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Surface plasmon resonance biosensor with high anti-fouling ability for the detection of cardiac marker troponin T

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ABSTRACT

Designing a surface recognition layer with high anti-fouling ability, high affinity, and high specificity is an important issue to produce high sensitivity biosensing transducers. In this study, a self-assembled monolayer (SAM) consisting of a homogeneous mixture of oligo(ethylene glycol) (OEG)-terminated alkanethiolate and mercaptohexadecanoic acid (MHDA) on Au was employed for immobilizing troponin T antibody and applied in detecting cardiac troponin T by using surface plasmon resonance (SPR). The mixed SAM showed no phase segregation and exhibited human serum albumin resistance, particularly with an antibody-immobilized surface. X-ray photoemission spectra revealed that the chemical composition ratio of OEG to the mixed SAM was 69% and the OEG packing density was 82%. The specific binding of troponin T on the designed surface indicated a good linear correlation ($R = 0.991$, $P < 0.0009$) at concentrations lower than 50 μg mL^{-1} with the limit of detection of 100 ng mL^{-1} using a SPR measuring instrument. It is concluded that the mixed SAM functions as designed since it has high detection capability, high accuracy and reproducibility, as well as shows strong potential to be applied in rapid clinical diagnosis for label-free detection within 2 min.

1. Introduction

Many transducers are being developed for biomedical diagnostics, which require high sensitivity, affinity, and specificity, and allow for label free and real-time biomolecular detection [1]. However, many of these devices have low selectivity and affinity for detecting targeted biomarkers. High-sensitivity transducers frequently give false-positive results that limit their biosensor utility. Therefore, the development of an anti-fouling surface as a recognition layer is still a major challenge for surface and analytical chemistry.

Antibody and antigen bindings are the most promising methodology for determining trace quantities of a specified biomarker in aqueous multi-biomolecular backdrop often seen in complex matrices [2], because an antibody has high affinity and selectivity for their antigen or their targeted molecular. Therefore, using antibodies can offset low affinity and low selectivity in designing biosensors. However, it cannot overcome the issue of protein fouling, which is the non-targeted molecular binding to chip surface, especially seen when detecting biomarkers in human fluids such as blood present due to illness or injury.

Myocardial infarction is typically diagnosed by integrating the history of the presenting illness and physical examination with electrocardiogram results and cardiac markers present in blood [3,4]. The cardiac markers are proteins that leak out of injured myocardial cells through their cell membranes into the bloodstream. Cardiac troponin T, a superior and clinical biomarker for myocardial injury [5–7], is released within 4–6 h of an attack of myocardial infarction and remains elevated for up to 2 weeks.

A label-free detection method of troponin T using surface plasmon resonance (SPR) technique has been investigated [8–10] previously. In previous publications, surface chemistries used carboxymethylated dextran and cysteamine to modify gold surface and detection limits of troponin T ranged from 30 ng mL^{-1}, 0.03 ng mL^{-1} to 0.05 ng mL^{-1}, respectively. However, these studies used a sandwich or multilayer method to increase the immobilized amount of cardiac troponin T antibody to enhance the signal. These ranges of detection for troponin T varied by a factor of 100 and there is a need to develop an efficient mixed SAM. The effectiveness of

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a bi-functional mixed SAM layer that also has non-fouling characteristics as well as high specific binding on the engineered surface has yet to be investigated. The anti-fouling surface exhibits high hydrophilicity and net molecular charge of zero, and is a hydrogen bond accepter not a donor [11]. Therefore, designing a recognition layer with anti-fouling ability is of great importance to improve the specific binding of biomarkers.

Oligo(ethylene glycol) (OEG) has been extensively employed for surface grafting to create protein-resistant surfaces [12–14]. The self-assembled monolayer (SAM) of OEG with high hydrophilicity, hydrogen bond accepter, and the zero net charge is suitable for creating the anti-fouling surface [15,16]. In particular, the OEG's surface with packing densities from 60% to 80% on Au showed excellent anti-fouling ability [17]. However, the OEG functional group is difficult to cross-link with biomolecules to retain its anti-fouling ability. Therefore, it should be combined with other molecules to create a mixed SAM [18–20]; however, the homogeneously mixed SAM with the above functionality was not easy to obtain due to the phase separation of thiol-molecules [21]. A well constructed chemically mixed SAM must take into account intermolecular hydrogen bonds and carbon chain length relating to van der Waals force [22,23]. To our knowledge, there are no previous investigations on applying a mixed SAM of OEG and mercaptohexadecanoic acid (MHDA) for creating an anti-fouling surface. The MHDA has COOH groups which can be used to react with NH_2 groups on the antibody.

