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Self-Assembled Human IgG Monolayers on Mixed Thiols Modified Gold Surfaces

ZHENGJIAN LV, JIANHUA WANG* and GUOPING CHEN

Key Laboratory of Biorheological Science and Technology, Ministry of Education and Institute of Biochemistry and Biophysics, College of Bioengineering, Chongqing University, Chongqing 400044, P.R. China

*Corresponding author: Fax: +86 23 65102507; E-mail: wjh@cqu.edu.cn; cqubio@hotmail.com

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The human IgG protein monolayers were fabricated on mixed thiols modified gold substrates by self-assembled monolayers method. The surface properties of protein monolayers were characterized by contact angle measurements, X-ray photoelectron spectroscopy and atomic force microscopy, respectively. It was found that the contact angles of the protein layers on the mixed thiols films containing 20 %, 40 %, 60 %, 80 %, 100 % of 16-mercaptohexadecanoic acid were 83.5 ± 3.3º, 56.7 ± 3.1º, 38.6 ± 2.9º, 25.8 ± 2.6º, 14.2 ± 2.3º, respectively. The atomic concentration of nitrogen of protein layers on pure 16-mercaptohexadecanoic acid film was counted to be 11.43 ± 0.56 %. Depth profile analysis showed the protein layer and 16-mercaptohexadecanoic acid film have the thickness of 7 nm and 2 nm, respectively. Furthermore, the adhesive forces between antibody and antigen layers with molar ratios of 16-mercaptohexadecanoic acid of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 were determined to be 11 ± 2, 158 ± 21, 325 ± 24, 589 ± 36, 814 ± 41, 883 ± 45 pN, respectively. In summary, these results suggested that the human IgG protein monolayers were successfully formed and the surface coverage of protein molecules increased parallel with the increasing of 16-mercaptohexadecanoic acid content. Thus, they demonstrated that the mixed thiols based self-assembled monolayers method is a reliable technique for fabricating protein monolayers with adjustable surface coverage and good controllability.

Key Words: Human IgG, Self assembled monolayers, Contact angle, X-ray photoelectron spectroscopy, Atomic force microscopy.

INTRODUCTION

Protein molecules immobilized on a solid support holds special importance in antibody-antigen based biosensors, biomaterials and biophysical researches[1-5]. Protein molecules may be immobilized *via* physical adsorption, or electrostatic entrapment, *etc*. Although these non-covalent immobilization methods have been successfully used to construct immunosensors, they have limited or no control over the orientation of protein molecules and have poor reproducibility and stability. In this context, a relative new method, namely the self assembled monolayers, is an attractive method to address the above mentioned problems by covalent coupling protein molecules on substrates. Among covalent binding methods, thiols based self assembled monolayers are extensively used for protein immobilization. Upon thiols based self assembled monolayers, the orientation, reproducibility and stability of protein molecules can be controlled by the high affinity of thiol groups for noble metal surfaces and the highly ordered nature of functionalized alkanethiol self assembled monolayers. The amine groups of lysine of protein molecules can be covalently coupled to the highly ordered ω-carboxyl functionalized alkane thiol self assembled monolayers simply by chemically activating the exposed carboxylic acid terminals with 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS)[6,7]. The immobilization of protein molecules on solid supports by thiols based self assembled monolayers can be achieved by immersing the thiols self assembled monolayers into the protein solution for 24 h. With respect to the support, gold is highly recommended for its wide availability, inertness and biocompatibility[8]. The mechanism of mixed thiols based self assembled monolayers and further bound to protein molecules is illustrated in Fig. 1.

Despite the easiness and the effectiveness of self assembled monolayers in protein immobilization, protein layers fabricated from pure thiols with exposed carboxyl tail groups will result in undesired densely packed surfaces, which are undesirable for many cases, for example, detecting single molecule recognition events. Therefore, controlling the surface coverage of protein layers is of great significance of protein immobilization. This can be accomplished by adjusting the molar ratio of two kinds of thiols, one with a carboxyl tail group and the other with an inert tail group that cannot be activated by 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide. These controlled protein surfaces

are promising for studies of surface related protein interactions. Hence, it is important to critically evaluate and characterize the protein layers with varied surface coverage so as to understand their performances.

