NANO EXPRESS

Preparation and Characterization of Covalently Binding of Rat Anti-human IgG Monolayer on Thiol-Modified Gold Surface

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Abstract The 16-mercaptohexadecanoic acid (MHA) film and rat anti-human IgG protein monolayer were fabricated on gold substrates using self-assembled monolayer (SAM) method. The surface properties of the bare gold substrate, the MHA film and the protein monolayer were characterized by contact angle measurements, atomic force microscopy (AFM), grazing incidence X-ray diffraction (GIXRD) method and X-ray photoelectron spectroscopy, respectively. The contact angles of the MHA film and the protein monolayer were 18° and 12°, respectively, all being hydrophilic. AFM images show dissimilar topographic nanostructures between different surfaces, and the thickness of the MHA film and the protein monolayer was estimated to be 1.51 and 5.53 nm, respectively. The GIXRD 2θ degrees of the MHA film and the protein monolayer ranged from 0° to 15°, significantly smaller that of the bare gold surface, but the MHA film and the protein monolayer displayed very different profiles and distributions of their diffraction peaks. Moreover, the spectra of binding energy measured from these different surfaces could be well fitted with either Au4f, S2p or N1s, respectively. Taken together, these results indicate that MHA film and protein monolayer were successfully formed with homogeneous surfaces, and thus demonstrate that the SAM method is a reliable technique for fabricating protein monolayer.

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Introduction

Well-ordered protein layers have great implications in biosensors [1–3], biomaterials [4, 5] and protein-based molecular recognition at single-molecule scale [6–8]. Based on self-assembled monolayer (SAM) method, a protein layer can be fabricated by binding proteins to a substrate either covalently (chemical coupling) or non-covalently (physical absorption) [9–12], but the covalent method is superior due to its good reproducibility and homogeneity in layer formation [13, 14]. In addition, it has been demonstrated that the substrate surface can be chemically modified easily and efficiently to tailor a specific protein layer. However, it is also known that the sensitivity and reproducibility of assays using such protein layers are strongly influenced by the layer's surface properties and protein immobilization. Thus, it is important to critically evaluate and characterize the protein layer at nanoscale in order to understand its performance.

In this study, a protein layer of rat anti-human IgG on a thiol-modified gold substrate as a model system was fabricated using SAM method and carefully characterized by multiple techniques. We used gold as substrate, a standard since SAM method has been developed two decades ago, because of its wide availability, inertness and biocompatibility [15]. The surface of the gold substrate was modified with a long carbon chain thiol, namely, 16-mercaptohexadecanoic acid (MHA), because

sulfur-containing molecules (thiols, sulfides and disulfides) have a strong affinity for gold and interact with it, yielding an Au–S bond.

In principle, fabrication of the above-mentioned model system is simple [16]. First, thiol-based SAM on gold substrate can be obtained by simply immersing the gold surface into a solution of the selected thiols, and the spontaneous reaction will produce a SAM ideally composed of tightly packed and well-ordered thiol molecule chains on the gold surface (MHA film). The MHA film is terminated with carboxyl groups that can be activated by the 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide (NHS). Then, the activated MHA film is subject to the protein solution for 12 h to form the protein layer. Notably, although the rat anti-human IgG protein has many free primary amine groups, the covalent binding to activated MHA film occurs most often with the amine group of lysine, which has been revealed by Koshland [17]. The mechanisms of surface modification and protein immobilization as described earlier are illustrated in Fig. 1.

A variety of techniques may be employed to analyze the thiol-based SAM and the protein monolayer such as quartz crystal microbalance [18], surface plasmon resonance (SPR) [19], atomic force microscopy (AFM) [20], X-ray photoelectron spectroscopy (XPS) [21], contact angle goniometry [4], grazing incidence X-ray diffraction method (GIXRD) [22] and fluorescence detection [23]. Among them, XPS and the GIXRD are usually used to analyze the state and distribution of chemical elements on different surfaces. Contact angle goniometry determines the bulky surface property at macro scale, whereas the AFM is capable of imaging proteins with nanometer resolution.