In this study, a densely packed SAM of homogeneous OEG and MHDA mixture on Au was developed by controlling the concentrations and mixing ratios of OEG and MHDA solution. The structure and chemical composition of the SAMs were characterized by X-ray photoemission spectroscopy (XPS) and atomic force microscopy (AFM). The performance of anti-fouling functionality was evaluated based upon its ability to resist human serum albumin (HSA) adsorption. The mixed SAM immobilized troponin T antibody was then applied for detecting troponin T by using the SPR technique.

2. Experimental

2.1. Materials

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS), MES buffer saline (0.1 mol L^{-1} of 2-[morpholino]-ethanesulfonic acid, 0.9% NaCl, pH 4.7) were purchased from Thermo Scientific (USA). (1-Mercaptoundec-11-yl)tetra(ethylene glycol) (OEG), 16-mercaptohexdecanoic acid (MHDA), cardiac troponin T antibody (38 kDa), troponin T, and HSA were obtained from Sigma Aldrich (USA). Dulbecco's phosphate buffer saline (D-PBS) was employed for the SPR experiments. Other reagents and solvents were purchased commercially and used without further purification.

2.2. Instrumentation

2.2.1. Contact angle of self-assembled monolayers

The water contact angle was measured using on a Dino-lite digital microscope (AnMo Electronics Corp.) mounted in the plane of the sample to show the droplet cross-section. The contact angle was based upon a 5 μL distilled water droplet on the chip surfaces. The contact angle was estimated from the cross-sectional image using software supplied the microscope manufacturer, Dino-Capture 2.0.

2.2.2. X-ray photoelectron spectroscopy

XPS (PHI 5000 VersaProbe, ULVAC-PHI Inc.) was used to measure the atomic compositions and to calculate the packing density. An Al K alpha anode of X-ray source at 15 kV and 25 W was used to stimulate photoemission. The spot size of 100 μm and the take-off angle of 45° were fixed. Typical pressure in the analysis chamber during spectral acquisition was 10^{-6} Pa. The electron gun was neutralized with Ar gas. The pass energy of 117.4 eV, step of 1 eV, and time/step of 50 ms $step^{-1}$ for wide scan, and the pass energy of 23.5 eV, step of 0.2 eV, and time step of 50 ms $step^{-1}$ were employed for the measurements. Data analysis software supplied by the XPS vendor was used to fit the spectra and to calculate elemental compositions from the peak areas. The peaks were fitted by using the Gauss–Lorentz mode.

2.2.3. Atomic force microscopy

The surface morphologies, topographical and phase images, and roughness of the surfaces were observed by an AFM (SPM-9500; Shimadzu Inc.). A silicon cantilever (Olympus, OMCL-AC160TS) was used for a dynamic mode and the scanning area was 2 μm × 2 μm. The surface roughness was calculated by employing root mean square (RMS) and Ra based upon the *Z*-range values.

2.2.4. Surface plasmon resonance measurement

SPR (Navi 200, BioNavis Ltd.) was employed for monitoring the recognition interaction between antibody immobilized surface and for detecting an antigen. The equipment was based on the Kretschmann configuration using attenuated total reflection and combined with a liquid flow analysis system. The sensor chip was attached to the prism base, and optical contact was established by using refractive index matching oil. A P-polarized light wave from a laser, incident light source, is directly at an internal angle of θ to the Au-prism interface. Before measurements began, the sensorgram was stabilized at room temperature by using D-PBS as a carrier buffer for 10 min. When the system reached a steady state, a 100 μL sample was injected at the flow rate of 100 μL min^{-1} for 1 min. The carrier buffer of D-PBS was then used for washing away unbound protein and it continued to flow for 10 min. The concentrations of troponin T in D-PBS were 50 μg mL^{-1}, 25 μg mL^{-1}, 10 μg mL^{-1}, 1 μg mL^{-1}, and 100 ng mL^{-1}. 1 mg mL^{-1} and 50 μg mL^{-1} of HSA dispersed in D-PBS were also introduced to evaluate the anti-fouling ability of the SAMs.

