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Immunosensor interface based on physical and chemical immunoglobulin G adsorption onto mixed self-assembled monolayers

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Abstract

An immunosensor interface based on mixed hydrophobic self-assembled monolayers (SAMs) of methyl and carboxylic acid terminated thiols with covalently attached human Immunoglobulin G (hIgG), is investigated. The densely packed and organised SAMs were characterised by contact angle measurements and cyclic voltammetry. The effect of the non-ionic surfactant, Tween 20, in preventing nonspecific adsorption is addressed by ellipsometry during physical and covalent hIgG immobilization on pure and mixed SAMs, respectively. It is clearly demonstrated that nonspecific adsorption due to hydrophobic interactions of hIgG on methyl ended groups is totally inhibited, whereas electrostatic/hydrogen bonding interactions with the exposed carboxylic groups prevail in the presence of surfactant. Results of ellipsometry and Atomic Force Microscopy, reveal that the surface concentration of covalently immobilized hIgG is determined by the ratio of COOH / CH_3-terminated thiols in SAM forming solution. Moreover, the ellipsometric data demonstrates that the ratio of bound anti-hIgG / hIgG depends on the density of hIgG on the surface and that the highest ratio is close to three. We also report the selectivity and high sensitivity achieved by chronoamperometry in the detection of adsorbed hIgG and the reaction with its antibody.

Keywords: Mixed self-assembled monolayers; Immunosensor; Human immunoglobulin G; Tween 20; Electrochemistry; Ellipsometry

1. Introduction

In the development of affinity biosensors, the fabrication of the interface plays a crucial role, since it determines the specificity, the reproducibility, and the stability of the entire sensor [1,2]. The nonspecific adsorption together with a proper bioreceptor orientation/distribution on the surface is an important factor that must be controlled in order to get the best biosensor performance. By the use of mixed SAMs, interfaces become tunable and the desired chemical properties [3] can be achieved.

Much work has been devoted [4–7] to the search of surfaces that minimize nonspecific adsorption of proteins, based on the understanding of protein adsorption mechanisms. It has been found that the major factors responsible for the interfacial activity and adsorption of proteins are the water structure-driven hydrophobic effect and the two types of interactions, electrostatic and strong hydrogen-bonding (often characterized by cooperative, multiple hydrogen bonds [8]). Surfaces with nonionic polyethylene oxide (PEO) grafts show greatly reduced protein adsorption [9], since they are able to minimize both electrostatic and hydrophobic interactions. The development of an interface of gold modified with tri(ethylene glycol)-terminated thiol has also been reported [10,11] as resistant to the nonspecific adsorption of some proteins; in this case, its laborious synthesis appears as a drawback in its use. In contrast, the addition of surfactants [12–14] during biocompounds immobilisation, such as sodium dodecyl sulphate (SDS) or polyethylene glycol sorbitan monolaurate (Tween 20) became common in biosensor preparation. However, most of the studies only refer to their blocking ability towards hydrophobic surfaces, during biomolecules covalent attachment [15].

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In the present work, the influence of the nonionic detergent, Tween 20, during the physical and covalent attachment of the bioreceptor, hIgG, in pure and mixed self-assembled monolayers containing methyl and carboxylic acid terminal groups, has been investigated by ellipsometry and Atomic Force Microscopy. The incorporation of both thiols enables a SAM interface with few linking groups for the covalent attachment [2,10,16] of hIgG, surrounded by hydrophobic moieties where physical adsorption is completely inhibited by the presence of Tween 20.

The adsorption of proteins and their biological performance at the electrode surfaces modified with self-assembled monolayers has been addressed by several techniques, such as chronoamperometry [17], ellipsometry [18], surface plasmon resonance [1,19,20], radiolabelling [21,22], quartz crystal microbalance [23,24], electrochemical impedance spectroscopy [25] and atomic force microscopy [15,26,27]. In the current paper, the biorecognition of both physically and covalently adsorbed hIgG towards its antibody in antiserum, over pure or mixed SAMs is evaluated by ellipsometry and chronoamperometry.

2. Experimental

2.1. Materials

hIgG, hIgG antiserum (anti-hIgG), human serum albumin antiserum (anti-HSA), Tween 20, 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma. 11-Mercaptoundecanoic acid and 1-Hexanethiol were purchased from Aldrich.