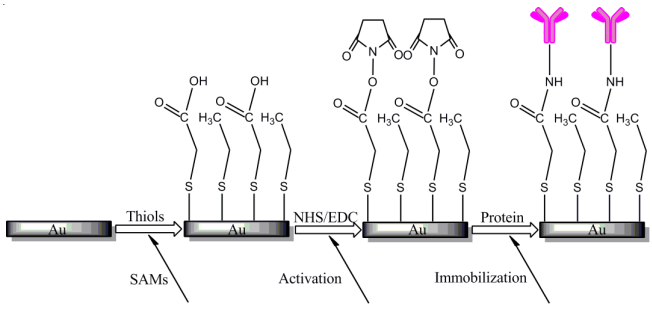


Fig. 1. A flow chart of the self assembled monolayers (SAMs) method used in this study. From left to right, the bare gold substrate is firstly modified by soaking it into the ethanol solution of 16-mercaptohexadecanoic acid and HDT for 24 h, the reaction system spontaneously yields an Au-S bond which enables the thiols covalently bound to gold substrate, resulting in the formation of well defined mixed thiols film. The mixed thiols film with exposed carboxylic acid terminals is then immersed into the protein solution after it is activated by NHS and EDC. The immobilization reaction is controlled at 4 °C and incubated for 8 h. Finally, an ideal protein monolayer on the mixed thiols-modified gold substrate is successfully fabricated. For simplicity, the structures of thiols are not entirely shown

A number of techniques may be employed to characterize the protein layers such as scanning electronic microscopy[9], infrared spectroscopy[10], quartz crystal microbalance[11], surface plasmon resonance[12], atomic force microscopy[13], X-ray photoelectron spectroscopy[14], contact angle goniometry[4], grazing incidence X-ray diffraction method[15] and fluorescence detection[16]. These methods provide useful data about surface properties from different aspects, for example, contact angle goniometry is a conventional and classical method which observes the wettability of the surface, whereas the atomic force microscopy is capable of probing protein molecules with force sensitivity down to pico-Newton. As for X-ray photoelectron spectroscopy, it is commonly used to analyze the state and concentration of chemical elements on different surfaces, as well as perform the depth profiling, which offers important information about the distribution of targeting elements.

We have previously fabricated and characterized antibody monolayers on 16-mercaptohexadecanoic acid (MHA) modified gold surfaces and further imaged the recognition events between antibody and antigen and probed the specific interactions between antibody and antigen by atomic force microscopy force measurements. These experiments were based on self-assembled protein monolayers on pure 16-mercapto hexadecanoic acid films, which led to high surface coverage of protein molecules and consequently had to use the Poisson statistical method to calculate the single molecule interaction forces. The rupture force for a single pair of antigen-antibody determined by such method was rather a statistical result than real single molecule interaction. To investigate the real single molecule interaction (whereby only one pair of antigen-antibody will be detected), we propose that one can control the surface coverage of protein molecules by mixing another inert thiol into the 16-mercaptohexadecanoic acid films. By adjusting the molar ratios of 16-mercapto hexadecanoic acid, one can obtain different surfaces with different amounts of protein exposed. The motivation of the present work was to verify the controllability of protein coverage onto mixed self assembled monolayers and to justify the accessibility of antibody to antigen varying with the protein coverage. Two thiols, 16-mercaptohexadecanoic acid and 1-hexadecanthiol (HDT) were selected as a model system to fabricate a series of self assembled monolayers with different molar ratios. The monolayers of human IgG on mixed thiols modified gold substrates were formed by linking protein molecules to the activated carboxylic acid terminals of 16-mercaptohexadecanoic acid and then further characterized by contact angle goniometry, X-ray photoelectron spectroscopy and atomic force microscopy, respectively.

EXPERIMENTAL

16-Mercapto hexadecanoic acid (MHA, $HS(CH_2)_{15}COOH$), 1-hexadecanthiol (HDT, $HS(CH_2)_{15}CH_3$), 1-ethyl-3-(dimethylaminopropyl) carbodi- imide hydrochloride (EDC, $C_8H_{17}N_3{\cdot}HCl$) and *N*-hydroxysulfosuccinimide (NHS, $C_4H_5NO_3$) were purchased from Sigma Aldrich Chemical Co. and used as received. Phosphate buffered saline (PBS, 140 mM NaCl, 3 mM KCl, pH 7.4) and ethanol (guaranteed grade) were purchased from Merck Co. and ultra pure water (resistivity of 18.2 MΩ cm) was obtained by the Millpore purification system. Human IgG and rat anti-human IgG were purchased from Biosun Co. (China).

Preparation of gold coated mica wafer: Gold substrates were prepared by vapour deposition of gold onto freshly cleaved mica in a high vacuum evapourator at -10^{-7} Torr. Mica substrates were preheated to 325 ºC for 2 h by a radiator heater before deposition. Evapouration rates were 0.1-0.3 nm/s and the final thickness of gold films was -200 nm. A chromium layer was also vapour deposited and sandwiched between the gold and mica to strengthen the adhesion between the surfaces. Gold coated, or bare gold substrates were annealed in H_2 frame for 1 min before use.

Formation of mixed self assembled monolayers: 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. A series of mixed thiols were prepared with molar ratios of 16-mercaptohexadecanoic acid: HDT of 0:1, 0.2:0.8, 0.4:0.6, 0.6:0.4, 0.8:0.2 and 1:0, respectively. Then self assembled monolayers were formed by immersing the bare gold substrates in 1 mM thiols in ethanol solution for 24 h. The formed self assembled monolayers with carboxyl tail groups were supersonicated in pure ethanol for 2 min to remove unbound thiols molecules, then rinsed sequentially with pure ethanol, ultra pure water and finally air dried in a N_2 stream.