Here, we present the method of preparation and fabrication of rat anti-human IgG protein layer on MHA-modified gold substrate, as well as its characterization by contact angle measurements, AFM, GIXRD and XPS, respectively.

Experimental

Preparation of Gold Substrates

Gold substrates were prepared by vapor deposition of gold onto freshly cleaved mica in a high vacuum evaporator at $\sim 10^{-7}$ Torr. Mica substrates were preheated to 325 °C for 2 h by a radiator heater before deposition. Evaporation rates were 0.1–0.3 nm/s, and the final thickness of gold films was ~ 200 nm. There is a chromium adhesion layer between gold and mica. Gold-coated or bare gold substrates were annealed in H_2 frame for 1 min before use.

Formation of SAM

The bare gold substrates were soaked into a hot piranha solution (v/v $H_2SO_4:H_2O_2 = 3:1$) for 30 min to clean the surface. The cleaning process was carried out with extreme care because piranha solution is highly reactive and may explode when in contact with organic solvents. Then SAM was formed by immersing the bare gold substrate in 1 mM 16-mercaptohexadecanoic acid ($HS(CH_2)_{15}CO_2H$, Sigma–Aldrich Chemical Co.) in ethanol solution (guaranteed grade, Merck Co.) for 24 h. The formed SAM was supersonicated in pure ethanol for 2 min to remove unbound thiol molecules, then rinsed sequentially with pure ethanol and ultra pure water and finally air-dried in a N_2 stream.

Protein Immobilization to SAM

Protein immobilization to SAM was carried out as described earlier with minor modification [24]. In brief, SAM with carboxylic acid terminal groups was activated by 2 mg/mL NHS (Sigma–Aldrich Chemical Co.) and 2 mg/mL EDC (Sigma–Aldrich Chemical Co.) in phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, pH 7.4, Merck Co.) solution for 1 h and subsequently rinsed thoroughly with ultra pure water and air-dried in N_2 stream. The activated

Fig. 1 Mechanism of protein covalently linked on a MHA-modified gold surface
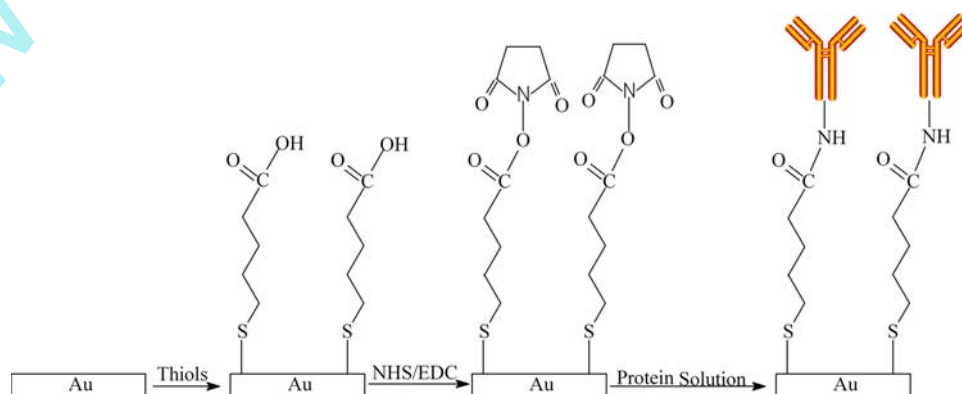
SAM was then immersed into 10 μg/ml rat anti-human IgG (Biosun Co., China) in PBS solution at 4 °C for 12 h. Finally, the prepared specimens of SAM with immobilized protein were stored in PBS solution at 4 °C before use.

Contact Angle Measurements

Contact angle of a surface was measured by the static sessile drop method using contact angle goniometry (Magicdroplet 200, Taiwan), and all measurements were performed under room temperature (∼25 °C) and ambient humidity. One microliter of Milli-Q water was deposited at random locations on the surface to be measured, and the angle between the baseline of the drop and the tangent at the drop boundary was measured on both sides of the drop. The results presented here are the average of at least five measurements.

