Stability of Mixed PEO−Thiol SAMs for Biosensing Applications

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The secret of a successful affinity biosensor partially hides in the chemical interface layer between the transducer system and the biological receptor molecules. Over the past decade, several methodologies for the construction of such interface layers have been developed on the basis of the deposition of self-assembled monolayers (SAMs) of alkanethiols on gold. Moreover, mixed SAMs of polyethylene oxide (PEO) containing thiols have been applied for the immobilization of biological receptors. Despite the intense research in the field of thiol SAMs, relatively little is known about their biosensing properties in correlation with their long-term stability. Especially the impact of the storage conditions on their biosensing characteristics has not been reported before to our knowledge. To address these issues, we prepared mixed PEO SAMs and tested their stability and biosensing performance in several storage conditions, i.e., air, N_2, ethanol, phosphate buffer, and H_2O. The quality of the SAMs was monitored as a function of time using various characterization techniques such as cyclic voltammetry, contact angle, grazing angle Fourier-transform infrared spectroscopy, and X-ray photoelectron spectroscopy. In addition, the impact of the different storage conditions on the biosensor properties was investigated using surface plasmon resonance. Via the latter technique, the receptor immobilization, the analyte recognition, and the nonspecific binding were extensively studied using the prostate specific antigen as a model system. Our experiments showed that very small structural differences in the SAM can have a great impact in their final biosensing properties. In addition it was shown that the mixed SAMs stored in air or N_2 are very stable and retain their biosensor properties for at least 30 days, while ethanol appeared to be the worst storage medium due to partial oxidation of the thiol headgroup. In conclusion, care must be taken to avoid SAM degradation during storage to retain typical SAM characteristics, which is very important for their general use in many proposed applications.

Introduction

Over the past century, biosensors have become an important research topic because they provide a promising and convenient alternative to conventional analytical methods for monitoring (bio)chemical substances in various fields such as medicine, environment, fermentation, and food processing.[1−4] In general, a biosensor consists of a biological layer which is connected to a transducer system by a chemical interface layer. Since this chemical layer constitutes the interface with the sample, this component strongly determines the stability, the specificity, and the reproducibility of the sensor as a whole. Especially, nonspecific signals due to interferents form a major problem in diagnostic applications, where an analyte at low concentration must be detected in the presence of an excess of nonspecific molecules. The construction of a specific and stable interface layer is therefore mandatory for the final biosensor application.

Over the past decade researchers have found that self-assembled monolayers (SAMs) of alkanethiols exhibit excellent properties for this particular purpose, because they are easy to prepare and form well-defined thin films on gold surfaces.[5−7] More specifically, mixed SAMs have often been applied for the covalent immobilization of biological receptors.[8−13] In this approach, two different alkanethiols are deposited simultaneously on the gold surface, creating a mixed SAM structure incorporating various functional groups. One thiol molecule carries a functional group that is capable of covalently binding the biological receptors (i.e., an antibody in immunosensor applications). The second thiol is a molecule incorporating a functional group (e.g., OH or polyethylene glycol) which shows excellent properties to reduce the nonspecific adsorption of undesired entities, such as serum proteins.[14−17] The incorporation of both alkanethiols in a unique

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mixed SAM enables full control over the number of functional groups, the steric hindrance, and the nonspecific adsorption. The resulting SAMs have proven to be very attractive as a platform for the development of immunosensor interfaces for real diagnostical applications.

In view of the excellent immunosensor properties of these mixed SAMs, it is surprising that so few data are available in the literature about their long-term stability. Those reports solely describe the characterization of the SAMs as a function of the storage time in various media without evaluating the final biosensor performance.[18−20] Nevertheless, for commercialization of such biosensors, these parameters are of the utmost importance and cannot be disregarded in our opinion.