Protein immobilization to mixed self assembled monolayers: Protein immobilization to self assembled monolayers was carried out as described before with minor modification[17]. In brief, self assembled monolayers with exposed carboxylic acid terminals were activated by 2 mg/mL N-hydroxysulfosuccinimide and 2 mg/mL EDC in phosphate buffered saline

solution for 1 h and subsequently rinsed thoroughly with ultra pure water and air dried in N_2 stream. The activated self assembled monolayers were then immersed into 10 µg/mL human IgG in PBS solution at 4 ºC for 12 h. Finally, the prepared specimens of self assembled monolayers with immobilized protein were stored in PBS solution at 4 ºC before use.

Contact angle measurements: Contact angles of the surfaces were measured by the static sessile drop method using contact angle goniometry (Magic droplet 200, Taiwan) and all measurements were performed under room temperature (-25 ºC) and ambient humidity. One µL drops of Milli-Q water were 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.

X-ray photoelectron spectroscopy measurements: X-ray photoelectron spectroscopy experiments were performed on a PHI Quantera SXM photoelectron spectrometer equipped with an AlK_α radiation source (1486.6 eV). The photoelectrons were analyzed at a take-off angle of 45º. 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). The N1s spectra were fitted using X-ray photoelectron spectroscopy PEAK Version 4.1, the binding energy of central peak was about 400 eV. Depth profile analysis (with a sputter depth of 15 nm) of the protein layer with pure 16-mercapto hexadecanoic acid was performed and the atomic concentration of nitrogen was determined for each protein layer.

Atomic force microscopy force measurements: Commercially available gold coated Si_3N_4 atomic force microscopy tips (Budget Sensors®, Innovative Solutions Bulgaria Ltd. Bulgaria) were used, of which the spring constants, calibrated by thermal fluctuation method[18], were 0.03-0.05 N/m. Functionalized atomic force microscopy tips with rat anti-human IgG coating were prepared similarly as described above. First, the atomic force microscopy tips were cleaned in the hot piranha solution for 30 min and then rinsed with ultra pure water. Subsequently, the tip surfaces were coated with thiols-based self assembled monolayers in the solution of pure 16-mercaptohexadecanoic acid and then activated in the solution of EDC and *N*-hydroxysulfosuccinimide. Finally, the tips were functionalized with rat anti-human IgG coating by incubating the activated tips in PBS solution of the protein at a concentration of 7 µg/mL and at 4 ºC for 8 h. The functionalized tips were stored in PBS at 4 ºC before use.

Adhesion forces between human IgG and rat antihuman IgG were measured by atomic force microscopy using Benyuan CSPM 5000 scanning probe microscope (Benyuan Co. China). When the functionalized atomic force microscopy tip scanned across the well ordered protein monolayer, at a given location, the tip was moved toward the surface of the monolayer and retracted. While the tip approached the monolayer surface it would deflect due to the antigen-antibody interaction force, which would be detected as a voltage-displacement signal and converted into a force-displacement curve[13,19]. Because the tip was considered an elastic cantilever, its deflection was determined by the force (F) exerted on it following Hooke's law, *i.e.*, $F = k \times d$, where d is the deflection, k is the spring constant of the cantilever tip. Generally, k should be small for atomic force microscopy in order to minimize measurement noise[13].

All force measurements were done by using contact mode atomic force microscopy with PBS as the medium between the tip and the protein monolayer and the retraction velocity was estimated to be 0.04 µm/s. From the force-displacement curve the adhesion force between the rat anti-human IgG on the substrate and the human IgG on the tip was calculated. Measurements were repeated many times at each of several randomly selected locations across the protein monolayer on the gold substrate.

The specificity of measured adhesion forces between the human IgG and the rat antihuman IgG were verified by conducting control and blocking experiments. The bare tips were used for control experiment. While in blocking experiment, the rat antihuman IgG functionalized tips were incubated with free human IgG before they were subjected to force measurements.

