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Short communication

Influence of immobilization protocol on the structure and function of surface bound proteins

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ABSTRACT

A new coupling strategy for biomacromolecules with (3-mercaptopropyl)trimethoxysilane (3MPTMS) and 11-(triethoxysilyl)undecanal (TESU) on gold surfaces is. This immobilization protocol was utilized for the enzyme horseradish peroxidase (HRP). To study the reactions and resulting structures, PM-IRRAS measurements were performed. PM-IRRAS shows there is structure preservation of the HRP when the new coupling strategy is used in contrast to non-specific adsorption on gold. The biological activity of adsorbed and immobilized HRP was measured by the enzyme catalyzed oxidation of 3,5,3′,5′-tetramethylbenzidine. Covalent immobilization of HRP on TESU film compared to physisorption of HRP shows higher enzyme activity on gold surfaces, confirming the structural preservation detected by PM-IRRAS.

1. Introduction

Technological advancement in areas such as chemical sensors, biosensors, biochips [1,2], and coating materials [3,4] requires a chemical and physical understanding of surface processes. When biomacromolecules such as enzymes are immobilized on support materials, it is essential to conserve the biological activity and accessibility of the catalytically active sites of the enzymes [5–7]. In this context, the formation of an organic layer is the most widely used strategy for the covalent immobilization of biomolecules on surface materials using self-assembled monolayers [8,9]. The formation of organic monolayer films by self-assembly is a common approach for modification of a variety of metals using alkanethiols [10] and alkoxysilanes [11] with different functional groups. The groups are often carboxyl groups with the disadvantage of the need for activation with N-hydroxysuccinimide (NHS) ester and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) [12]. The aim of the work presented here is to introduce an alternative immobilization strategy, using the example of horseradish peroxidase (HRP).

The thiosilane (3-mercaptopropyl)trimethoxysilane (3MPTMS) is used to functionalize a gold surface via the mercapto group. The hydrolyzable methoxysilyl groups of the surface immobilized 3MPTMS are used to graft the aldehyde terminated organosilane 11-(triethoxysilyl)undecanal (TESU) via a sol–gel reaction on the surface [13]. The resulting aldehyde modified surfaces were used to immobilize p-aminophenol as a model molecule or HRP as a biomacromolecule having a known secondary structure and an easily measurable bioactivity. The amine groups of lysine residues from the HRP can react with the surfaces' aldehyde groups forming a Schiff's base (imine) which is further reduced via reductive amination to a stable secondary amine using sodium cyanoborohydride $NaCNBH_3$ [14,15]. Water, alcohols, and other oxygen nucleophiles typical for biological fluids do not compromise the yields of reductive amination [16,17]. Scheme 1 illustrates the overall strategy for surface immobilization described in this work.

In order to analyze the adsorption phenomena and adhesion processes of biomolecules on the surfaces, a variety of measurement techniques can be used including infrared reflection absorption spectroscopy (IRRAS) [18], time of flight secondary ion mass spectrometry (ToF-SIMS) [19], X-ray photoelectron spectroscopy (XPS) [19], and atomic force microscopy (AFM) [20]. Of these measurement techniques, IRRAS is a particularly sensitive and nondestructive technique for acquiring information about, for example, the formation of chemical bonds with the substrate, the conformation, hydrogen-bonded structures, and the orientation of functional groups in the adsorbed substances on the metal surfaces [21]. IRRAS is only partially suitable for samples with an ultrathin film (thickness less than 10 nm) as the measurement time necessary to get an acceptable signal-to-noise ratio becomes several hours [22] and the long measurement time often leads to spectral artifacts due to small changes in the atmospheric composition, especially the water vapor content. For this reason, PM-IRRAS was developed [23]. The polarization modulation (PM) is a very efficient way of discriminating near-surface absorptions from the isotropic strong absorptions occurring in the sample environment [22]. Due to PM, the sample with thin film is also the