To address this need, we performed a full characterization of a mixed SAM in different dry (air and N_2) and wet (ethanol, H_2O, and phosphate-buffered saline (PBS)) storage conditions over a period of 30 days. The surface characterization of the mixed SAMs was performed using various characterization tools, i.e., contact angle (CA), cyclic voltammetry (CV), grazing angle Fourier-transform infrared spectroscopy (GA-FTIR), and X-ray photoelectron spectroscopy (XPS). In addition, the changes in molecular architecture derived from these characterization methods were correlated with the immunosensing properties of the mixed PEO−thiol SAM. Finally, the immunosensor performances were investigated using surface plasmon resonance spectroscopy (SPR). In this respect, we targeted the detection of prostate specific antigen (PSA) as a model antigen. More specifically we investigated the influence of the SAM storage in the different dry and wet media on the three most important immunosensor parameters, i.e., the immobilization degree of an anti-PSA monoclonal antibody, the specific PSA recognition signals, and the nonspecific adsorption.

Experimental Section

Materials. All materials and reagents were used as commercially received. Carboxylic acid-capped hexa(ethylene glycol) undecanethiol ($SHC_{11}(PEO)_6COOH$) was obtained from SensoPath Technologies, and (1-mercapto-11-undecyl)tri(ethylene glycol) ($SHC_{11}(PEO)_3OH$) was synthesized as described previously.[21] Ultrapure ethanol and 1-piperazineethanesulfonic acid 4-(2-hydroxyethyl) monosodium salt (HEPES) were purchased from Riedel-DeHaën. 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanolamine were obtained from Biacore. Human prostate specific antigen (PSA) and normal female serum were obtained from Scipac, while the PSA66 monoclonal antibody (anti-PSA) was purchased from Fujirebio Diagnostics. $K_4[Fe(CN)_6]$, $K_3[Fe(CN)_6]$, KCl and Tween 20, ethylenediaminetetraacetic acid (EDTA), and glycine−HCl were received from Sigma-Aldrich.

Preparation of Gold Substrates and Mixed SAMs. The gold substrates for CV, GA-FTIR, CA, and XPS were fabricated by electron beam evaporation of 10 nm Ti and 100 nm Au on a polished 6 inch silicon wafer baring 1.2 μm thermally grown SiO_2. The same method, with 2 nm Ti and 50 nm Au, was followed for preparing the gold SPR samples. Prior to self-assembly, cleaning of these substrates involved rinsing with acetone and incubation for 15 min in an UV/O_3 chamber provided with an ozone-producing mercury grid lamp (BHK Inc.) to remove all organic contaminants on the gold substrates.[22] Immediately after being cleaned, the gold substrates were immersed in the appropriate mixture of 1 mM thiols in ethanol. This solution was obtained by mixing 1 mM $SHC_{11}(PEO)_6COOH$ and 1 mM $SHC_{11}(PEO)_3OH$ at a volume ratio of 5:95 v/v %. After 3 h of SAM deposition, the substrates were rinsed with ethanol, dried under a stream of N_2, and stored at room temperature in separate falcon tubes, in a dark cabinet, containing the different dry (i.e., air and N_2) and wet (i.e., ethanol, H_2O, and phosphate buffer) storage media.

Instrumentation. CA measurements were performed on 1 μL sessile drops of ultrapure water with an OCA 20 system from Dataphysics using SCA 20 software. The reported CA values were averaged over at least five distinct spots on two separate samples.

CV experiments were performed with a homemade electrochemical cell with a Pt counter electrode and a Ag/AgCl micro reference electrode (Microelectrodes Inc.). The setup uses a Gamry Instruments potentiostat with Framework software. All experiments were performed in a 1 mM $K_3Fe(CN)_5/K_4Fe(CN)_6$ solution with 0.1 M KCl as the background electrolyte. The CV response was found to be independent of the scan number. Therefore, current responses were only shown for the seventh scan.

GA-FTIR measurements were performed on a Bruker IFS 66V/S over a wavenumber range 4000−500 cm^{-1} in combination with Opus software. The spectra are the result of the Fourier transformation of 2048 interferometric scans obtained with a resolution of 1 cm^{-1}.

XPS spectra were collected on a Theta300 spectrometer (Thermo Electron Corp.) equipped with a monochromatized aluminum Kα source. The constant pass energy was set at 100 eV. All binding energies were referenced to the CH_x component of the C 1s peak at 284.6 eV. All spectra were fitted using software developed at the FUNDP-LISE, using mixed Gaussian−Lorentzian functions and a Shirley background.

