Stable Self-Assembled Monolayers for sensitive biosensor interfaces

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ABSTRACT

The controlled interaction of (bio)molecules with various types of surfaces, nanoparticles and nanostructures has become increasingly important for advanced biosensors and biochips. Our aim is to construct stable and well characterized interface chemistries based on pre-activated mixed self-assembled monolayers (SAMs) of alkane thiols. To allow a full exploration of this interface chemistry, first we have investigated the characteristics and biosensing properties of different SAMs on gold substrates. The detection of the cancer marker prostate specific antigen (PSA) was used as a model system. Secondly, we have functionalized gold nanoparticles using such SAMs. Functionalized nanoparticles can be used for enhancing the biosensor performance and constructing nano-(bio)-assemblies.

This study clearly demonstrated that pre-activated SAMs have advanced properties and can be used for various applications.

Keywords: self-assembled monolayers, biosensor, nanoparticles

1 INTRODUCTION

Biosensors have become an important research topic over the past century because they provide a rapid and convenient alternative to conventional analytical methods for monitoring (bio)-chemical substances in various fields such as medicine, environment, fermentation, and food processing. In general, a biosensor consists of a biological recognition layer which is connected to a transducer system by a chemical interface layer. Since the chemical interface layer constitutes the interface with the sample, this component mainly determines the specificity, the reproducibility, and the stability of the whole sensor. In particular, non-specific signals due to interferents constitute a major problem in diagnostic applications, where an analyte with a low concentration has to be detected in the presence of much larger concentrations of non-specific molecules. The construction of a specific and stable affinity interface layer is therefore mandatory for real biosensor applications.

Over the last decade researchers have found that self-assembled monolayers (SAMs) of alkanethiols on gold have excellent properties for this purpose, because they are easy to prepare and form well ordered structures in a controlled and reproducible manner [1]. Moreover mixed SAMs have been applied for the immobilization of biological receptors [2]. In this approach, two different alkanethiols are spontaneously deposited on the gold surface, creating a mixed SAM structure. One carries a functional group to covalently bind the biological receptors (i.e. an antibody in immunosensor applications). The second one are polyethylene oxide (PEO)-containing thiols, which have excellent properties concerning non-specific adsorption of undesired entities, such as serum proteins, on SAMs. The incorporation of both alkanethiols in a mixed SAM approach enables a full control over the number of functional groups, steric hindrance and non-specific adsorption.

In our research we go one step further by using preactivated SAMs. They have an interesting additional property in the way that they are able to couple biological receptors covalently to the sensor surface, without the need for extra coupling reagents. In the end, these pre-activated mixed SAMs can be the ultimate interface chemistry for the development of biosensor interfaces.

In this paper, we describe first the SAM formation on Au substrates. Their characteristics and biosensing properties were analyzed. The detection of the marker PSA was used as a model system. PSA has been identified as a valuable biomarker to screen prostate cancer, the most common cancer in men world-wide [3].

Secondly, we have investigated the SAM functionalization of gold nanoparticles to better suit the integration with biological systems. By modifying the nanoparticles surface, one can obtain enhanced aqueous solubility and biocompatibility. This has gained widespread interest in biotechnological systems. With selected biomolecules bound to nanoparticle surfaces, new 'hybrid' nanostructures can be obtained for applications such as biosensing and imaging, or nanostructures can be embedded in other biocompatible materials to modify material properties or impart new functionalities.

2 MATERIAL & METHODS

2.1 Reagents

All materials and reagent were used as commercially received. 2-(2-(2-(2-(2-(2-(11-mercapto-undecyloxy)-ethoxy)-ethoxy-)-ethoxy)- ethoxy)- ethoxy)- ethoxy-acetic acid (11-PEO6-COOH) was obtained from SensoPath Technologies and 2-(2-(2-(11-mercapto-undecyloxy)-

ethoxy)-ethoxy)-ethanol (11-PEO3-OH) was synthesized according to procedure described in literature [2]. 2-(2-(2-(2-(2-(2-(11-mercaptoyldisulfanyl-undecyloxy)-ethoxy)-ethoxy-)-ethoxy)- ethoxy)- ethoxy)- ethoxy- aldehyde (11-PEO6-CHO) and 2-(2-(2-(2-(2-(2-(11-mercaptoyldisulfanyl-undecyloxy)-ethoxy)-ethoxy-)-ethoxy)- ethoxy)- ethoxy)- ethoxy- N-hydroxysuccinimide (1-PEO6-NHS) were obtained from Prochimia. Ultrapure ethanol was purchased from Riedel-DeHaën and tetrahydrofuran (THF) was optained from Acros Organics. 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), and ethanolamine were received from Biacore®. Human prostate specific antigen and normal female serum were obtained from Scipac, while PSA66 monoclonal antibody was perchased from Fujirebio Diagnostics.