RESULTS AND DISCUSSION

Surface modification and protein immobilization: Self assembled monolayers method is extensively used for its simplicity and easiness, nevertheless, there are some aspects that required to be considered carefully in order to form satisfactory protein monolayers on self assembled monolayers modified substrates[8,20-22]. These include, but not limited to 1) gold was used as substrate because it is chemically inert and thiols bind to it with a high affinity. Gold deposited mica has ultra-flat surface, which is desirable for self assembled monolayers; 2) 16-Mercapto hexadecanoic acid and HDT were used to form mixed thiols self assembled monolayers because they have the same carbon chain but different terminal groups, which is favourable to compare the surface properties of self assembled monolayers when varying the molar ratios of these two thiols. Also, their long main carbon chains can be served as a spacer to minimize interference between the protein molecules and the gold substrates; 3) Protein immobilization was carried out in PBS buffer solution at 4 ºC and pH = 7.4 because pH and temperature may both affect protein activity; 4) The coated protein layers should not only provide optimally orientated protein molecules, but also give minimal steric hindrance to the protein molecules so that they can expose their specific binding sites. 5) The protein concentration was important for forming uniform protein monolayers. The adequate protein concentration of 10 µg/mL was found for forming uniform layers in this study and above this concentration the proteins might aggregate and form irregular layers. Considering self assembled monolayers method has been proven capable of ensuring the activity, mobility and stability of protein molecules and all experimental aspects addressed properly as described above, the method presented here can be used to prepare tailorable sample surfaces of biological molecules. Indeed, the topography of protein modified surfaces prepared using this method had been examined by atomic force microscopy imaging and confirmed satisfactory[23].

Wettability of the surfaces: Theoretically, the contact angle (θ) of the films formed by pure 16-mercapto hexadecanoic acid will lower than 10º due to the existence of highly hydrophilic carboxyl acid terminals, while the films with HDT will slightly higher than 110º because of the highly hydrophobic terminal methyl group[24-26].The θ of the bare gold surface and the pure HDT film were determined to be 58.2 ± 3.4º (data not shown) and 117.6 ± 4.2º, respectively (Fig. 2). The former data are consistent with results from other studies[27,28]. Since the θ increases with the increasing carbon chain length, it is not surprising that the latter data was somewhat higher than the values of thiols with short-to-moderate carbon chain lengths.

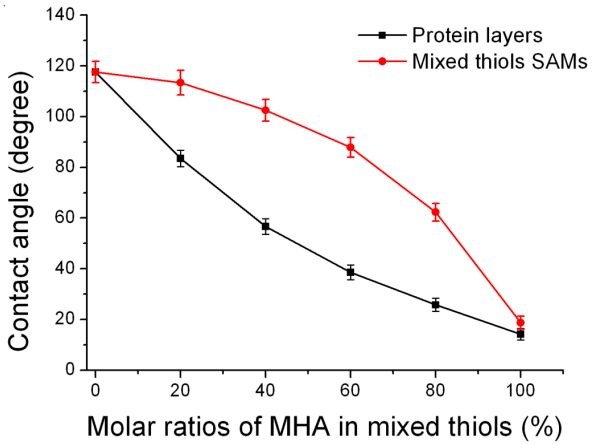


Fig. 2. The contact angles of six mixed thiols (red dots) films which contain 0, 20, 40, 60, 80 and 100 % of MHA, were determined to be 117.6 ± 4.2º, 113.4 ± 4.9º, 102.5 ± 4.3º, 87.9 ± 3.9º, 62.3 ± 3.5º and 18.8 ± 2.6º, respectively. The black squares denote the contact angles of human IgG monolayers above the mixed thiols films. The contact angles of these six different protein surfaces were found to be 117.6 ± 4.2º, 83.5 ± 3.3º, 56.7 ± 3.1º, 38.6 ± 2.9º, 25.8 ± 2.6º and 14.2 ± 2.3º, respectively.

The θ of mixed thiols films were gently decreased with the decreasing of 16-mercapto hexadecanoic acid in mixed thiols when the molar ratios of 16-mercapto hexadecanoic acid were higher than 50 %, whereas dramatic decrease was observed when the molar ratios of 16-mercapto hexadecanoic acid were lower than 50 %. In contrast, the θ of protein layers above the mixed thiols films have the opposite tendency, suggesting protein molecules significantly altered the surface wettability. Notably, the θ of the protein layer was measured to be 14.2 ± 2.3º, which was very close to that of the pure 16-mercapto hexadecanoic acid film (18.8 ± 2.6º). The high hydrophilicity of protein surface was further verified by measuring a series of films of mixed thiols. It was found that the θ of the protein layers on the mixed thiols films containing 20, 40, 60 and 80 % of 16-mercapto hexadecanoic acid were 83.5 ± 3.3º, 56.7 ± 3.1º, 38.6 ± 2.9º, 25.8 ± 2.6º, respectively (Fig. 2, black dots), each data point was significantly smaller than its counterpoint of the mixed thiols films (Fig. 2, red dots). These results suggested that both the 16-mercapto hexadecanoic acid film and the protein monolayers have hydrophilic surfaces.

As mentioned above, the θ of the pure 16-mercapto hexadecanoic acid film should lower than 10º. But our experimental data (18.8 ± 2.6º) showed that 16-mercapto hexadecanoic acid film exhibit a hump (which means the θ of -COOH end group surfaces may exhibit higher than it should be). This phenomenon was also observed by Lee *et al.*[29,30]. They suggested the hump probably stems from conformational changes of the carboxylate groups upon partial ionization and offered two hypothesis to rationalize the existence of the hump. The first hypothesis (the disorder theory) states that the onset of ionization induces a sufficient degree of disorder in the monolayer to cause hydrophobic methyl groups to be exposed at the surface. The second one proposes that the hump originates from stable hydrogen bonding at intermediate values of pH, which results in the exposure at the surface of carbonyl groups or methyl groups rather than the more hydrophilic -COOH or COO^- groups.