Biological immunosensing experiments were performed using the Biacore2000 surface plasmon resonance in combination with Biacore2000 Control Software, version 1.3. The operating temperature was set at 20 °C. Prior to an experimental run, the surface was cleaned by two 5 μL injections of 10 mM glycine−HCl (pH 2.2). Afterward, a continuous flow of 5 μL/min of Hepes-buffered saline (HBS) (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween20, pH 7.4) was maintained during the antibody coupling. Covalent immobilization of an anti-PSA monoclonal antibody on the mixed SAMs was accomplished via random coupling via their primary amines. Hereby, the carboxylic groups of the SAMs were activated by injecting 50 μL of a solution containing 0.1 M NHS and 0.4 M EDC in a 1:1 ratio. Next, 132 μL of antibody solution (500 μg/mL in 10 mM acetate buffer pH 5) was injected, followed by a pulse of 50 μL of 1 M ethanolamine, pH 8.5, to block the remaining NHS esters. Two 5 μL injections of 10 mM glycine−HCl (pH 2.2) were introduced to remove the noncovalently bound antibodies from the surface. Analyte solutions containing different PSA concentrations were prepared in HBS buffer and injected over the antibody-activated surface at a flow rate of 20 μL/min for 6.5 min. Between each PSA injection, the antibody-activated surface was regenerated by two pulses of 10 mM glycine−HCl (pH 2.2). A similar procedure was performed for 2× diluted normal female serum in HBS to evaluate the occurrence of nonspecific adsorption.

Results and Discussion

SAM Characterization as a Function of Storage Condition and Time. Understanding the stability of thiol monolayers on gold is essential to determine the range of applications in which they can be used. For example, stability is of the utmost importance, if SAMs need to be applied as a biosensor interface in real diagnostic applications. Therefore, we evaluated the stability of a 5% mixed PEO−thiol SAM (Figure 1) after preservation in various storage media. The characterization of

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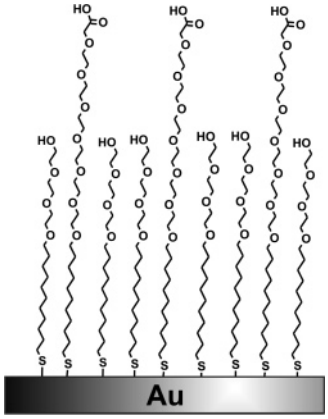

Figure 1. Schematic representation of the 5% mixed PEO−thiol SAM deposited onto a gold sensor surface.

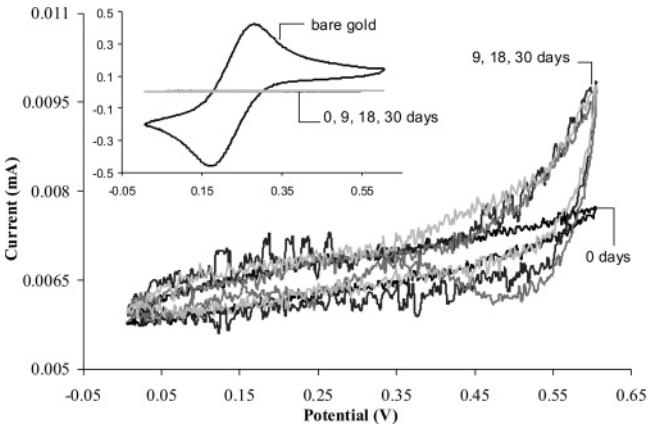

Figure 2. Cyclic voltammogram of the mixed PEO−thiol SAM after immersion for 0, 9, 18, and 30 days in N_2. The insert shows all CV's, plus the one of the uncoated bare Au samples.

the SAMs was performed using various techniques, i.e., CA, CV, GA-FTIR, and XPS. These complementary characterization tools allow us to identify minor changes in the SAM quality and to correlate these changes to the applied storage conditions.

