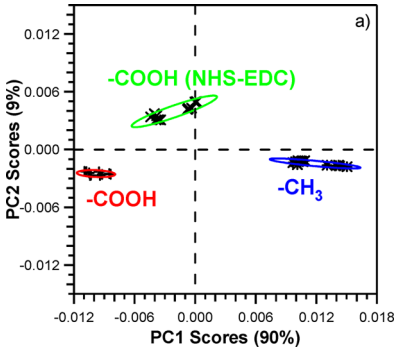

Figure 3. PCA calculations results (scores from PC1 and PC2) on −COOH-, −COOH- activated with NHS-EDC, and $-CH_3$-terminated SAMs after Anti-GDH adsorption.

The loadings for both PCs are presented in Table 1. On PC1, large positive loadings are obtained, indicating a strong separation of the corresponding samples. The positive scores in PC1 are attributed to the $-CH_3$-terminated surfaces after adsorption of Anti-GDH. Amino acids (a.a.) corresponding to peaks giving positive PC1 loadings are proline, valine, arginine (or histidine), serine, and threonine. These amino acids are the most exposed after adsorption of Anti-GDH on $-CH_3$-terminated SAMs. For negative loadings, very small values are observed, indicating a weaker separation of the corresponding samples on the scores plot (−COOH with or without activation). The corresponding a.a. are cysteine, arginine, or methionine.

Table 1. Most Important Positive (Pos.) and Negative (Neg.) Loadings from PC1 and PC2

PC1 (90% of variance) loadings					
pos.	mass	a.a.	neg.	mass	a.a.
0.83	70.07	Pro	−0.05	46.99	Cys
0.38	72.08	Val	−0.04	43.02	Arg
0.16	110.08	Arg/His	−0.01	127.10	Arg
0.16	60.05	Ser	−0.01	58.99	Cys
0.15	74.06	Thr	−0.01	61.01	Met
PC2 (9% of variance) loadings					
pos.	mass	a.a.	neg.	mass	a.a.
0.63	72.08	Val	−0.35	46.99	Cys
0.19	81.04	His	−0.27	69.03	Thr
0.14	110.07	Arg/His	−0.26	74.06	Thr
0.13	127.10	Arg	−0.23	70.07	Pro
0.13	56.05	Lys/Phe/Met	−0.18	61.01	Met

PC2, with only 9% of variance, still gives a small separation between Anti-GDH adsorbed onto −COOH- or $-CH_3$-terminated SAMs on one hand (negative scores and loadings), and onto activated −COOH surfaces on the other hand (positive scores and loadings). Characteristic proline and threonine peaks, already identified for $-CH_3$ surfaces separation in PC1, are evidenced in the negative PC2 loadings. Cysteine and methionine, which allowed separation of the two −COOH surfaces in PC1 (negative scores), are also observed in negative PC2 loadings. On the other hand, the peak corresponding to valine has positive loadings in PC2 (specific to activated −COOH surfaces), but was previously attributed to the separation of $-CH_3$ surfaces (positive PC1 loadings). This could explain the fact that samples corresponding to the activated −COOH surfaces on scores plot (Figure 3) tend to shift to positive values on the PC1. Taking into account the PC1 negative loadings and the PC2 positive loadings that separate the adsorption of Anti-GDH on the activated −COOH-terminated surface from the nonactivated one, arginine together with histidine peaks appear to make most of the separation for these two surfaces.

Testing Biorecognition of GDH by Anti-GDH. Before the biorecognition capabilities of Anti-GDH adsorbed onto the three surfaces were tested, a blocking step was performed using a milk solution to avoid nonspecific recognition of the antigen (i.e., adsorption on the substrate). Milk was chosen because it is a mixture of different proteins with a variety of molecular weights and electrostatic properties that will maximize adsorption on any region of the SAM-coated gold surfaces that is not covered by Anti-GDH. It is considered that after milk adsorption the entire surface of the substrate is covered with either Anti-GDH or proteins contained in milk. From both PM-IRRAS (Figure 1) and QCM-D (Figure 2), the signal of adsorbed proteins is increased after milk adsorption. In PM-IRRAS, an increase of the amide I and II bands area of 30% is observed for all surfaces. In QCM-D, the changes in frequency after milk adsorption on Anti-GDH covered surfaces vary between 40% (for $-CH_3$ surfaces) and 60% (for activated −COOH surfaces). Those differences could be explained by the fact that in QCM-D the water trapped in the protein layer is also measured.