Gold film (200 nm) on glass (1.1×1.1 cm^2) with a pre-layer of chromium (2–4 nm) purchased from Arrandee, was used as substrate for mixed SAMs preparation.

2.2. Buffer and solutions

Phosphate-buffered saline (PBS: (8.0 mM $Na_2PO_4 \cdot 1.14$ mM KH_2PO_4, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) was prepared in Millipore water (18 MΩ cm at 25 °C). Solutions of NHS (0.05 M) and EDC (0.2 M) were prepared in Millipore water immediately before use. HIgG was diluted with PBS to obtain two different concentrations, 0.1 and 0.05 mg/ml, with and without the addition of 1% Tween 20 (v/v). Anti-hIgG serum and anti-HSA serum were diluted with PBS to 0.1 mg/ml with the addition of 1% Tween 20.

2.3. Gold surface modification

Prior to use, the gold slides were annealed in the cold part of a Bunsen flame and quenched in ultra pure water. This treatment produces a flat gold surface with predominant (111) orientation, as confirmed by STM. The average roughness factor of the substrates (1.2) was estimated by the iodine chemisorption method [28] and is in agreement with reported values for similar thin gold electrodes [29,30]. The mixed monolayers were prepared by substrate immersion in 1 : 1, 1 : 5, 1 : 10, and 1 : 20 11-Mercaptoundecanoic acid : 1-hexanethiol solutions in ethanol, in a total thiol concentration of 1 mM, for 24 h, and then removed, thoroughly rinsed with ethanol and pure water, and dried under a stream of nitrogen.

Mixed monolayers will be designated throughout the paper by the corresponding COOH percentage in the deposition solution: 50%, 20%, 10% and 5%.

2.4. Covalent immobilization of hIgG

The gold substrates modified with mixed SAMs were immersed into 0.05 M NHS and 0.2 M EDC solution for 15 min. After rinsing with water, the substrates were then immersed in 0.1 mg/ml hIgG solution containing 1% (v/v) Tween 20, for 30 min, followed by copious rinsing with water and drying with nitrogen.

2.5. Contact angle measurements

The measurements were conducted using the Sessile drop method. De-ionized water (4 μl) was gently dropped on the SAMs and the contact angle was read directly using a goniometer.

2.6. Electrochemical studies

All electrochemical measurements were performed using a PARSTAT 2263 electrochemical work station produced by PerkinElmer, and conducted in an one-compartment Teflon electrochemical cell, fitted with a Saturated Calomel Electrode (SCE) and a Platinum wire as reference and counter electrodes respectively. The gold substrates were mounted at the bottom of the electrochemical cell using an O-ring, which defines a geometric area of 0.57 cm^2. The electrolyte solution, NaOH 0.5 M or $K_4Fe(CN)_6$ 1 mM in KCl 1 M, was de-aerated with nitrogen (99.999%) for 30 min. All measurements were performed at room temperature (20±2 °C).

2.7. Ellipsometry

Ex situ ellipsometric data were obtained with a rotating analyzer type ellipsometer SE 400 (SENTECH Instruments GmbH, Berlin, Germany) fitted with a He–Ne laser (λ=632.8 nm). The measurements were carried out at an angle of incidence of 70°.

2.8. Atomic Force Microscopy

The measurements were performed in a Nanoscope IIIa Multimode AFM Microscope (Digital Instruments, Veeco) in *tapping* mode using etched silicon probes (oscillation frequency of about 300 kHz).

3. Results and discussions

3.1. Characterization of SAMs

3.1.1. Cyclic voltammetry

Electrochemical reductive desorption is one of the most used methodologies to characterize modified surfaces with self-

assembled monolayers [31–33]. The cyclic voltammograms recorded at 20 mV s^{-1} in 0.5 M NaOH of the different modified electrodes under study are depicted in Fig. 1. The sharp cathodic peak typically represents the SAM reductive desorption from gold (111) and is seen to occur at −1.03 and − 0.94 V for the COOH- and CH_3-terminated SAM respectively, while the desorption of the mixed system, with 50% of 11-Mercaptoundecanoic acid, takes place at intermediate values, i.e. − 0.99 V. In this case, the appearance of only one main desorption peak points to the absence of phase separation of the pure components in the binary SAMs, which can be indicative of a good distribution of linking groups on the surface.