Determination of atomic concentrations of nitrogen and depth profile analysis by X-ray photoelectron spectroscopy: It is the consensus that protein contains 12-16 % of nitrogen elements. So it is efficient to characterize protein layers by determining the amounts of nitrogen in each surface. To this end, the atomic concentration of nitrogen of the protein layer immobilized on pure 16-mercapto hexadecanoic acid film was determined and the result showed it had the relative content of 11.43 ± 0.56 %, which was close to theoretical prediction.

In addition, depth profile analysis of the protein layer on pure 16-mercapto hexadecanoic acid film was conducted and the results were summarized in Fig. 3. From 0 to 7 nm of sputter depth, the atomic concentrations of carbon, oxygen, nitrogen and sulfur elements were about 68, 15, 11 and 3 %, respectively. Such distribution of elements can be attributed to the existence of homogeneously immobilized protein molecules onto the 16-mercapto hexadecanoic acid film. Nevertheless, small amount of gold (about 3 %) was still detected in the protein surface, suggesting defects occurred during self assembled monolayers formation[31,32]. The thickness of protein layer was estimated to be 7 nm. It is consistent with the values reported in literature which were determined either by X-ray photoelectron spectroscopy or other methods[33-35], suggesting the protein molecules exhibited compact conformations other than elongated conformations (20 nm length)[36]. As the sputter depth increased to 9 nm, the atomic concentrations of oxygen and nitrogen steadily declined, while the atomic concentration of carbon dramatically rose. Such observation may be accounted for the detection of 16-mercapto hexadecanoic acid film beneath the protein layer. The thickness of 16-mercapto hexadecanoic acid film approximated 2 nm, which were in line with other studies[37-39]. When sputtered to 12 nm, there were almost pure gold, but trace amount of carbon and oxygen were still detected, indicating minor contamination happened prior to measurement.

Force sensing by atomic force microscopy: As human IgG molecules were successfully immobilized on the mixed thiols films and the atomic force microscopy tips were functionalized by rat antihuman IgG, the specific interaction forces between human IgG and rat antihuman IgG can be sensed by atomic force microscopy force measurements. The antigen surfaces were firstly imaged, the antigen molecules were pinpointed by analyzing the height and volume of surface particles, the discerned antigen molecules were then

subjected to force measurements. The specificity of interaction was verified by performing control and blocking experiments. The adhesive forces of control and blocking experiment were determined to be 13 ± 4 and 54 ± 12 pN (Fig. 4, red and blue lines), respectively. Considering the noise of the instrument, they could be assigned to no interaction and nonspecific interaction.

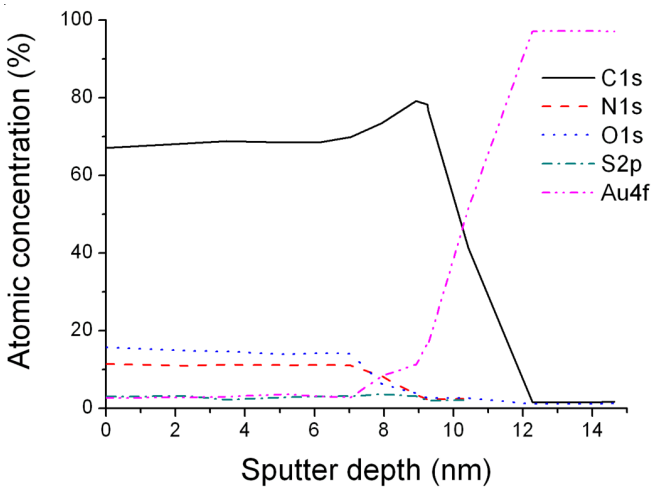


Fig. 3. Depth profile analysis of the protein layer immobilized on pure MHA film. The sputter depth ranged from 0 to 15 nm and the atomic concentrations of C, N, O and S elements were recorded accordingly.