2.3. Preparation of biosensor chips

2.3.1. Preparation of mixed self-assembled monolayers

A 10 mmol L^{-1} OEG and a 3 mmol L^{-1} MHDA were dissolved in absolute ethanol. The mixed OEG/MHDA solution was adjusted with the MHDA/OEG molar ratio of 0.3 at the concentrations of 0.3 mmol L^{-1} for MHDA and 1 mmol L^{-1} for OEG. The Au chips for the SPR measurements were cleaned by UV-ozone irradiation (λ_{irr} = 254 and 185 nm; UV/Ozone Cleaner™, Bioforce Nanoscience Co. Ltd.) for 20 min and were extensively rinsed with ultrapure water followed by absolute ethanol. The cleaned chips were then immersed in the OEG/MHDA solution overnight at 37 °C, which were washed several times with absolute ethanol and dried under nitrogen blow. Following this same procedure, the OEG and MHDA monolayers were prepared as controls.

2.3.2. Antibody immobilization

Cardiac troponin T antibody was immobilized on the carboxyl group of MHDA through the EDC/NHS active sites. The chips with the mixed SAM were soaked at room temperature into the MES buffer including EDC/NHS (2 mmol L^{-1}/5 mmol L^{-1}) for 15 min and activated at carboxyl groups of MHDA. The chips were then immersed in the antibody solution (10 μg mL^{-1}) for 30 min at room temperature and the unreactive antibody was washed with D-PBS. The antibody-immobilized chips were stored in D-PBS under 4 °C until use. A schematic procedure for creating the mixed SAM and for immobilizing troponin T antibody is shown in Fig. 1.