AFM Imaging

All AFM images were acquired using Benyuan CSPM 5000 scanning probe microscope (Benyuan Co., China) equipped with a 1.6-μm E scanner. Commercial Si_3N_4 cantilevers (BudgetSensors) with resonant frequency of 200 KHz were used. AFM worked with tapping mode in PBS buffer solution at typical scanning rate of 2.0 Hz.

GIXRD

The GIXRD experiments were performed on a Rigaku D/max 2500pc X-Ray diffractometer, Cu Kα radiation and graphite monochromator operated at 40 kV, 100 mA. The grazing incidence angle was set at 1.5° for the bare gold and the protein monolayer and 0.5° for the MHA film. The diffraction data of samples were collected with step scanning method. Qualitative phase analysis of each sample was performed using the MDI Jade 5.0 software program.

XPS

XPS experiments were performed on a PHI Quantera SXM photoelectron spectrometer equipped with an Al Kα radiation source (1486.6 eV). The photoelectrons were analyzed at a take-off angle of 45°. Survey spectra were collected over a range of 0–1400 eV. During the measurements, the base pressure was lower than 6.7×10^{-8} Pa (ultra high vacuum). All spectra were fitted using XPSPEAK Version 4.1, an XPS peak-fitting program.

Results and Discussion

Surface Modification and Protein Immobilization

Although SAM method is relatively simple and easy to do, there are some aspects need to be considered in order to form an ideal protein monolayer [3, 15, 16, 25]. These include, but not limited to (1) gold substrate was used because it binds thiols with a high affinity and is chemically inert; (2) 16-mercaptohexadecanoic acid with long carbon chain was used because it is flexible to serve as a spacer to minimize the interference between protein

Fig. 2 Contact angle measurements of protein monolayer immobilized on a MHA film (**a**), on a film of mixed thiols (1-dodecanethiol to 16-mercaptohexadecanoic acid at 1:1 molar ratio) (**b**) and of the same mixed thiols film itself (**c**)
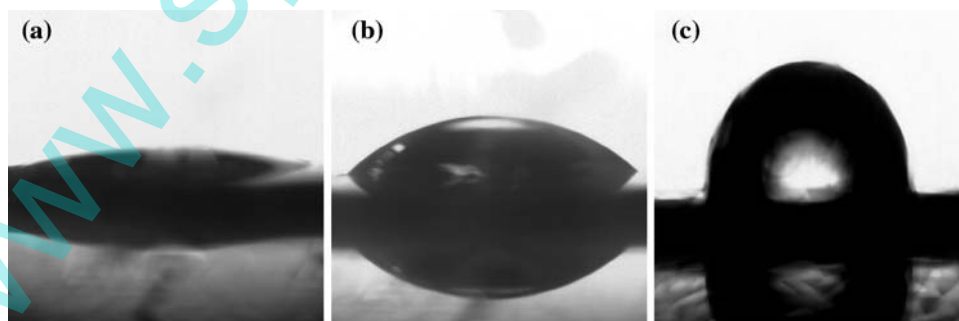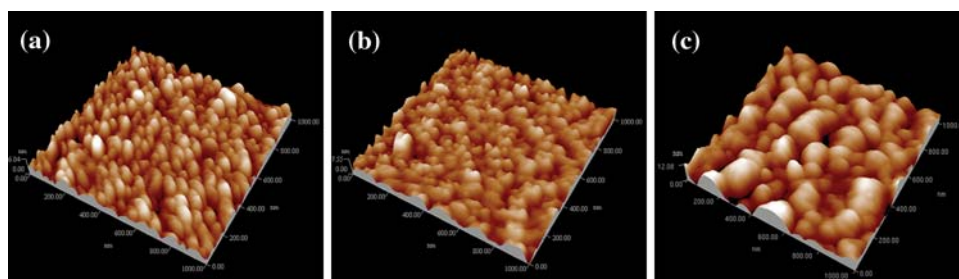