First the SAM stability was characterized using CV and CA measurements. These methods have previously been reported as evaluation tools for the stability of SAMs and allow us to identify potential differences in surface coverage and surface wettability. Figure 2 shows the CV results for the mixed SAM immediately after deposition (0 days) and after storage for 9, 18, and 30 days in N_2. Compared to the uncoated bare Au sample, the cyclic voltammogram of the mixed SAMs shows a highly reduced oxidation and reduction current which reveals a strong passivation of the gold electrode surface. This indicates that the mixed SAM forms a densely packed monolayer. On the other hand, after storage for 9, 18, and 30 days in N_2, the peak current densities slightly increased as compared to the initial behavior but remained more or less stable over the storage period. Similar results were obtained for the SAMs stored in air, ethanol, H_2O, and PBS (data not shown). Comparable conclusions could be drawn from the CA measurements. The initial water CA for the mixed PEO−thiol SAM, 31° ± 2°, corresponds well to previously reported values[23] and supports the expected hydrophilic nature of the mixed PEO−thiol SAM. After storage of the samples, a small increase in the CA was observed as a function of storage time for all conditions (Table 1). This can most probably be explained by a change in surface structure or surface contamination. The latter observations are in contrast with previously reported data.[24−27] For example, Flynn et al.[27] have investigated the stability of a PEO-functional alkanethiol SAM on gold in PBS. After 15 days of preservation, they observed an abrupt change in the CA values. Furthermore, their CV results showed a significant deviation from the initial behavior after 7 days of storage and the current densities of both the anodic and cathodic peaks continued to increase over the course of 35 days. These discrepancies in comparison with previous studies can most probably be attributed to a combination of several possible causes. A proper cleaning of the Au substrate, an optimized SAM deposition procedure, the use of long alkanethiols (creating more lateral van der Waals interactions), and the introduction of a long PEO chain with terminal carboxylic end groups (creating more hydrogen bonding) may increase the SAM stability.[24,27−30] In addition, since the major mechanism for degradation of alkanethiol SAMs is dependent on the availability of oxygen in the presence of UV light,[19,29] all preservations were performed in complete darkness.

For a more detailed characterization of the SAMs, XPS and GA-FTIR measurement were performed. These characterization methods allow us to evaluate potential changes in the atomic composition and chemical nature of the thiol SAMs on the gold substrate. The low-resolution survey and the high-resolution (S 2p, O 1s, C 1s) XPS spectra of the mixed PEO−thiol SAM were recorded, which confirmed the presence of the expected elements in the alkanethiol monolayer by the occurrence of carbon, oxygen, and sulfur peaks compared to a bare gold sample. When the mixed PEO−thiol SAM was preserved in dry conditions (i.e., air and N_2), only minor changes were observed, indicating a high stability of the SAM in these media. As discussed before, the observations are in contrast with previously reported literature data.[29,31−33] For example Schoenfisch et al.[33] have investigated the stability of a CH_3-functionalized alkanethiol in air. A rapid and complete loss in intensity of the thiolate sulfur 2p peak (∼162 eV) occurred upon air exposure for 24 h, and a new sulfur 2p peak appeared at 168 eV. They explained this observation by the rapid oxidation of the sulfur headgroup upon air exposure. This oxidation could not be revealed at all from our XPS measurements on SAMs stored under dry conditions after 4 weeks of storage (Table 2). Only a small amount of carbon contamination can be distinguished which was attributed to an increase in the *C*−C peak area and a decrease in the *C*−O peak area, both in the C 1s subregion (data not shown).

In contrast to the high stability of the SAM in dry storage conditions, some minor changes were observed in the SAM preserved in the wet storage media. The XPS results (Table 2) clearly indicate the higher amount of O and S present on the SAM samples stored in ethanol for 4 weeks compared to the other wet storage media. A closer look into the S 2p region showed the appearance of a new peak arising at 168 eV. As