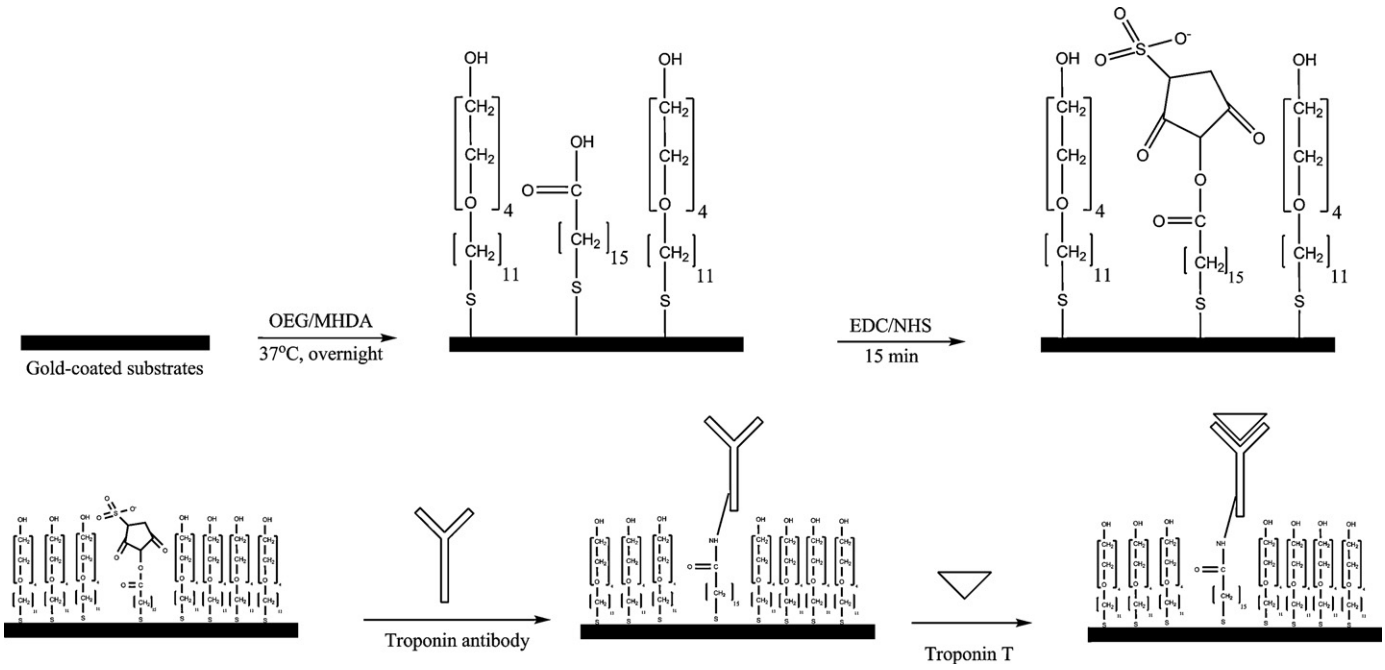

Fig. 1. Schematic diagram of surface modification for the mixed SAM of OEG and MHDA and immobilization method of antibody binding carboxyl groups of MHDA on Au.

3. Results and discussion

3.1. Characterization of self-assembled monolayers

To exhibit anti-fouling ability of the SAMs, the wettability, hydrophilic and hydrophobic properties, are crucial factors. Table 1 shows the contact angles of water droplets on the bare Au, OEG, MHDA, OEG/MHDA, and antibody fixed OEG/MHDA SAMs. The Au surface exhibited a value of 70.9°. After the formation of OEG and MHDA SAMs on Au, the contact angles were decreased to 53.9° and 30.2°, respectively. The value of OEG SAM in the present study is higher than those at 30–40° reported previously [24,25], but nearly matched the values reported for the SAMs terminated with [–$(OCH_2CH_2)_3$–OH] and [–$(OCH_2CH_2)_4$–OH] at 59.6° [26] and 61.0° [27]. The MHDA SAM exhibited less hydrophobicity [28,29] compared with the OEG SAMs. The MHDA SAM usually shows a contact angle of about 10°, but our result was 30.2°. This difference may be attributed to the nanostructure of the Au surface. According to the Wenzel's equation [30], the microstructure of a surface amplifies the natural tendency of the surface. However, the Au surface was sufficiently smooth which allows for neglecting topography effects. Therefore, the contact angle is thought to exhibit the characteristics attributed to OEG and MHDA. In addition, the contact angle of the mixed SAM at 40.2° was between the values of OEG and MHDA SAMs, which were attributed to the content of OEG and MHDA. After immobilizing the antibody on the mixed SAM, the value was shifted from 40.2° to 48.2°. This change was significant due to the antibody that contains hydrophobic domains of the side chain of amino acids.

AFM topographical and phase images in Fig. 2 revealed how the different surfaces impacted the morphology. The result indicated that Au, OEG, MHDA and OEG/MHDA SAMs had atomically smooth surfaces. The Ra and RMS values of the SAMs were 0.423 nm and 0.547 nm for the Au, 0.28 nm and 0.36 nm for OEG, 0.528 nm and 0.812 nm for MHDA, and 0.417 nm and 0.527 nm for OEG/MHDA coated surfaces, respectively. While the OEG and MHDA SAMs showed some voids judging from the topographical images, the OEG/MHDA SAM showed few voids and no phase segregation. It indicated that the structure of the mixed SAM was homogeneous and had a high packing density.

In order to verify the OEG and MHDA ratio on Au, XPS was used to confirm the atomic composition of the chip surface. Fig. 3 shows the C 1s peaks of OEG, MHDA, and the mixed SAM. The C 1s peaks of OEG on Au have two distinct peaks at 286.5 eV and 285.0 eV assigned to the C–O and CH_2 functional groups [31]. On the other hand, the C 1s peak of MHDA on Au has three peaks at 289.1 eV, 286.0 eV, and 285.0 eV assigned to the C=O, C–O, and CH_2 functional groups [32]. The C 1s peaks of OEG and MHDA were deconvoluted into the corresponding functional groups, which was in good agreement with their binding energies. Particularly, the area ratio of C=O to C–O of MHDA SAM was calculated to be 1.04, which nearly matched the theoretical compositional ratio of 1.0. Therefore, for the mixed SAM, by subtracting the area under the C–O peak from that under the C=O peak, the result then represents the ratio of OEG molecules. Thus, the OEG/(OEG + MHDA) ratio is able to be determined based upon the peak fitted areas of C–O and C=O. The compositional ratio was calculated to be 0.69 ± 0.05 which nearly coincides with the theoretical ratio at 0.77 (10/13).