Fig. 3 3D topographies of the bare gold substrate (**a**), the MHA film (**b**) and the protein monolayer (**c**) recorded by tapping mode AFM in PBS buffer solution. The scanning size is 1 μm × 1 μm

molecules and gold substrate and (3) the pH, temperature and ion strength may affect the protein activity. Therefore, in the present study, the temperature and pH for protein immobilization conditions were controlled at 4 °C and 7.4, respectively, in PBS. In addition, the modified protein layer should not only provide optimal orientation but also minimal steric hindrance to the protein molecules so that they can mimic their natural state. The SAM method has been proven capable of ensuring the activity, mobility and stability of protein molecules [15, 26]. Furthermore, although it has been proven that 1 mM thiol and immersion for 24 h are sufficient for forming well-ordered thiol film [25], it should be noted that the protein concentration is also important. We found that 10 µg/ml was an adequate protein concentration to form uniform layer, and higher concentration may cause protein aggregation. When all considered properly, the method presented here can be a reliable one for biologic sample preparation.

Characterization of Bare Gold, MHA Film and Protein Monolayer

The contact angles of the bare gold surface and the MHA film were determined to be 58° and 18° (data submitted to elsewhere), respectively. These data are consistent with results from other studies [27–29]. Whereas the contact angle of the rat anti-human IgG monolayer was measured to be 12° (Fig. 2a), which was very close to that of the MHA film. In order to verify that the measured hydrophilicity is due to the presence of the protein monolayer instead of the MHA film underneath it, further measurements were made on the protein monolayer on a film of mixed thiols (1-dodecanethiol mixed with 16-mercapto-hexadecanoic acid at 1:1 molar ratio). It was found that the contact angle of the protein monolayer on the mixed thiols film was 36.5° (Fig. 2b), which was significantly smaller than that of the mixed thiols film itself (97°, Fig. 2c). These results suggest that both the MHA film and the protein monolayer have hydrophilic surface.

The 3D topographies of the bare gold substrate, the MHA film and the protein monolayer are shown in Fig. 3. The surface roughness of the bare gold substrate was calculated to be 1.06 nm (value of root mean square), suggesting good surface uniformity. Dissimilar nanostructures were observed between the three different surfaces, suggesting that successful modification occurred during each step of the SAM formation. This is also supported by the Z bar variation (equiv. to height) of the three different surfaces, which increased from 6.04 nm for the bare gold substrate, to 7.55 nm for the MHA film and 12.08 nm for the protein monolayer, respectively. Although it is recognized that height information from tapping mode AFM is not exactly the height of a molecule [30], it still allows