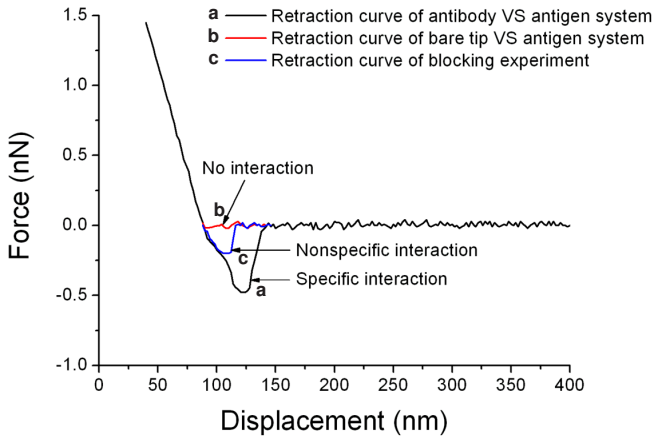
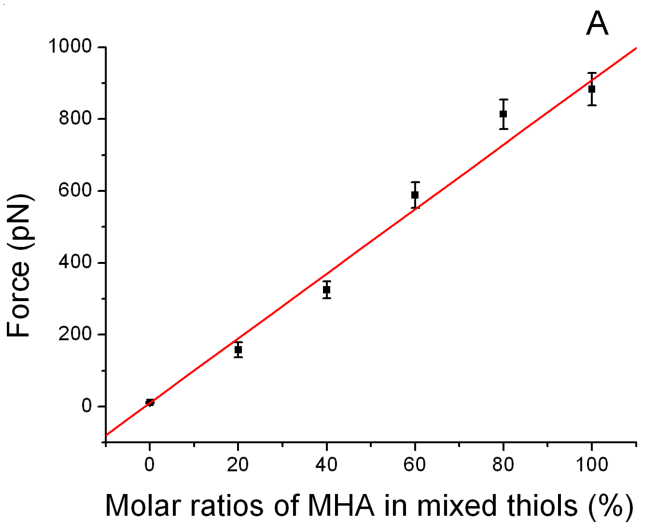
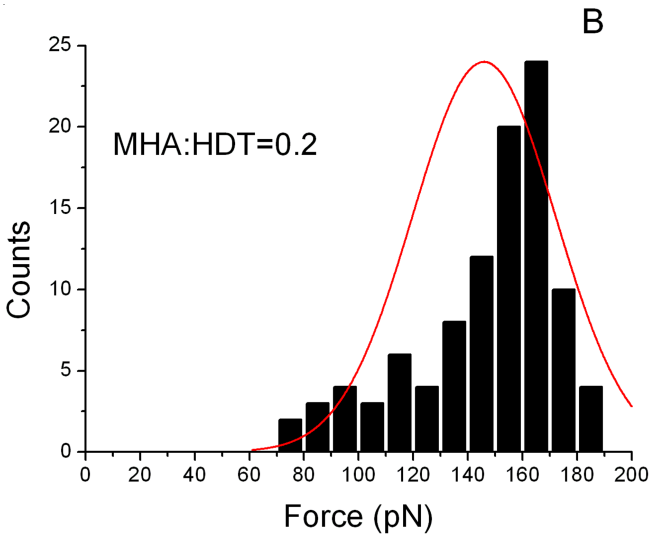


Fig. 4. A typical force-displacement curve recorded by AFM. When the antibody functionalized AFM tip is far from the antigen surface, there is no force detected. As the tip approaches, there are attractive forces between antigen and antibody, when they are close enough, the tip will jump onto the antigen surface. On the contrary, when the tip retracts, the AFM cantilever will not jump off the antigen surface immediately due to the existence of strong interaction forces between antigen and antibody until the exert forces are larger than the specific interaction forces. When the distance separating the tip and antigen layer is large enough, the AFM tip will back to the equilibrium state. Finally, the determined interaction forces are called rupture forces.

The determined adhesive forces between antibody and antigen layers with molar ratios of 16-mercaptohexadecanoic acid of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 were 11 ± 2, 158 ± 21, 325 ± 24, 589 ± 36, 814 ± 41 and 883 ± 45 pN, respectively (Fig. 5). As shown in Fig. 5, a linear relationship was observed between adhesive forces and molar ratios of 16-mercapto hexadecanoic acid (R = 0.9952), implying more antigen molecules accessible, more antigen-antibody bonds formed, further resulted in larger specific interaction forces. More specifically, at low molar ratio of 16-mercapto hexadecanoic acid (0.2), the adhesive forces between antibody and antigen (158 ± 21 pN) was very close to the single rupture force between human IgG and rat antihuman IgG. (14 4 ± 11 pN)[40], suggesting the human IgG molecules were sparsely distributed on the surface. However, with high molar ratio of 16-mercapto hexadecanoic acid (1.0), the adhesive force of the interaction system was found to be 883 ± 45 pN, which was also six times larger than the above determined value, suggesting the human IgG molecules were densely packed on the surface. As the contents of 16-mercapto hexadecanoic acid increased, the pairs would manifold between antibody functionalized tips and antigen layers. This phenomenon was also observed in previous studies when protein layers were fabricated using thiols based self assembled monolayers, in which the number of pairs were calculated to be couples to tens[41-43].