The structure of pure OEG SAM was generally dependent on the solvent particularly water content in ethanol and the temperature [31]. Furthermore, the packing density of OEG is crucial to achieve the anti-fouling ability of its surface, which is attributed to the hydration of OEG functional groups [31]. The density of OEG was previously defined as the ratio of the area under the O 1s peak from the mixed SAM to that of pure OEG [17,32]. Thus, in this study, the O 1s peak at 532 eV was used to determine the packing density of

Table 1
Contact angle measurements of Au and after surface treatments.

Surface	Au	OEG	MHDA	Mixed SAM of OEG and MHDA	Antibody-immobilized
Contact angle (°)	70.9	53.9	30.2	40.2	48.2

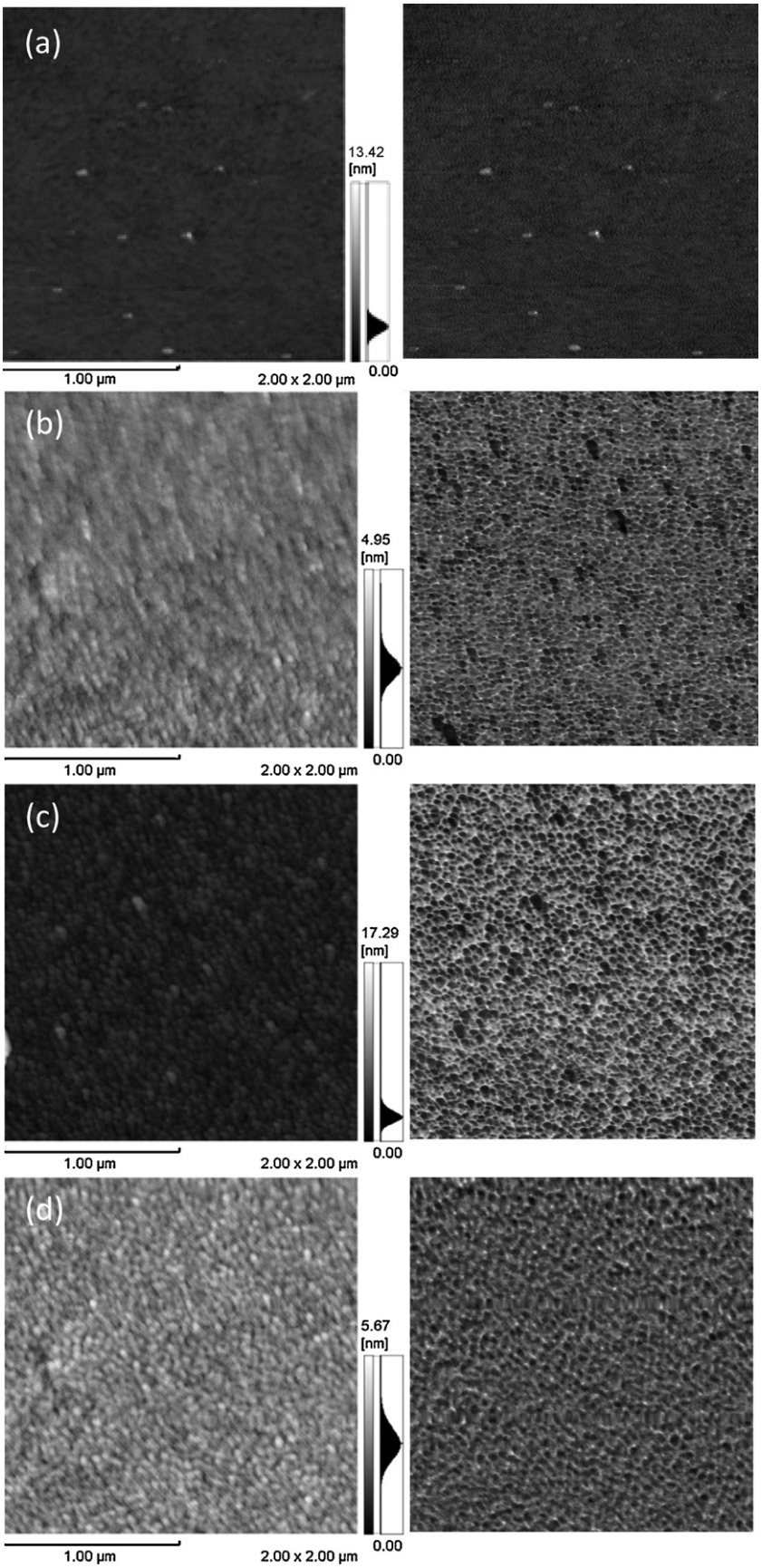