Upon GDH interaction with $-CH_3$-terminated SAMs, PM-IRRAS and QCM-D showed no protein adsorption, indicating the absence of molecular recognition. No frequency shifts occur in QCM-D upon flowing antigen solution, and even a slight decrease of the amide bands area from 3.8 au to 3.7 au, possibly due to the removal of Anti-GDH or milk proteins upon rinsing and drying, is observed in PM-IRRAS. On −COOH-terminated surfaces, the amide bands area increased from 2.8 to 3.2 au in PM-IRRAS and a frequency shift of 2.0 ± 1.0 Hz was observed in QCM-D after flowing GDH solution. In the case of activated −COOH surfaces, the measured amide bands area increased by 0.9 au and the measured frequency shift was 4.6 ± 0.5 Hz after GDH exposure. Biorecognition of GDH by the specific antibody was thus effective on both −COOH-terminated SAMs but not on the $-CH_3$ one. Those results will be further analyzed in the Discussion section by comparing these values to the quantity of adsorbed Anti-GDH and by normalizing the results with the mass of the corresponding protein (antibody or antigen). But first, the orientation of Anti-GDH on the different surfaces will be discussed based on ToF-SIMS and PCA results.

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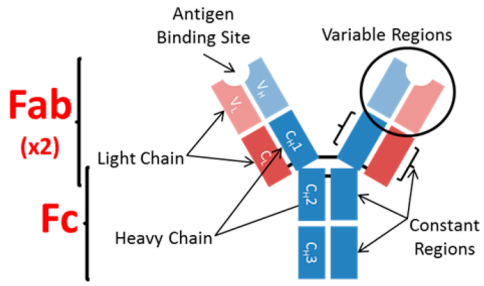

	number of a.a.			%age		
a. a.	C_H2	C_H3	Fc	C_H2	C_H3	Fc
Val	14	6	20	13.1	5.6	9.3
Pro	10	9	19	9.3	8.4	8.9
Lys	9	9	18	8.4	8.4	8.4
Thr	9	9	18	8.4	8.4	8.4
Ser	9	8	17	8.4	7.5	7.9
Glu	8	7	15	7.5	6.5	7.0
Asn	3	9	12	2.8	8.4	5.6
Phe	8	4	12	7.5	3.7	5.6
Gln	5	6	11	4.7	5.6	5.1
Others	32	40	72	29.9	37.4	33.6
Total	107	107	214	100.0	100.0	100.0

Figure 4. General representation of an antibody (Mouse IgG1) and table of the most represented a.a. in the constant fragment (Fc).

DISCUSSION

Orientation of the Antibody. As pictured in Figure 4, antibodies can be described as Y-shaped proteins with the two Fab fragments at the top side of the Y. Those are both composed of two variable parts, V_L and V_H, and two constant ones, C_L and C_H1, (the L and H subscript refer to light and heavy chains in the antibody, respectively; Figure 4). On the other side of the antibody, the Fc fragment is composed of the associated C_H2 and C_H3 regions from the two different heavy chains. The antibody used in this study is a monoclonal mouse IgG1 whose exact a.a. sequence of its Fab fragment is not determined. Nevertheless, the sequence of the Fc fragment is the same for all IgG1 and can be retrieved in the UniProt database.[39] Most of the amino acids present in the C_H2 and C_H3 regions (forming the Fc fragment) are shown in the table inserted in Figure 4. Apart from Arg or His, all the a.a. explaining the separation of Anti-GDH physisorbed on $-CH_3$-terminated SAMs are among the highest concentration a.a. in the Fc fragment. Proline, valine, serine, and threonine concentrations in the Fc fragment are 8.9, 9.3, 7.9, and 8.4%, respectively. This strongly indicates that, on the $-CH_3$ surfaces, adsorbed Anti-GDH antibodies mostly expose the Fc region, i.e., they are preferentially attached upside down, the binding sites being in contact with the SAM and not accessible for further Ag recognition. In addition to the suggested orientation, conformational changes of the antibody upon adsorption should be accounted for in the PCA separation. Because of the lack of information about the Anti-GDH exact structure, it will be unfortunately difficult to thoroughly discuss them here.