It is well known that the peak potential, E_p, of the alkanethiols reductive desorption changes with the length of alkyl chain and also with the terminal group nature. The dependence of E_p on the first parameter has been interpreted [31] in terms of the required energy to establish a potential gradient through the monolayer able to induce the ion flux to the electrode, which supports the reductive reaction. Accordingly, as the alkyl chain length increases, the potential to observe the desorption becomes more negative. Another important contribution for the E_p value is the adsorbed thiols intermolecular interactions; it is widely confirmed that the chain–chain van der Waals interaction increases with the alkyl chain length. In the case of COOH-terminated thiol, along with the chain–chain attractive interaction, the electrostatic repulsive interaction among the carboxylic acid groups should be taken into account due to the complete dissociation of the carboxylic acid groups into carboxylate ions in 0.5 M NaOH solution. Since the 11-Mercaptoundecanoic acid chain length is about twice that of the 1-Hexanethiol, this shall be the major factor for the observed 90 mV difference between the corresponding main desorption peaks.

It is worth to note that all the voltammograms in Fig. 1 exhibit one main cathodic peak followed by a much smaller one at more negative potential values. The second peak is most probably related with the desorption from more energetic sites with other gold crystallinities, present in small fraction after the flame-annealing process, such as (110), where thiols are strongly adsorbed in the pseudo-four-fold hollow sites [29,30].

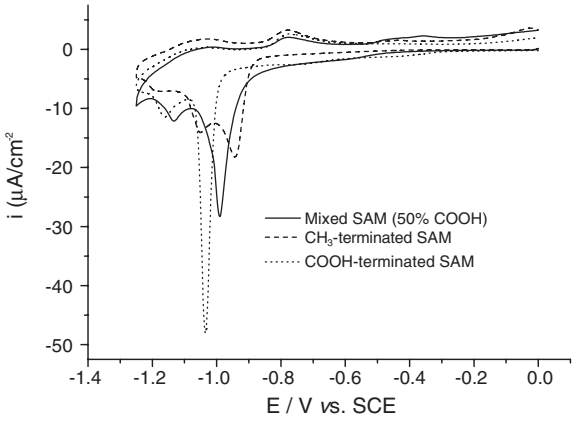

Fig. 1. Cyclic voltammograms for gold electrodes modified with SAMs in 0.5 M NaOH at 20 mV s^{-1}.

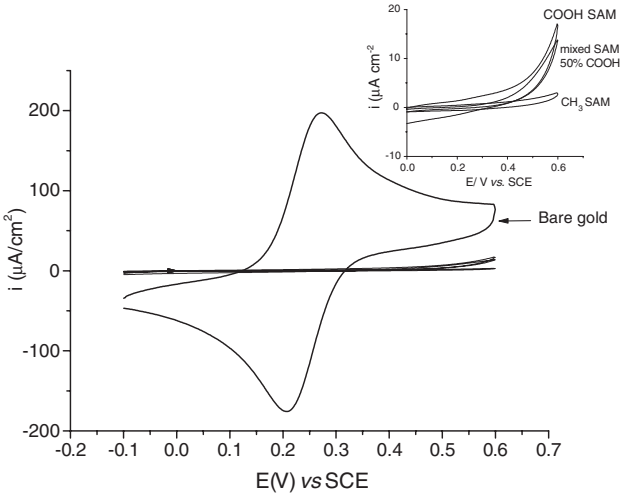

Fig. 2. Cyclic voltammograms at bare gold electrode and gold electrode coated with pure COOH, CH_3 and mixed 50% COOH SAMs in $K_4Fe(CN)_6$ 1 mM + KCl 1 M at 100 mV s^{-1}. The inset shows the three different SAMs in detail.

The amount of adsorbed thiols can be evaluated through the charge involved in the reductive desorption [32]. From the data shown in Fig. 1, the surface concentrations on gold surfaces have been estimated as 7.1×10^{-10} and 6.8×10^{-10} mol cm^{-2} for COOH-terminated thiol and CH_3-terminated thiol SAMs respectively, while 7.4×10^{-10} mol cm^{-2} has been found for the total thiolate species in mixed SAM. The presence of only one main reduction peak for the desorption of mixed SAMs rules out the possibility of estimating the ratio of methyl and COOH on the surface by electrochemical stripping of the monolayer. Surface coverage values are close to the geometrically retrieved for a close-packed monolayer, 7.6×10^{-10} mol cm^{-2} [31], indicating that the monolayers formed by the alkanethiols must display such type of organization.