2.2 SAM-functionalized gold substrates

Gold on silicon surfaces were fabricated by electron beam evaporation of 10 nm Ti and 100 nm Au on a polished 6" Silicon wafer with 1.2 µm thermally grown SiO_2. 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. Immediately after cleaning, the gold substrates were immersed in the appropriate thiol solutions of 1 mM in ethanol or THF. After 3h of SAM deposition, the substrates were rinsed with ethanol or THF and dried under a stream of nitrogen.

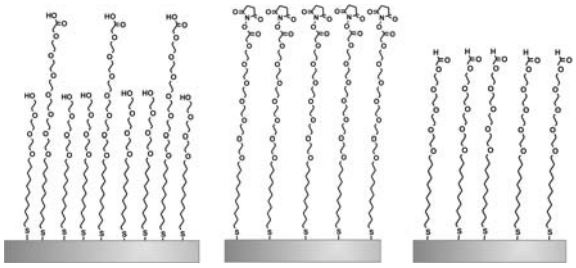

Figure 1: Schematic representation of the 5% COOH, the 100% NHS and 100% CHO SAM, respectively.

2.3 SAM-functionalized gold nanoparticles

Gold nanoparticles were prepared via the 'in-situ' synthetic method. In this procedure, gold nanoparticles are capped and stabilized with the desired self-assembled monolayer during a one-pot nanoparticle synthesis. In the applied 'in-situ' procedure, 132 µl of 1 M of the desired thiol compound was added to 520 µL (0.3 M) gold salt ($HAuCl_4$) in H_2O. The solution was diluted to 10 mL in H_2O. Subsequently, the reduction was performed by adding 20 µL of 14 mM sodiumborohydride ($NaBH_4$) under vigorous stirring. After 2 h of stirring, the gold nanoparticle solution was centrifuged at 5000 rpm at room temperature (Eppendorf centrifuge 5804R). The supernatant was removed and the nanoparticles were resuspended in H_2O. Centrifugation and washing was repeated 3 times.

2.4 Characterization techniques

2.4.1 Grazing Agle-Fourier-transform infrared Spectroscopy

The Grazing Angle-Fourier-transform Infrared Spectroscopy (GA-FTIR) measurements were performed on a Bruker IFS 66V/S instrument over a wavenumber range of 4000 cm^{-1} to 500 cm^{-1} and analysed with Opus software. The spectra are the result of the Fourier-transformation of 2048 interferometric scans at a resolution of 1 cm^{-1}. A deuterated SAM of HS-$(CD_2)_{17}$-CD_3, was used as background sample.

2.4.2 UV-vis spectroscopy

UV-Vis spectroscopy was carried out using a Shimadzu UV-1601PC instrument with a slit width of 2 nm and a data interval of 0.5 nm. Data was evaluated with Shimadzu-UV software.

2.4.3 Transmission electron spectroscopy

The nanoparticle size and morphology were examined using a Tecnai transmission electron microscopy (TEM) at accelerating voltages up to 300 kV. The nanoparticle samples were dried on a carbon grid.

2.4.4 Surface Plasmon Resonance

The Biacore2000® Surface Plasmon Resonance (SPR) instrument operates at a constant temperature of 20 °C. The binding of proteins onto the modified gold support is monitored on-line and is reported as resonance units (RU). A sensor signal of 1 RU corresponds to the adsorption of a protein mass of 1 pg/mm². The data were evaluated with the biacore2000® Control Software Version 1.3.

A continuous flow of Hepes Buffered Saline (HBS) (10 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid, 150mM NaCl, 3.4 mM ethylenediaminetetraacetate and 0.005 % Tween20, pH 7.4) was maintained during the measurements. Covalent immobilization of PSA66 monoclonal antibody was accomplished via coupling to their primary amines. An antibody solution of 132 µl (500 µg/ml in 10 mM acetate buffer pH 5) was injected, followed by an injecting of 50 µL ethanolamine (1 M, pH 8.5) to block the remaining activated groups. Two 5 µL injections of 10 mM glycine.HCl (pH 2.2) were introduced to remove the non-covalently bound antibodies from the surface. Analyte solutions, containing different PSA concentrations were prepared in HBS and injected over the antibody-activated surface at a flow rate of 20 µl/min for

6.5 minutes. Between each PSA injection, the antibody-activated surface was regenerated by two pulses of 10 mM glycine.HCl (pH 2.2). This procedure was repeated wtih ½ diluted normal female serum in HBS to evaluate the occurrence of non-specific adsorption.