On the −COOH-terminated surfaces (before or after activation), most of the a.a. responsible for their separation in PCA (Cys, Arg, Met or His) are not present in high concentration in the Fc fragment. One can thus deduce that another part of the antibody is preferentially exposed on those surfaces, i.e., the Fab fragments, whose a.a. sequence is unfortunately not known. Such an orientation is expected to lead to better biorecognition features. Moreover, the separation of the −COOH-terminated surfaces in PC2 (only 9% of variance) suggests slight differences in the preferential orientation of the antibodies on these two surfaces. Results of biorecognition tests on −COOH-terminated surfaces will be interpreted in light of these suggested preferential orientations in Influence of Orientation on Biorecognition.

In a study by Wang et al.,[30] the authors tracked the orientation of an adsorbed IgG using ToF-SIMS and PCA. They compared the mass spectra after adsorption of the whole Anti-hCG antibody to those of the Fab or Fc fragments alone on several SAMs surfaces. They were then able to conclude that the antibodies adopted various orientations, end-on or head-on, on the −COOH- or $-NH_2$-terminated SAMs. Our approach is a real case study analyzing the adsorption of the whole GDH antibody (we do not have access to its separated Fab or Fc fragments). However, because the a.a. sequence for the Fc fragments of mouse IgG1 is known, we were able to identify the most likely orientations of the antibodies on the various investigated surfaces.

Influence of Orientation on Biorecognition. No recognition of GDH by adsorbed Anti-GDH was observed on $-CH_3$-terminated surfaces. On the basis of previously discussed ToF-SIMS and PCA data, Anti-GDH adsorbed onto this surface is likely oriented preferentially with its Fc fragments facing up. This is consistent with the absence of GDH biorecognition because Fab fragments are not accessible to target recognition. Let us add that adsorption on such hydrophobic surfaces may modify antibody conformation resulting in a loss of bioactivity. Hydrophobic interactions are indeed partly responsible for the secondary structure of proteins.[40,41] Moreover, the structure of GDH shows the presence of both hydrophilic and hydrophobic regions on its surface (see PDB structure 1HRD for an analogue of the GDH used in this study[42]). On a hydrophobic $-CH_3$-terminated surface, the anti-GDH likely interacts via its hydrophobic epitopes.

In the case of both −COOH-terminated surfaces, GDH was recognized by the immobilized Anti-GDH as evidenced by PM-IRRAS and QCM-D. In order to quantify this biorecognition, the ratio of the numbers of GDH versus Anti-GDH proteins was calculated (Ag/Ab). The GDH in use for this study is formed of 449 a.a. and has a molecular mass of ≈50 kDa (PDB structure 1HRD).[42] As for Anti-GDH, the exact a.a. composition is unknown, but IgG1 antibodies are generally considered to weigh ≈150 kDa. In PM-IRRAS, the area of amide bands is proportional to the quantity of adsorbed proteins; the ratio GDH/Anti-GDH is then calculated using eq 2

$$\frac{N_{\mathrm{GDH}}}{N_{\mathrm{Anti\text{-}GDH}}} = \frac{(A_{\mathrm{GDH}} - A_{\mathrm{milk}})m_{\mathrm{Anti\text{-}GDH}}}{A_{\mathrm{Anti\text{-}GDH}}m_{\mathrm{GDH}}} \quad (2)$$

where N_x is the number of protein x, A_n is the area of the amide I and II bands at the corresponding n step, and m_x is the mass of the x protein.

In QCM-D, considering that those measurements remain in the limits of validity of the Sauerbrey model, and assuming that the amount of trapped water molecules is similar for both proteins, Anti-GDH and GDH, the delta in frequency at each

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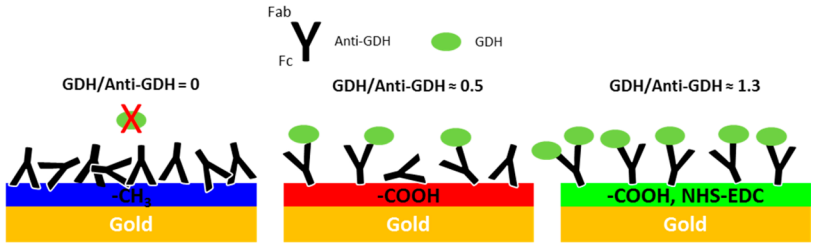

Figure 5. Schematic representation of the physisorption or grafting of Anti-GDH on $-CH_3$- or −COOH-terminated SAMs and consequences on the GDH biorecognition. The milk blocking step is not represented for sake of clarity.

step can be correlated to the quantity of adsorbed proteins. The GDH/Anti-GDH ratio could thus be calculated using eq 3

$$\frac{N_{GDH}}{N_{Anti\text{-}GDH}} = \frac{\Delta F_{GDH} m_{Anti\text{-}GDH}}{\Delta F_{Anti\text{-}GDH} m_{GDH}} \quad (3)$$

where N_x is the number of protein x, ΔF_n is the delta in frequency at the corresponding n step, and m_x is the mass of the x protein. Results are summarized in Table 2 and show that calculated ratios are in good agreement for both techniques.