The electrochemical behavior of bare gold electrode and gold electrodes coated with the three above mentioned SAMs towards the redox conversion of $K_4Fe(CN)_6$ in 1 M KCl has been studied and the obtained cyclic voltammograms are presented in Fig. 2. The well defined oxidation and reduction peaks denoted at the bare gold electrode, are suppressed at the modified gold electrodes showing that all the three SAMs provide a good barrier to the charge transfer, as expected for densely packed monolayers on gold electrodes [2].

3.1.2. Contact angle measurements

Contact angle measurements with water provided information on the hydrophobic nature of the SAMs under study: the contact angle for CH_3-terminated SAM (93 ± 1°) demonstrate its hydrophobicity, while for a pure COOH SAM the obtained value, 30 ± 1°, reveals its hydrophilic character, in conformity with data reported in the literature [34]. The value observed for a mixed SAM, with 20% of 11-Mercaptoundecanoic acid in the deposition solution, was slightly lower than the one for a pure CH_3-terminated SAM, but still in agreement with a highly hydrophobic SAM (87 ± 1°).

3.2. Proteins physical adsorption on SAMs

In order to study the influence of the different surface properties on the protein physical adsorption on the prepared SAMs (Au/COOH, Au/COOH : CH_3, Au/CH_3) hIgG has been used as a model protein for the related experiments. The modified gold substrates were immersed for 30 min into 0.1 mg/ml hIgG solution with and without the addition of 1% Tween 20. The surfaces were then characterized by ellipsometry. The ellipsometric parameters were measured before and after hIgG adsorption and the values have been converted into surface concentration, Γ, using estimated thickness data [35] and according to the following equation [36],

$$\Gamma\ (\text{ng mm}^{-2}) = k \times \text{thickness (nm)}$$

where k is the density of the protein (1.36 g ml^{-1}). The surface concentration values obtained for adsorbed hIgG, with and without the addition of Tween 20 into the protein solution, onto the mixed and pure COOH and CH_3 SAMs are compiled in Table 1.

In the absence of Tween 20, hIgG adsorbs on the three types of SAMs. The hIgG coverage is 0.386 μg cm^{-2} on COOH-terminated SAM and 0.506 μg cm^{-2} on CH_3-terminated SAM, while for all mixed SAMs the average coverage is 0.48 μg cm^{-2}. These results support the enhancement of physical adsorption of hIgG onto hydrophobic SAMs.

It can be seen in Table 1 that hIgG physical adsorption, possibly due to electrostatic interactions, still occurs on pure COOH-terminated SAM and on mixed SAM with 50% of 11-mercaptoundecanoic acid, even though 1% of Tween 20 has been added into the hIgG solution. However, it can be noticed that the coverage of hIgG on those two SAMs are lower when 1% Tween 20 is added to the solution. Indeed, in the presence of Tween 20, no hIgG physical adsorption on CH_3-terminated SAM and mixed SAMs with 20%, 10% and 5% of 11-mercaptoundecanoic acid have been detected by ellipsometry.

The total inhibition of protein adsorption onto highly hydrophobic SAMs by Tween 20 has been confirmed by AFM. Fig. 3 shows a gold modified electrode with a pure CH_3 SAM after contacting with a hIgG solution without (a) and with (b) the addition of surfactant. The absence of globular features (hIgG single molecules and agglomerates) in Fig. 3b demonstrates that Tween 20 is able to completely block the hIgG physical adsorption on hydrophobic SAMs. It was reported [12] that detergents are able to remove the protein from the surface through the formation of aggregates of detergent or aggregates

Table 1
Surface concentration of hIgG adsorbed physically on SAMs in the presence and absence of Tween 20

SAMs	Surface concentration of physically adsorbed hIgG/μg cm^{-2}					
	COOH	50% COOH	20% COOH	10% COOH	5% COOH	CH_3
HIgG	0.386	0.495	0.495	0.422	0.514	0.506
HigG/Tween 20	0.354	0.128	~0	~0	~0	~0