qualitative identification of different species on surfaces based on their relative difference in height [7]. The thickness of the MHA film was estimated to be 1.51 nm, which was smaller than theoretical prediction, This discrepancy may be due to tilting of the MHA molecules [31] and system error of AFM. The thickness of rat anti-human IgG
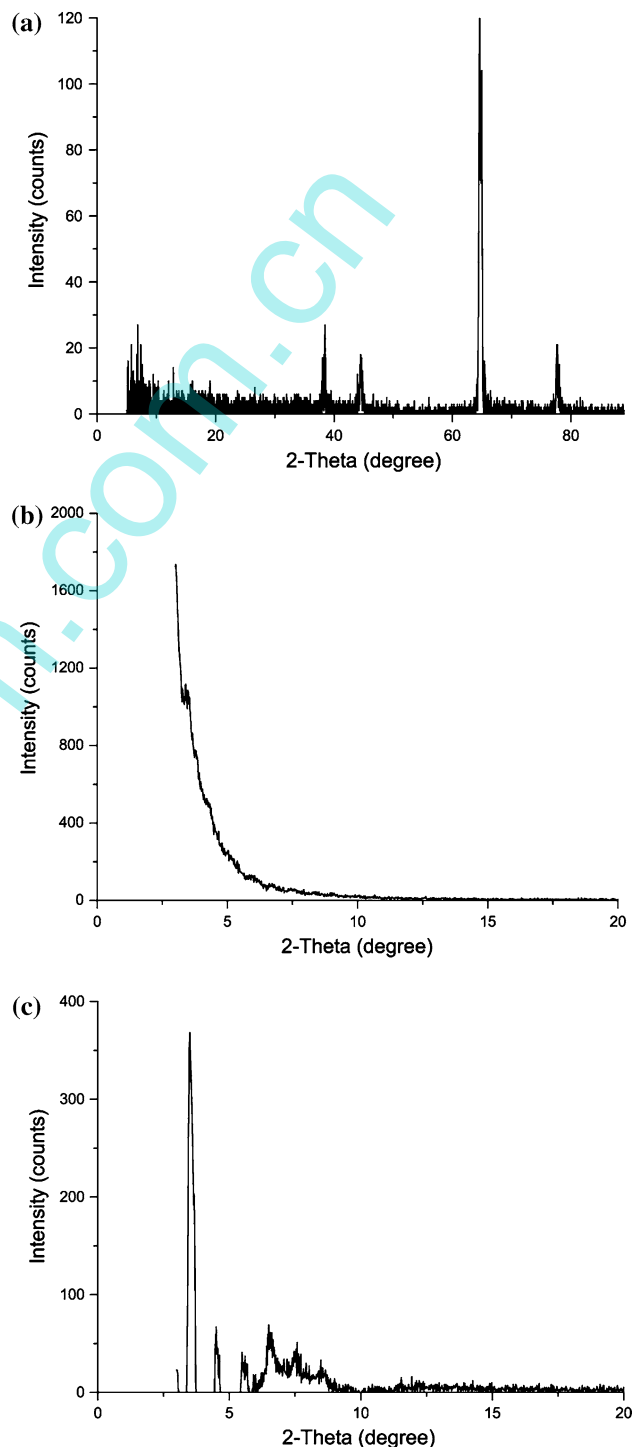
Fig. 4 GIXRD spectra of the bare gold (a), the MHA film (b) and the protein monolayer (c)
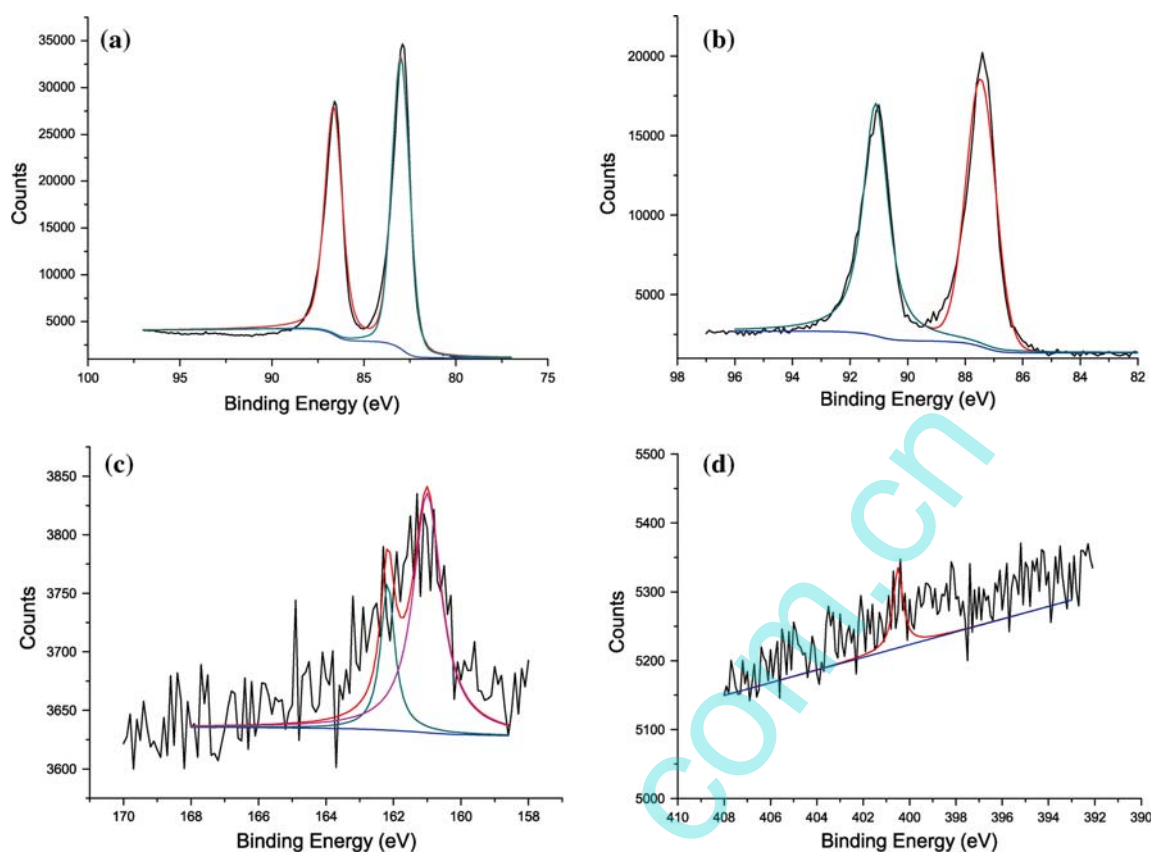
Fig. 5 Binding energy spectra of Au4f of the bare gold substrate (**a**), Au4f of the MHA film (**b**), S2p of the MHA film (**c**) and N1s of the protein monolayer (**d**)