3 RESULTS

3.1 Surface chemistry

A schematic presentation of the three monolayers included in this study namely, 5% COOH, 100% NHS and 100% CHO SAM, is shown in Figure 1. The structural characterization of the monolayers was performed by GA-FTIR spectroscopy (Figure 2). The bands at 2863 and 2920 cm^{-1} are ascribed to the symmetric and asymmetric CH_2-streching bands of the C_{11}-methylene units, respectively. These stretching vibration modes are very sensitive to the packing density and to the presence of gauche defects, which makes them ideally suited as probes to determine the SAM quality. The obtained values for the three different SAMs indicate well-ordered alkanethiolate films [4]. The C-O-C strechings vibration gives a very strong absorption band at 1132 cm^{-1}. The ether CH_2-twisting modes occur at 1261 cm^{-1}. The end-groups of the SAMs all have a C=O functionality which is observed in the spectrum around 1730 cm^{-1}.

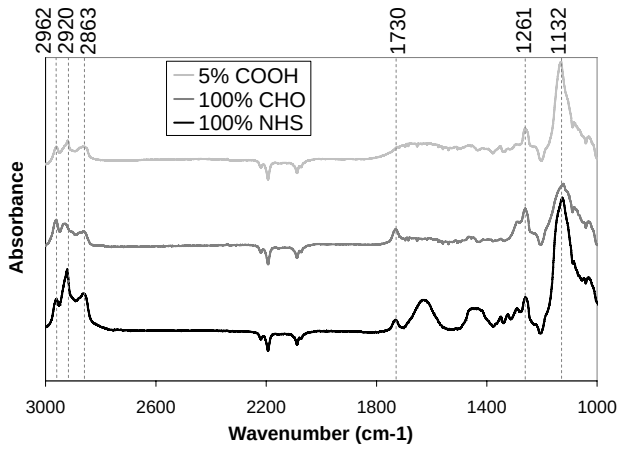

Figure 2: FTIR-spectra of the 3 different SAMs

The performed CV results, as the FTIR data, demonstrated that all SAMs form densely packed monolayers after the standard 3 h deposition time (data not shown).

3.2 Antibody-immobilization chemistry

SPR was used to investigate the random covalent immobilization of antibody via coupling of their primary amines (i.e. lysine residues). Three different coupling chemistries were investigated. In the first strategy, the random coupling procedure attaches the antibodies via their lysine residues to the activated COOH-groups of the 5% COOH SAM via EDC/NHS chemistry. The second strategy involves the coupling of the lysine residues to the CHO-groups of the 100% CHO SAM followed by a reduction of the initially formed shiff base by cyanoborohydride. In the third strategy, the lysine residues were attached to the NHS-ester functional groups of the 100% NHS SAM in one step.

These coupling procedures can result in antibodies oriented in different orientations on the sensor-surface. Whereas antibody orientation plays a significant role in the antigen binding's efficiency [5], we have studied the orientation of the antibody immobilization onto the different SAMs by recognition of an Fc- and Fab-specific antibody. For the investigated SAMs the RU immobilization values together with the recognition signals of the Fc-and Fab-specifiec antibody are given in Table 1.

surface	RU_{PSA66}	$RU_{FC\ (1\mu g/mL)}$	$RU_{Fab\ (1\mu g/mL)}$
5% COOH	5082 ± 346	652 ± 77	416 ± 126
100% CHO	425 ± 176	-	-
100% NHS	3773 ± 570	542 ± 75	421 ± 119

Table 1: Immobilization values and Fc-and Fab-specific antibody recognition signals expressed in RU.

Using the formula derived previously [6], we can estimate the surface mass density for a densely packed monolayer, taking into account all the possible antibody orientations. One may consider the antibody to be a lens-shaped spheroid and densely packed in either upright or in horizontal position. Thus a calculated value of 1300 RU is expected for a monolayer with antibodies in lying position to 6500 RU for a monolayer of antibodies in upright position [6]. When comparing the immobilization behavior, we noticed that on the 100% CHO SAM no full monolayer of antibodies could be obtained. This is probably due to the reduction step, which is too harsh for the coupled antibodies. In the case of the 5% COOH and 100% NHS SAM, one can notice a full coverage with all of the immobilized antibodies in the same orientation, as indicated by the RU values for the recognition of the Fc- and Fab-specific antibodies.