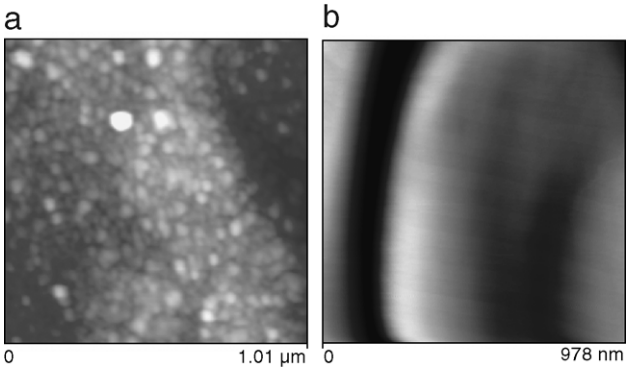

Fig. 3. AFM images of gold modified CH_3-terminated SAM after immersion in hIgG solution without (a) and with (b) the addition of 1% Tween 20.

of protein and detergent. From both ellipsometric and AFM measurements there is no evidence for Tween 20/hIgG agglomerates on the surface, which supports this blocking mechanism.

To ascertain the blockage effect of Tween 20 for the physical adsorption of biomolecules in serum on hydrophobic SAMs, the gold substrates modified with CH_3-terminated SAM and mixed SAMs (20%, 10% and 5% of 11-mercaptoundecanoic acid) were immersed, for 30 min, into anti-hIgG serum added of 1% Tween 20. No physical adsorption of biomolecules on these SAMs has been detected by ellipsometry or observed by AFM. Thus, Tween 20 can be considered effective to minimize the physical adsorption of hIgG by hydrophobic interaction on hydrophobic surface. On the other hand, the physical adsorption of hIgG observed on the COOH-terminated SAM and on the mixed SAM with 50% of 11-mercaptoundecanoic acid, is very likely the consequence of electrostatic interactions between hIgG and most probably charged SAMs. According to the literature, it is expected that surface COOH, with a pK_a of about 6.5 [37] is, at least, partially deprotonated at pH 7.4. At this pH it is possible that hIgG can adsorb electrostatically to the SAM, since the value is close to its reported pI (between 5.0 and 8.0) [38].

3.3. Protein covalent adsorption on SAMs

For the covalent protein immobilization, the COOH terminal groups in mixed SAMs were previously activated with the coupling agents (EDC/NHS) followed by immersion in hIgG PBS solution in the presence of Tween 20.

The surface concentration, evaluated by ellipsometry, of covalently attached hIgG onto mixed SAMs prepared from 20%, 10% and 5% COOH, were 0.109, 0.082 and 0.068 μg cm^{-2}, respectively (Table 2). The coverages are considerably lower than those achieved by physical adsorption (Table 1) without Tween 20 (close to 0.5 μg cm^{-2}), but significant when compared with non-measurable values obtained with the addition of surfactant. These observations indicate that hIgG adsorption only occurred at the surface carboxylic groups and corroborate the powerful effect of Tween 20 in preventing physical non-specific adsorption due to hydrophobic interactions.

Table 2
HIgG and anti-hIgG surface concentration values obtained on pure SAMs (COOH and CH_3-terminated thiols) and on mixed SAMs (20%, 10% and 5% of COOH-thiol in the deposition solution)

SAMs	Surface concentration/μg cm^{-2}		
	hIgG	Anti-hIgG	Anti-hIgG / hIgG
COOH [a]	0.381	0.517	1.35
CH_3 [a]	0.503	0.571	1.14
20% COOH [b]	0.109	0.272	2.49
10% COOH [b]	0.082	0.218	2.65
5% COOH [b]	0.068	0.218	3.20

[a] IgG physical adsorption without the addition of Tween 20.
[b] Covalent immobilization of hIgG with addition of Tween 20.

The difference between surface concentrations of hIgG on the mixed SAMs is not very large, yet it increases with the ratio of the COOH-terminated thiol in the deposition solution.

AFM is used to examine the distribution of hIgG molecules on the modified surfaces. Before hIgG adsorption AFM images of pure an mixed SAMs cannot be distinguished; however, there are significant differences in the distribution of the covalently immobilized protein in mixed SAMs, as demonstrated in Fig. 4, for the 20% and 10% COOH SAMs. In Fig. 4b (10% COOH SAM) there is a large fraction of the surface that is not covered by IgG molecules, which should correspond to the hydrophobic domains of CH_3-terminated SAMs. In contrast, the AFM image of 20% COOH SAM (Fig. 4a) shows a highly protein covered surface, as the result of the presence of a greater number of available COOH binding sites. These observations agree with the ellipsometric data, since the larger amount of protein was obtained for the 20% COOH SAM. It is worth to point out, that the amount of protein on the surface depends not only from the number of COOH moieties but also from their distribution on the surface. Nevertheless, there is no doubt that distinct ratios of COOH : CH_3 in SAM deposition solution are responsible for different protein patterns on the surface.