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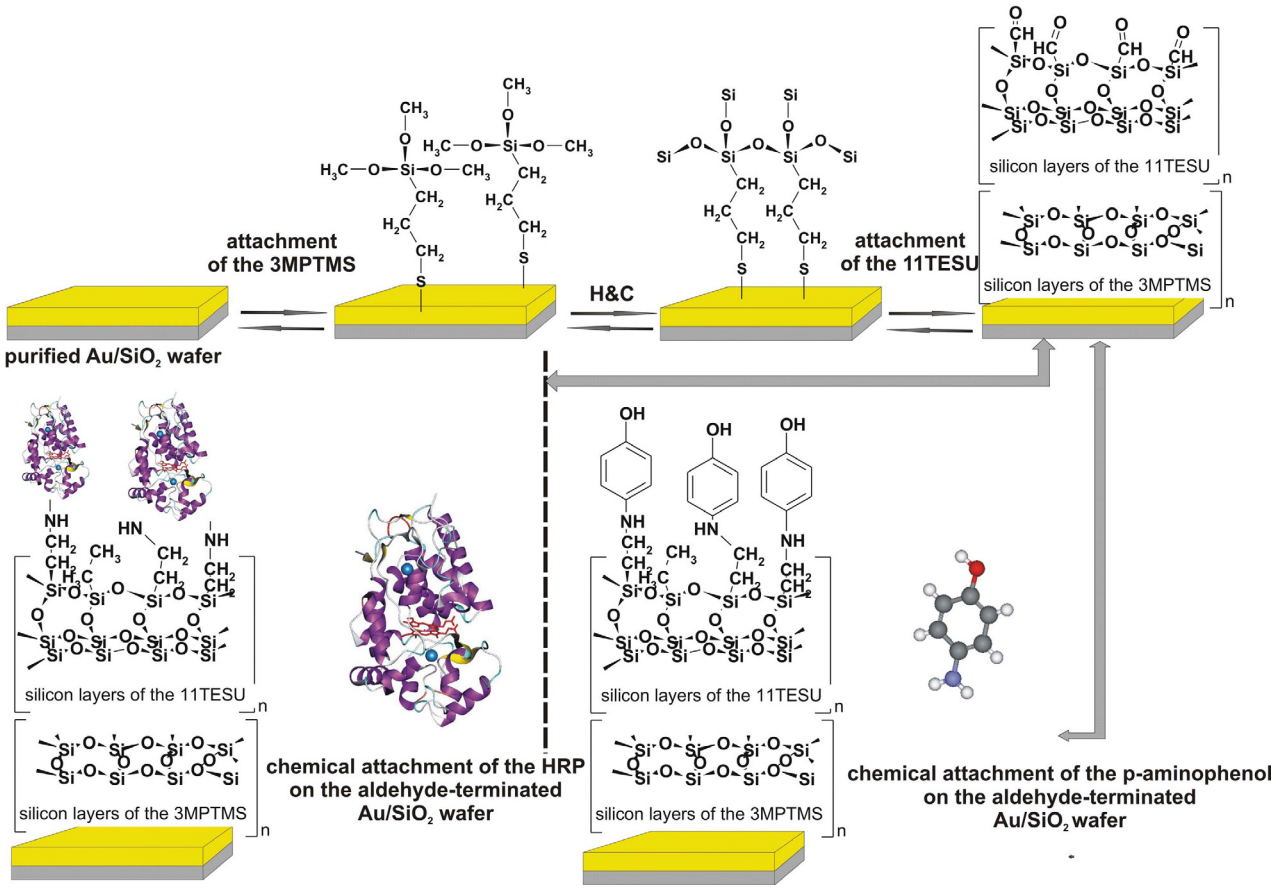

Scheme 1. Strategy for the immobilization of the biomolecule HRP and the model compound p-aminophenol on the gold surface (H&C = hydrolysis and condensation).

reference sample measured at the same time. Therefore, omnipresent contaminations such as hydrocarbons are also detected. They are often overlooked in conventional IRRAS experiments but their presence is, for example, known from XPS experiments.

There are several works that use the enzyme HRP to determine the catalytic activity at interfaces [24–27]. In this study we have also chosen HRP as the model enzyme for the readily detectable oxidation of 3,5,3′5′-tetramethylbenzidine diamine to 3,5,3′5′-tetramethylbenzidine diimine (Scheme 2). Josephy et al. [28] described the mechanism of oxidation of 3,5,3′,5′-tetramethylbenzidine using HRP.

2. Experimental

2.1. Materials

Au/SiO_2 wafers were obtained from Si-Mat Silicon Materials (diameter 100 mm, orientation P/Bor ⟨1 0 0⟩, thickness 525 ± 25 μm, single polished). (3-Mercaptopropyl)trimethoxysilane (3MPTMS, 95%) and 11-(triethoxysilyl)undecanal (TESU, 90%) were purchased from ABCR (Karlsruhe, Germany). Horseradish peroxidase (HRP, Pcode: 101161191), triethylamine (TEA, 99.5%), methanol (≥99.9%), ethanol (96%), sodium hydrogen carbonate (≥99%, $NaHCO_3$), sodium carbonate (≥99%, Na_2CO_3), and sodium cyanoborohydride (5M, $NaBH_3CN$) were purchased from Sigma–Aldrich (Steinbach, Germany). One Step Ultra TMB-ELISA was acquired from Pierce Protein Research Products (ThermoScientific, Bonn, Germany). Water was obtained from an UHQ II ELGA water purification system (18 MΩ/cm).

2.2. IR spectra

ATR-IR spectra were recorded between 4000 and 650 cm^{-1} on a Bruker Equinox 55 FT-IR-spectrometer (Bruker Optik, Germany) equipped with Golden Gate™ ATR unit A531G (Specac) with a resolution of 4 cm^{-1} and 32 scans. For the PM-IRRAS experiments a polarization modulation accessory (Bruker PMA-50) was used with MCT detector and a photoelastic modulator controller (PEM-90 controller) from Hinds Instruments (HINDS model II/Zn50 with a nominal frequency of 50 kHz, retardation range for half wave 1–10 μm, and aperture 14 mm) coupled with a Bruker Tensor 27 FT-IR spectrometer. The spectra were an average of 45 min recording time and were taken at a spectral resolution of 4 cm^{-1} and an incident angle of 83.5°.

2.3. Immobilization protocol

3MPTMS layers were formed at room temperature (RT) on the gold surface by immersion in a solution of 20 mM 3MPTMS in methanol for 2 h (Scheme 1). Then the silanized substrate was rinsed with methanol for 1 h at a shaking frequency of 100 min^{-1} using an orbital shaker and dried under a nitrogen stream. The samples were then dried in an exsiccator (200 mbar) for 16 h. The hydrolysis and condensation of the 3MPTMS on the substrates were carried out at 80 °C by immersing the 3MPTMS-modified surfaces in 0.1 M HCl for 1 h, 2 h, and 3 h. Subsequently, the prehydrolyzed 3MPTMS layer was cleaned with double distilled water and dried in a nitrogen stream for about 1 min.

The silanization process was carried out by incubating the prehydrolyzed 3MPTMS layer in a 96% ethanol solution containing 2% TESU and 1% TEA for 2 h at RT. Then the surfaces were rinsed with ethanol for 10 min at 100 min^{-1} using an orbital shaker and heated for 2 h at 110 °C. The TEA acts as catalyst for the hydrolysis and condensation