Fig. 2. AFM topographical and phase images of: (a) Au, (b) OEG, (c) MHDA, and (d) OEG/MHDA mixed SAM. Left is the topographical images and right is the phase images.

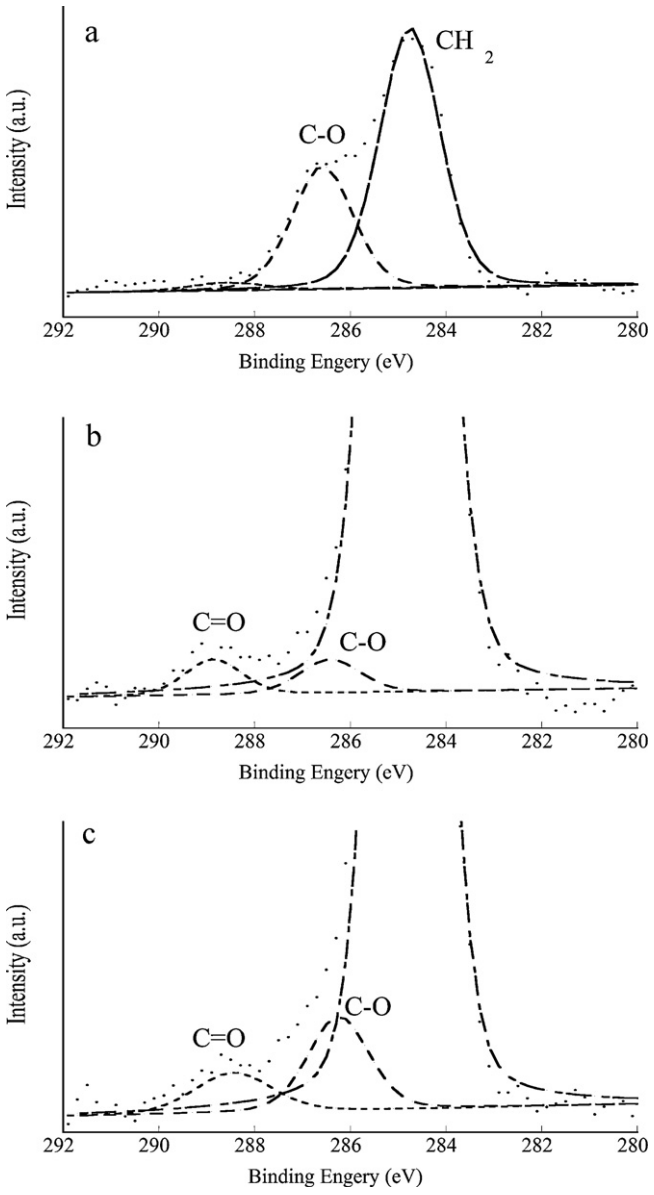

Fig. 3. XPS of C 1s peaks of: (a) OEG, (b) MHDA, and (c) the mixed SAM of OEG and MHDA. The peak was fitted by using the Gauss–Lorentz mode under a similar half width. The dots represent experimental data and the dashed lines are curve-fitted results.

OEG in the mixed SAM. The packing density of OEG was determined as follows; the O 1s peak area of OEG in the mixed SAM was calculated from the compositional ratio of 0.69, which was then divided by the O 1s area of pure OEG. The packing density was thus calculated to be 82%, indicating that the mixed SAM fabricated would have anti-fouling ability [17].