3.4. Detection of interaction between hIgG and anti-hIgG

It is known that the detection of adsorbed proteins and protein interactions on surfaces requires sensitive methods, since the amount per unit area is usually extremely low. In this work, both ellipsometry and chronoamperometry have been used to detect the interaction of immobilized human IgG with its antibody.

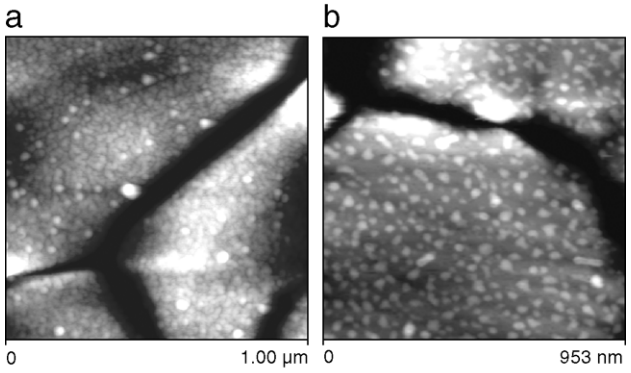

Fig. 4. AFM images of hIgG covalently attached on gold modified with (a) 20 and (b) 10% (11-mercaptoundecanoic acid in the deposition solution) SAMs.

3.4.1. Ellipsometric measurements

Table 2 compiles surface concentration values, obtained by ellipsometry, for immobilized hIgG and further interaction with anti-hIgG in the presence of 1% Tween 20, as well as the ratio between the two layers. It is worth noting that without the addition of Tween 20, non-specific adsorption between anti-hIgG and exposed hydrophobic sites on the surface would take place. From the data analysis, it is clear that the ratio of anti-hIgG / hIgG is higher for the covalently adsorbed hIgG on mixed SAMs than for the physically adsorbed molecules on pure SAMs, where the ratio is closer to one. On mixed SAMs, each hIgG molecule is able to bind more than one polyclonal anti-hIgG, and the ratio increases with decreasing content of $HOOC(CH_2)_{10}SH$ in the deposition solution. The differences may arise from the combination of two factors, steric hindrance on pure COOH and CH_3 SAMs, since surface concentrations of hIgG are considerably higher than those on the mixed monolayers and the fact that hIgG might be oriented in a favourable position for antibody interaction due to covalent immobilisation.

3.4.2. Chronoamperometric measurements

Chronoamperometry is a sensitive method which does not require the labeling of reactants [17]; however, the label-free detection is unable to distinguish specific bound compounds from nonspecific adsorbed molecules, which in the present case is overcome with the incorporation of mixed hydrophobic SAMs and Tween 20.

Fig. 5 shows the current transients obtained under polarization at $E = 300$ mV, in $K_4Fe(CN)_6$ 1 mM + KCl 1 M solution, for the CH_3-terminated SAM modified gold electrode, before and after immersion in 0.05 mg/ml hIgG solution over 5 s, and then immersion for 30 min in 0.1 mg/ml anti-hIgG serum with the addition of 1% Tween 20. An obvious decrease in the current is observed, at the applied potential where the oxidation of $Fe(CN)_6^{4-}$

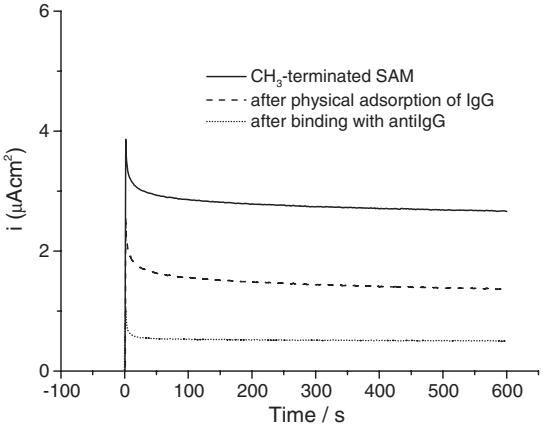

Fig. 5. Chronoamperometric assays of gold electrode coated with CH_3-terminated SAM before and after hIgG physical adsorption for 5 s, and then bound with anti-hIgG. Measurements were performed in $K_4Fe(CN)_6$ 1 mM + KCl 1 M at constant potential, $E = 300$ mV.