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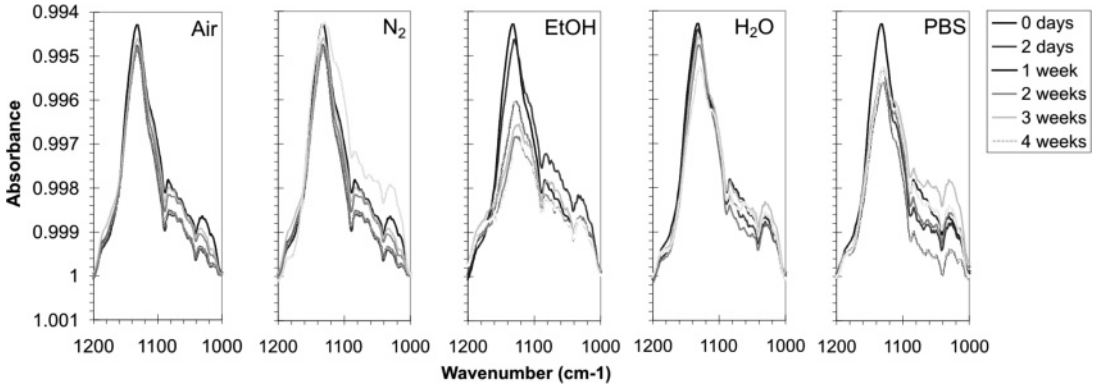

Figure 3. GA-FTIR spectrum of the C−O/C−C stretching region upon exposure to different media as a function of time.

Table 1. Contact Angle Data (deg) for the Mixed PEO−Thiol SAM Deposited on a Au Substrate after Storage for 0−30 days in the Different Storage Media

	storage media				
storage time (days)	air	N_2	EtOH	H_2O	PBS
0	31 ± 2	31 ± 2	31 ± 2	31 ± 2	31 ± 2
1	34 ± 2	32 ± 1	30 ± 1	35 ± 1	35 ± 2
9	34 ± 1	33 ± 1	33 ± 2	31 ± 1	35 ± 2
18	35 ± 1	35 ± 2	35 ± 1	35 ± 1	36 ± 1
30	37 ± 2	34 ± 1	38 ± 3	36 ± 1	35 ± 1

Table 2. XPS Surface Elemental Concentrations and Peak Areas of Simulated Peaks in the S 2p Region, after Storage for 4 Weeks into Different Storage Media (Air, N_2, Ethanol, H_2O, PBS)

	surface elemental concns (%)					
				S		
storage medium	Au	C	O	S−Au (161 eV)	S−H/S−C (163 eV)	SO_4/SO_3 (168 eV)
air	24.2	55.6	18.0	1.0	1.2	
N_2	25.1	55.5	17.4	0.8	1.2	
EtOH	22.1	54.1	20.6	1.4	1.6	0.2
H_2O	23.0	57.2	18.0	0.7	1.1	
PBS	23.8	57.0	17.4	0.6	1.2	

previously reported, this peak can be assigned to oxidized sulfur species (SO_4 and SO_3).[32,34−36] This oxidation of the sulfur head groups can give rise to conformational loss and as a consequence could create a less dense monolayer on the gold substrate. Due to this phenomenon, the sulfur signal is attenuated less by the overlying carbon chains. The higher amount of sulfur species observed for the SAM stored in ethanol could also suggest that some thiol molecules were desorbed from the Au surface through oxidation and as a consequence are located at the outermost SAM surface.

To support these XPS observations, FTIR spectroscopy was applied to investigate possible conformational changes in the SAM after storage. No changes were observed in the GA-FTIR spectra for the mixed PEO−thiol SAM after preservation in dry conditions. The two absorption bands at ∼2855 and ∼2920 cm^{-1}, which are ascribed to the symmetric and asymmetric CH_2-stretching bands of the C_{11}-methylene units, indicate a well-ordered alkanethiol film for all dry storage conditions[37] (data not shown). Upon exposure to H_2O and PBS, only minor changes were observed in the initial band height of the C−O/C−C stretching vibration at ∼1132 cm^{-1} (Figure 3). This suggests only a minor change in PEO conformation, probably induced by water entrapment in the SAM during storage. In contrast, upon storage in ethanol, the initial band height of the C−O/C−C stretching vibration (Figure 3) and the ether CH_2 twisting band at ∼1261 cm^{-1} (data not shown) decrease significantly. In addition, the bandwidth of the C−O stretching vibration increases considerably. This observation suggests that the PEO chain undergoes a transition from a highly uniform structure into a less dense amorphous structure.[38] The same effects can be observed for the samples stored in ethanol in the CH stretching modes region (data not shown). The $\nu_a(CH_2)$ band shifts to a position of 2922 cm^{-1}, compared to the initial band position of 2920 cm^{-1}. These observations could not be observed upon storage in the 2 other wet storage media (H_2O and PBS). Probably the different solubility of the thiols plays an important role. The mixed thiols dissolve more easily in the following order of storage media: ethanol > H_2O > PBS.