3.3 Antigen binding behavior

SPR was also used to evaluate the efficiency of the antibody-antigen binding in function of the immobilization strategy and concentration. Since the amount of antibody immobilized varies in the different immobilization strategies, different antigen (PSA) recognition signals were observed (Figure 3). In the case of the 100% CHO SAM, the recognition signals were very low compared to the RU values obtained for the other two SAMs. This is likely due to the incomplete antibody immobilization.

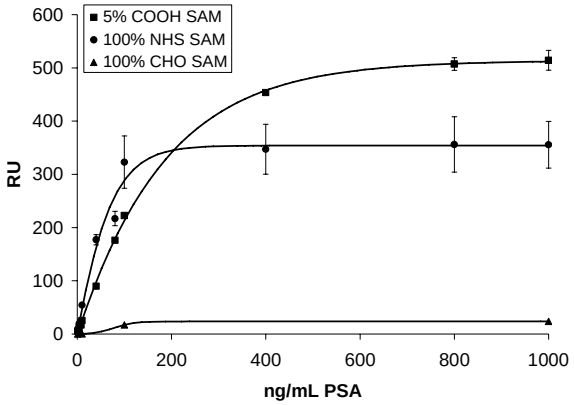

Figure 3: PSA recognition signals on the three investigated SAMs.

For the other two investigated SAMs the binding efficiency of the surface was determined using the response due to the amount of antibody immobilized and the subsequent antigen binding:

$$\text{Binding Efficiency} = \frac{RU_{PSA} MW_{PSA66}}{2RU_{PSA66} MW_{PSA}}$$

where RU_{PSA} is the sensorgram response upon antigen binding, MW_{PSA66} is the molecular weight of the PSA antibody, RU_{PSA66} is the response due to the initial antibody immobilization, and MW_{PSA} is the molecular weight of the antigen PSA. We obtained similar values for the 5% COOH (25%) and the 100% NHS SAM (24%). The difference between the recognition signals is seen in Figure 3, were the sensitivity is higher on the pre-activated 100% NHS SAM surface.

The SPR system was also used to evaluate the occurrence of non-specific adsorption on the 5% COOH and 100% NHS SAM by investigating the binding of ½ diluted normal female serum. We obtained the same amount of non-specific binding (250 ± 40 RU) (Data not shown.)

3.4 Functionalization of gold nanoparticles

The UV-visible spectrum of gold nanoparticles is known to exhibit a strong surface plasmon absorbance band at about 520 nm. The resonance frequency of nanoparticles has been found to depend upon its size, shape, material properties, surrounding media, and proximity to other nanoparticles [7]. Thiol-functionalized gold nanoparticles showed a characteristic surface Plasmon peak around 520 nm (Figure 4), confirming the formation of gold nanoparticles in the presence of the different SAMs.

The TEM images of the thiol-functionalized gold nanoparticles further reveal the formation, the size, the shape and distribution of the gold nanoparticles. Figure 4 shows the TEM images of two of the synthesized nanoparticles (5% COOH- and 100% CHO- gold nanoparticles). One can verify that spherical nanoparticles were formed with an average size of 50 nm and 30 nm for the 100% CHO-gold-nanoparticles and 5% COOH-gold-nanoparticles, respectively.

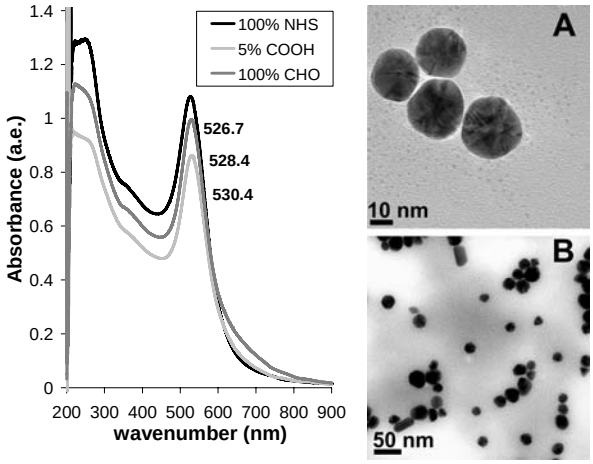

Figure 4: left: UV-vis absorption spectrum of the different synthesized gold nanoparticles. Right: TEM images of the (A) 5% COOH- and (B) 100% CHO- gold nanopartikels.

4 CONCLUSION

In this paper we have demonstrated the use of pre-activated SAMs (100% NHS) in different applications (functionalization of surfaces and gold nanoparticles). Our results show that the binding and recognition efficiency is as good or even better as compared to the mixed self-assembled monolayers (5% COOH SAM) especially at low analyte concentrations. In conclusion, we can point that these pre-activated SAMs, by reducing the amount of surface modification steps, can be very useful for many applications.

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