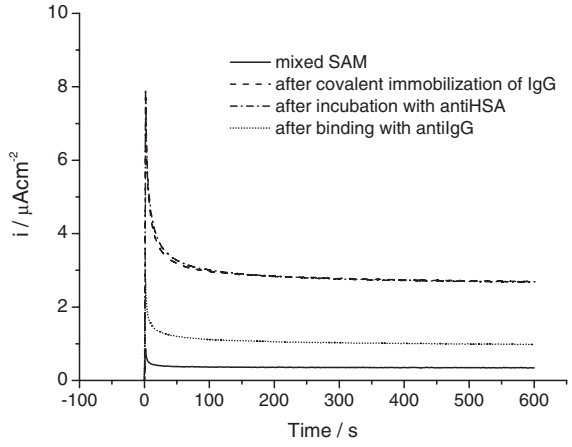

Fig. 6. Chronoamperometric assays of gold electrode coated with mixed SAMs before and after hIgG covalent immobilization, then incubation with control anti-HSA serum and finally bound with anti-hIgG. Measurements were performed in 1 mM $K_4Fe(CN)_6$ in 1 M KCl at constant potential, E=300 mV.

occurs, after hIgG physical adsorption; incubation with anti-hIgG serum causes a further decrease. This result demonstrates that although a large decrease of the current of the redox couple $Fe(CN)^{4-}_6$ / $Fe(CN)^{3-}_6$, was observed by cyclic voltammetry after the deposition of CH_3-terminated SAM (Fig. 2), chronoamperometry is sensitive enough to detect current changes either due to little amount of adsorbed hIgG or to the interaction between hIgG and anti-hIgG on the surface of the electrode.

Electrochemical data have been supported by ellipsometric measurements, using the same surface modification conditions. The amount of hIgG adsorbed on CH_3-terminated SAM, after 5 s physical adsorption and of the complex layer of hIgG/anti-IgG were 0.150 and 0.313 μg cm^{-2}, respectively.

The chronoamperometric curves observed in $K_4Fe(CN)_6$ 1 mM+KCl 1 M for the mixed SAM with 20% of 11-mercaptoundecanoic acid, before and after covalent immobilization of hIgG are shown in Fig. 6. Instead of the expected decrease in the current, the hIgG covalent immobilization causes an increase in the electrical signal. This might be explained by a decrease in the number of negatively charged carboxylic groups, as a result of the covalent protein coupling.

Fig. 6 also shows the chronoamperometric responses of the chemically bound hIgG after immersion in 0.1 mg/ml anti-HSA serum added of 1% Tween 20 for 30 min. It is clearly observed that no decrease in the current occurs after incubation with anti-HSA serum, indicating that the nonspecific adsorption from antiserum is totally blocked by the Tween 20. In contrast, a notable decrease in the current takes place as a result of hIgG binding with its antibody after immersion in 0.1 mg/ml anti-hIgG serum in the presence of 1% Tween 20, revealing again the appropriateness of chronoamperometry to detect the immunosensor specificity.

4. Conclusions

The feasibility of using SAMs of alkanethiols as interface for biosensors in combination with a non-ionic detergent, Tween 20, was successfully investigated in this paper. SAMs were prepared from 1-hexanethiol, 11-mercaptoundecanoic acid, and from the mixture of these two thiols. The electrochemical results confirmed the presence of stable, densely packed and organized pure and mixed monolayers, which provide good barrier to the electron transfer of soluble $K_4Fe(CN)_6$.

Physical and covalent adsorption of hIgG molecules on pure and mixed SAMs was detected by ellipsometry. It was proved that Tween 20 could completely block the physical adsorption of biomolecules on hydrophobic CH_3-terminated SAMs and mixed SAMs with few COOH terminal groups. Covalent attachment in mixed SAMs yields a better distribution of hIgG on the surface and probably their adequate orientation, since the adsorbed molecules could bind more antibody than the physically immobilized hIgG in pure SAMs. It was shown that a lower ratio of COOH-terminated thiol in SAM deposition solution (5% COOH and 95% CH_3) exhibits the most favorable interface regarding hIgG immobilization and its antibody recognition. In this case, the average ratio of anti-hIgG / hIgG was about three.

It was also demonstrated that the specific interaction of physical and covalently attached hIgG with its antibody in the antiserum can be efficiently detected by chronoamperometric measurements.

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