Table 2. Calculated Ratios of the Recognized GDH over the Quantity of Anti-GDH on −COOH-Terminated SAMs Weighed by Their Respective Masses (50 and 150 kDa per Unit) Obtained from PM-IRRAS and QCM-D Measurements

	PM-IRRAS	QCM-D
−COOH	0.6 ± 0.3	0.4 ± 0.1
−COOH, NHS-EDC	1.3 ± 0.5	1.2 ± 0.2

In the case of the Anti-GDH physisorbed on −COOH surfaces, only one GDH appears to be recognized per two adsorbed antibodies (GDH/Anti-GDH ratio ≈ 0.5). When grafting the antibodies to the surface, more than one GDH protein per grafted Anti-GDH is recognized (ratio ≈ 1.3). This suggests differences in the orientation of the physisorbed or grafted antibodies as shown earlier by the distinction obtained on PC2 in PCA calculations. In the case of grafted antibodies, a covalent bond is formed between acids on the surface and amines of the Anti-GDH, leading to a better biorecognition. In Figure 4, one can see that lysine residues, an amine terminated a.a., are present in high concentrations in the Fc fragments (8.4%). They could therefore interact preferentially with activated acid moieties on the surface and force the antibody to adopt an orientation with its Fab fragments up. In the case of physisorbed Anti-GDH, lower biorecognition is observed. Weaker interactions are involved here. The main ones would be electrostatic interactions between acid moieties of the surface and amine from the antibody, but H-bond or hydrophobic interactions should also intervene. The antibody is thus prone to adopt various orientations after adsorption (tilted or lying on the surface with no or less access to the Fab fragments for the antigen). Moreover, weaker interactions with the surface could lead to the displacement in the solution of Ab/Ag complexes by competition. Figure 5 shows a scheme summarizing the results obtained in each case.

CONCLUSION

The interaction of a monoclonal antibody Anti-GDH, with three SAMS terminated by $-CH_3$, −COOH, and −COOH activated by NHS-EDC was investigated using PM-IRRAS and QCM-D. The orientation of the formed layer was explored by ToF-SIMS and PCA. PM-IRRAS and QCM-D results showed that on $-CH_3$-terminated SAMs, Anti-GDH adsorbed in greater quantities, possibly due to hydrophobic interactions. However, ToF-SIMS and PCA data suggested that in this case anti-GDH was oriented upside down, leading to no biorecognition of the target, GDH. Those results were obtained using the amino acid sequence of the Fc fragment of the antibody but could also result from conformational changes in the structure of the antibody due to the strong hydrophobic interactions.

In both other cases, physisorbed or grafted Anti-GDH on −COOH surfaces, no clear orientation was determined, but PCA results suggested an orientation different from the one observed upon adsorption on $-CH_3$-terminated SAMs (PC1). Moreover, some differences in orientation between the physisorbed and the grafted antibodies were observed (PC2). Biorecognition measurements showed clearly that GDH recognition mostly takes place when the Anti-GDH is grafted to the −COOH-terminated surface. This was explained by the formation of covalent bonds favoring the preferential heads-up orientation of Anti-GDH. Orientation of Anti-GDH physisorbed on the surface is governed by weaker electrostatic interactions; some of the measured biorecognition could then be in competition with displacement of Ab/Ag complexes in the solution.

This study combined macroscopic measurements of biorecognition, in the liquid phase or after drying the surface, to a characterization of adsorbed antibodies at a molecular level. It explains the well-known, but so far rarely fully interpreted, role of the surface underlying the immobilized probe on a biosensor. These results underline the power of the ToF-SIMS technique applied as a direct probe in determining protein orientation on a surface in order to predict its bioactivity.

ASSOCIATED CONTENT

Supporting Information

Characterization of the activation of the −COOH layer, full description of peaklists used in PCA calculations, and full QCM-D measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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