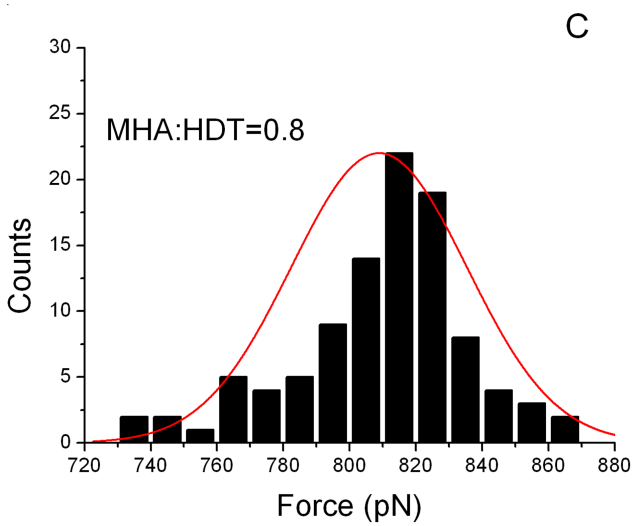


Fig. 5. Adhesive forces determined between rat antihuman IgG functionalized AFM tips and human IgG immobilized on mixed thiols modified gold substrates (A). The adhesive forces for different protein layers with molar ratios of MHA in mixed thiols of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 were 11 ± 2, 158 ± 21, 325 ± 24, 589 ± 36, 814 ± 41 and 883 ± 45 pN, respectively. The red line is the linear fit of determined adhesive forces (R = 0.9952) and the errors are ± SD. Also, the force distributions of MHA:HDT = 0.2 and MHA:HDT = 0.8 are shown in B and C, respectively. In these two cases, the most probable force values were 130-180 pN and 790-840 pN, respectively. The red lines in B and C are Gaussian fits

Conclusion

A series of mixed thiols films with different molar ratios of 16-mercapto hexadecanoic acid were formed by self assembled monolayers method and human IgG monolayers binding onto these films were fabricated and characterized by contact angle measurements, X-ray photoelectron spectroscopy, atomic force microscopy force measurements, respectively. Results showed that both the 16-mercapto hexadecanoic acid film and the protein monolayer were highly hydrophilic, whereas the HDT film was hydrophobic. The X-ray photoelectron spectroscopy measurements showed the atomic concentrations of nitrogen of protein layers increased with the increasing of molar ratios of 16-mercapto hexadecanoic acid. In addition, depth profile analysis of X-ray photoelectron spectroscopy displayed the atomic distributions of the protein layer on pure 16-mercapto hexadecanoic acid film. Both the atomic concentrations and the thickness of constitutive layers were agreeable to other experimental results. Moreover, atomic force microscopy force measurements were performed to sense the specific interaction forces between antibody functionalized tips and antigen layers, the results showed that the determined adhesive forces were linear with the molar ratios of 16-mercapto hexadecanoic acid, or more precisely, the amount of free antigen molecules on the surfaces of protein layers. At low molar ratio of 16-mercapto hexadecanoic acid, the adhesive force was very close to rupture force which required to separate single antigen-antibody pair, while at high molar ratio of 16-mercapto hexadecanoic acid, multiple interactions may occur. Taken together, these results suggested that using the presented method, protein molecules can be successfully bound to mixed thiols modified gold substrates with good reproducibility and adjustability. Therefore, this mixed thiols based method may provide a well suitable approach for protein immobilization and further a scaffold for single molecule detection.