In conclusion, the traditional characterization tools only reveal significant changes after storage in ethanol, while storage in other media showed only minor effects on the SAM characteristics. For ethanol, partial oxidation of the thiol headgroup occurred, suggesting that a less dense and less ordered SAM was formed as a function of time. The performed characterization only gives an indication but no definite answer on questions such as, e.g., "Will the SAM after storage still be useful to perform biosensing experiments?". To learn more, SPR was used to investigate the effect of the SAM stability on the immunosensor properties.

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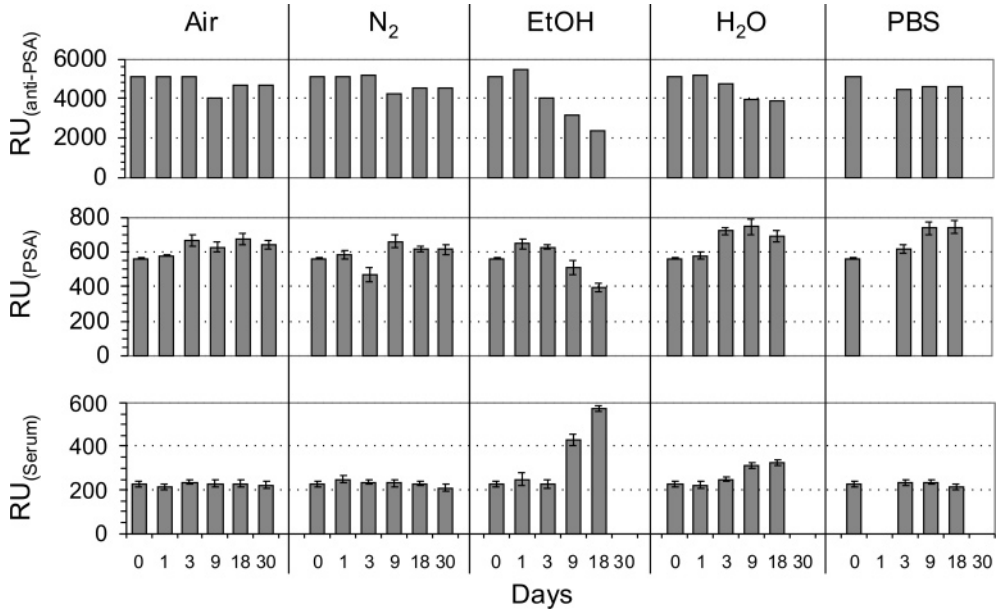

Figure 4. SPR signals for the mixed PEO−thiol SAM, after storage in the five different storage media, for a period of 30 days. In the graph, the anti-PSA immobilization levels are shown together with the recognition signals of 1 μg/mL PSA and the nonspecific adsorption of normal female serum.

Immunosensing Properties of the Mixed PEO−Thiol SAM. The aim of the performed SPR experiments is to investigate the biosensing performance after preservation of the mixed PEO−thiol SAMs in the different storage media. In addition, we correlated the observed changes in the SAM characteristics, as described above, with these biosensor properties. As a model system, we studied the direct detection of prostate specific antigen (PSA) by an anti-PSA monoclonal antibody. Figure 4 gives an overview of the anti-PSA immobilization degree, the direct recognition signals of 1 μg/mL PSA in HBS (saturation level), and the nonspecific binding of 1/2 diluted female serum, as a function of time and storage condition.