monolayer was found to be 5.53 nm, consistent with the usual large size of antibody proteins. Nevertheless, the AFM images directly revealed well-ordered MHA film and protein monolayer.

Figure 4 shows the GIXRD spectra of the bare gold substrate (a), the MHA film (b) and the protein monolayer (c), respectively. The spectra of the bare gold substrate are quite agreeable with that of standard Au. In contrast, the GIXRD spectra of the MHA film and the protein monolayer show strong diffraction peaks with smaller 2θ degrees (between $0°$ and $15°$) than that of the bare gold surface. However, the protein monolayer displayed a series of strong diffraction peaks at 2θ degrees range of $0°$–$10°$, compared with the MHA film. These differences in the profile and peaks distribution of the X-ray diffraction spectra between these surfaces suggest that the two steps to form SAM protein monolayer had successfully accomplished.

Figure 5 shows all the XPS spectra of the three different surfaces, namely, Au4f spectra of the bare gold substrate (a), Au4f spectra of the MHA film (b), S2p spectra of the MHA film (c) and N1 s spectra of the protein monolayer (d), respectively. XPS analysis demonstrated that for the MHA film and the protein monolayer, there were no noticeable chemical elements other than the one expected based on their chemical configuration. The high resolution spectra of Au can be well fitted with a doublet structure centered at 86.6 and 82.9 eV. After the MHA modification, the Au4f spectra shifted its peaks to 87.46 and 91.11 eV, indicating chemical shifts. With respect to sulfur spectra, no detectable peaks above 164 eV were found. This means that no unbound thiol molecules presented on the MHA film, indicating that the MHA modification was adequately performed [32]. There are two peaks centered at 162.17 and 161 eV, and the 162.17 eV peak should be attributed to the interaction between the MHA and the gold surface that decreases the binding energy [4, 32]. However, the 161 eV peak could be considered to an additional C–S bond formation, which does not affect the binding energy [33]. Nitrogen spectra can be well fitted with a structure centered at 400.55 eV, suggesting protein molecules covalently immobilized on the MHA film.

Conclusions

In this work, the MHA film and rat anti-human IgG monolayer on gold substrates were fabricated by SAM method and characterized by contact angle measurements,

AFM imaging, GIXRD and XPS, respectively. Both the MHA film and the protein monolayer were highly hydrophilic, and dissimilar nanostructures were formed on all the three different surfaces as revealed by AFM imaging. Although both the MHA film and the protein monolayer displayed smaller GIXRD 2θ degrees than the bare gold substrate, the two modified surfaces exhibited different profiles and distributions of their X-ray diffraction peaks. Moreover, the binding energy spectra of the three different surfaces could be well fitted with either Au4f, S2p or N1s, respectively. Together, the results suggest that using the presented method, protein molecules can be successfully bound to thiol-based modified gold substrates with good reproducibility and homogeneity for both fabricated thiol film and protein monolayer. Therefore, this covalent modification method may provide a highly reproducible, and well-suitable approach for protein immobilization.

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