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REFERENCES

1. F. Cecchet, A. Duwez, S. Gabriel, C. Jerome, R. Jerome, K. Glinel, S. Demoustier-Champagne, A.M. Jonas and B. Nysten, *Anal. Chem.*, **79**, 6488 (2007).
2. W. Lee, B. Oh, Y.M. Bae, S. Paek, W.H. Lee and J. Choi, *Biosens. Bioeletron.*, **19**, 185 (2002).
3. N.K. Chaki and K. Vijayamohanan, *Biosens. Bioeletron.*, **17**, 1 (2002).
4. M.C. Martins, B.D. Ratner and M.A. Barbosa, *J. Biomed. Mater. Res. A*, **67**, 158 (2003).
5. H. Kim, H. Cho, J.H. Park, S. Kim, S. Paek, J. Noh and H. Lee, *Colloids Surf. A*, **313-314**, 541 (2008).
6. R.K. Smith, P.A. Lewis and P.S. Weiss, *Progr. Surf. Sci.*, **75**, 1 (2004).
7. K.L. Carraway and D.E. Jr. Koshland, *Methods Enzymol.*, **25**, 616 (1972).
8. J.C. Love, L.A. Estroff, J.K. Kriebel, R.G. Nuzzo and G.M. Whitesides, *Chem. Rev.*, **105**, 1103 (2005).
9. Y. Sasaki, Y. Suzuki and T. Ishibashi, *Science*, **263**, 62 (1994).
10. R. Adato, A.A. Yanik, J.J. Amsden, D.L. Kaplan, F.G. Omenetto, M.K. Hong, S. Erramilli and H. Altug, *Proc. Natl. Acad. Sci. USA*, **106**, 19227 (2009).
11. K. Nakano, T. Yoshitake, Y. Yamashita and E.F. Bowden, *Langmuir*, **23**, 6270 (2007).
12. E. Ostuni, B.A. Grzybowski, M. Mrksich, C.S. Roberts and G.M. Whitesides, *Langmuir*, **19**, 1861 (2003).
13. P. Hinterdorfer and Y.F. Dufrene, *Nat. Methods*, **3**, 347 (2006).
14. P. Facci, D. Alliataa, L. Andolfia, B. Schnyderb and R. Kotz, *Surf. Sci.*, **504**, 282 (2002).
15. Z. Lv, J. Wang, L. Deng and G. Chem, *Nanoscale Res. Lett.*, **4**, 1403 (2009).
16. L.S. Jang and H. Keng, *Biomed. Microdevices*, **10**, 203 (2008).
17. J. Wakayama, H. Sekiguchi, S. Akanuma, T. Ohtani and S. Sugiyama, *Anal. Biochem.*, **380**, 51 (2008).
18. J.L. Hutter and J. Bechhoefer, *Rev. Sci. Instrum.*, **64**, 1868 (1993).
19. B. Cappella and G. Dietler, *Surf. Sci. Rep.*, **34**, 1 (1999).
20. S. Ferretti, S. Paynter, D.A. Russell, K.E. Sapsford and D.J. Richardson, *TrAC Trends Anal. Chem.*, **19**, 530 (2000).
21. G.B. Demirel and T. Caykara, *Appl. Surf. Sci.*, **255**, 6571 (2009).
22. Z. Lv, J. Wang, G. Chem and L. Deng, *Int. J. Biol. Macromol.*, **47**, 661 (2010).
23. L. Li, S. Chen, S. Oh and S. Jiang, *Anal. Chem.*, **74**, 6017 (2002).
24. P.A. DiMilla, J.P. Folkers, H.A. Biebuyck, R. Harter, G.P. Lopez and G.M. Whitesides, *J. Am. Chem. Soc.*, **116**, 2225 (1994).
25. P.E. Laibinis, C.D. Bain, R.G. Nuzzo and G.M. Whitesides, *J. Phys. Chem.*, **99**, 7663 (1995).
26. H. Ann, M. Kim, D.J. Sandman and J.E. Whitten, *Langmuir*, **19**, 5303 (2003).
27. T. Ishida, S. Tsuneda, N. Nishida, M. Hara, H. Sasabe and W. Knoll, *Langmuir*, **13**, 4638 (1997).
28. P.E. Laibinis and G.M. Whitesides, *J. Am. Chem. Soc.*, **114**, 1990 (1992).
29. T.R. Lee, R.I. Carey, H.A. Biebuyck, G.M. Whitesides, *Languir*, **10**, 741 (1994).
30. S. Tam-Chang, H.A. Biebuyck, G.M. Whitesides, N. Jeon and R.G. Nuzzo, *Languir*, **11**, 4371 (1995).
31. C. Schonenberger, J.A.M. Sondag-Huethorst, J. Jorritsma and L.G.J. Fokkink, *Langmuir*, **10**, 611 (1994).
32. J.A.M. Sondag-Huethorst, C. Schonenberger and L.G.J. Fokkink, *J. Phys. Chem.*, **98**, 6826 (1994).

33. F. Hook and B. Kasemo, *Anal. Chem.*, **73**, 5796 (2001).
34. F. Caruso, D.N. Furlong and P. Kingshott, *J. Colloid Interf. Sci.*, **186**, 129 (1997).
35. C. Zhou, J.M. Friedt, A. Angelova, K.H. Choi, W. Laureyn, F. Frederix, L.A. Francis, A. Campitelli, Y. Engelborghs and G. Borghs, *Langmuir*, **20**, 5870 (2004).
36. F.A. Denis, P. Hanarp, D.S. Sutherland, J. Gold, C. Mustin, P.G. Rouxhet and Y.F. Dufrene, *Langmuir*, **18**, 819 (2002).
37. N. Faucheux, R. Schweiss, K. Lutzow, C. Werber and T. Groth, *Biomaterials*, **25**, 2721 (2004).
38. N.L. Abbott, J.P. Folkers and G.M. Whitesides, *Science*, **257**, 1380 (1992).
39. M.D. Porter, T.B. Bright and D.L. Allara, *J. Am. Chem. Soc.*, **107**, 3559 (1987).
40. Z. Lv, J. Wang, G. Chen and L. Deng, *Nanoscale Res. Lett.*, **5**, 1032 (2010).
41. Y. Jiang, C. Zhu, L. Ling, L. Wan, X. Fang and C. Bai, *Anal. Chem.*, **75**, 2112 (2003).
42. T. Han, J.M. Williams and T.P. Beebe, Jr., *Anal. Chim. Acta*, **207**, 365 (1995).
43. J.M. Williams, T. Han and T.P. Beebe, Jr., *Langmuir*, **12**, 1291 (1996).