For the dry storage media (air and N_2), the SPR data did not reveal many changes as a function of preservation time. The immobilization degree remains close to the initial value of ~5000 RU or 5000 μg/cm^2, which is representative for a densely packed monolayer of antibodies.[39] In time, only an insignificant increase in the recognition values was observed, whereas the nonspecific binding of serum proteins (~230 RU) remains constant. When comparing this with the surface characterization, we can conclude that the immunosensor properties remain of high quality in these storage conditions. The small amounts of surface contamination observed via CA and XPS measurements do not seem to cause any changes in the immunosensor properties, probably by the extra cleaning steps with glycine prior to the SPR measurements.

Similar as for the previous characterization methods, the SPR measurements for the SAMs stored in H_2O and PBS reveal only minor changes as a function of time. The immobilization levels stayed relatively close to the initial value, and a slight increase in the PSA recognition signals was observed. The nonspecific binding of serum proteins only reveals a small difference between these two media. In PBS, no changes occurred, while for H_2O a slight increase in nonspecific binding was observed which can most probably be explained by the slightly lower immobilization levels. In contrast to these media, the biosensor properties were heavily affected by storage in ethanol. For ethanol we observed a pronounced decrease in immobilization level, which drops from a value of ~5000 RU to a value of ~2400 RU after 18 days of preservation. Due to this low immobilization level, a large decrease in recognition value is obtained and the nonspecific binding increases by a factor of 3 (Figure 4).

These changes in biosensor properties observed for storage in ethanol were related to the earlier conclusions drawn from GA-FTIR and XPS measurements. Upon exposure to ethanol thiol end group oxidation is known to occur in combination with a solubility effect.[40] As stated earlier, the solubility (desorption) of the SAM is higher in the order ethanol > H_2O > PBS. In the case of ethanol, oxidation of the thiol end groups is most obvious. This oxidation will cause desorption of the thiol molecules from the Au surface. As a consequence, a less dense amorphous structure is created, which most likely caused a lower accessibility of the COOH end groups. Due to this phenomenon, serum proteins can be entrapped in the less dense structure and a reduced amount of antibodies can be coupled to the sensor surface which lowers the PSA detection level.

In conclusion, the minor differences in SAM density and polyethylene oxide conformation as shown by CA, CV, XPS, and GA-FTIR have a significant impact on the final biosensing properties, especially after storage in ethanol. As a result, we obtained the lowest antibody immobilization and PSA recognition signals and the highest nonspecific binding values for ethanol. Storage in N_2, air, and PBS had minor effects on the final biosensing properties of the mixed PEO−thiol SAMs, and these can therefore be considered as the best media for long-term storage.

Conclusion

In this study, we evaluated the structure and stability of mixed PEO−thiol SAMs on gold and examined the effects arising from their exposure to various storage media on the immunosensor properties. The study was performed on a mixed PEO−thiol SAM containing both COOH and OH functions because of its well-known and excellent properties for the construction of biological interfaces for immunosensor applications. The evalu-

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ation of their stability in different storage conditions was performed with various characterization tools such as CA, CV, GA-FTIR, and XPS. We showed that the deposited SAMs give rise to hydrophilic, ordered, and densely packed monolayers. Over time, the deposited SAMs remain stable upon dry exposure to air and N_2 over a period of 30 days. Only a small amount of carbon contamination was observed from XPS and CA analysis. Upon exposure to wet storage conditions, the stability in H_2O and PBS was shown to be acceptable, as indicated by the minor changes in GA-FTIR and XPS spectra. On the other hand, ethanol was identified as the worst storage medium. The GA-FTIR and XPS spectra both reveal significant changes after long-time storage in ethanol. It was concluded that partial oxidation of the sulfur headgroup occurred which most probably causes conformational loss and thiol desorption from the Au surface. As a consequence, a less dense and amorphous SAM is obtained as a function of time. These differences in SAM characteristics were found to have a large impact on the immunosensor properties as measured by SPR. Due to the disordered structure of the SAM, the immobilization degree of the monoclonal antibody decreases. In addition, the direct recognition signals decreases and the nonspecific binding of serum components increases. In conclusion, this study will contribute to the development of SAMs with enhanced stability and will as such allow for a more successful use in many proposed applications.

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