3.2. Antibody immobilization

Fig. 4 exhibits the sensorgram of the immobilization process of cardiac troponin T antibody. D-PBS was initially introduced for determining the background level, and the MES buffer including EDC/NHS was flowed to activate the carboxyl group of MHDA [32]. After activation, the MES buffer was replaced by PBS in order to determine the density of active sites. Finally, the antibody was introduced and the un-reactive antibody was rinsed away with D-PBS. The result indicated that the amount of active sites on the surface was 0.009 ng mm^{-2} or 3.1×10^{-5} mole mm^{-2}

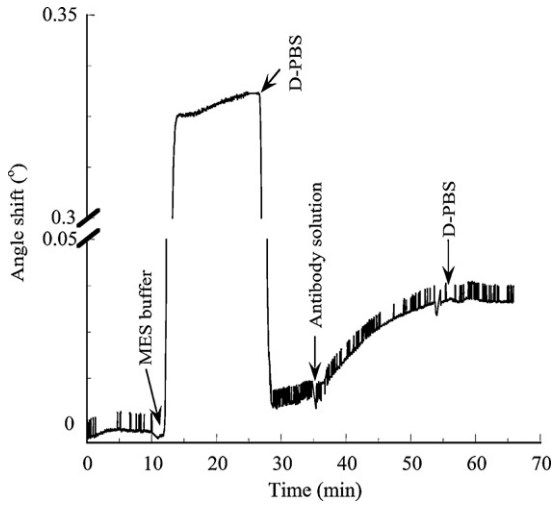

Fig. 4. Sensorgram of the immobilization process for cardiac troponin T antibody. Initially, D-PBS was introduced for stabilizing a baseline and then the MES buffer was flowed and activated the carboxyl groups of MHDA of which surface was rinsed with D-PBS. The antibody binds to the surface active sites, which was rinsed with D-PBS again.

and the amount of protein immobilized was 0.028 ng mm^{-2} or 7.4×10^{-7} mole mm^{-2}.

The AFM topographical and phase images in Fig. 5 clearly suggested that the cardiac troponin T antibodies were immobilized on the mixed SAM. In the topographical image, the white dots represent antibodies binding to carboxyl groups of MHDA. The Ra value of the surfaces before and after antibody immobilization was apparently increased from 0.42 nm to 0.87 nm, and the RMS was also increased from 0.53 nm to 1.18 nm. There are no visual defects even after the immobilization process. These results suggest that this procedure succeeded in antibody immobilization.

3.3. Anti-fouling ability evaluation

To characterize the anti-fouling ability, 1 mg mL^{-1} HSA was used for verifying the non-specific adsorption compared with surfaces of the bare Au, the mixed SAM, and already immobilized troponin antibody. The sensorgrams of these experiments are illustrated in Fig. 6. The sensorgrams obtained on three different surfaces were apparently distinguishable and the antibody immobilized chip had the best anti-fouling ability. The resonance angle shift of bare Au, the mixed SAM, and antibody immobilized chip was 0.166, 0.048 and 0.025, respectively. While the mixed SAM reduced the protein fouling by 71%, the chip immobilized with the antibody reduced the protein fouling by 85%. According to the literature [33,34], the adsorption of 1 ng of protein on the surface may lead to a shift in the angle of about 0.1° and the amount of non-specific binding protein on antibody-immobilized surface was 0.025 ng mm^{-2}. These results indicated that the mixed SAM had a good anti-fouling functionality, especially after antibody immobilization.

3.4. Measurements of biomarker protein, troponin T

From the above results, the mixed SAM exhibited high anti-fouling ability and was applied for detecting troponin T. Five different concentrated troponin T solutions from 50 μg mL^{-1} to 100 ng mL^{-1} were detected by SPR (Fig. 7). The results of 50 μg mL^{-1}, 25 μg mL^{-1}, 10 μg mL^{-1}, 1 μg mL^{-1} and 100 μg mL^{-1} were 0.27°, 0.15°, 0.03°, 0.014° and 0.006° angle shifts, respectively. In order to identify the non-specific binding ability, 50 μg mL^{-1} of HSA solution was also employed as a control. There was no

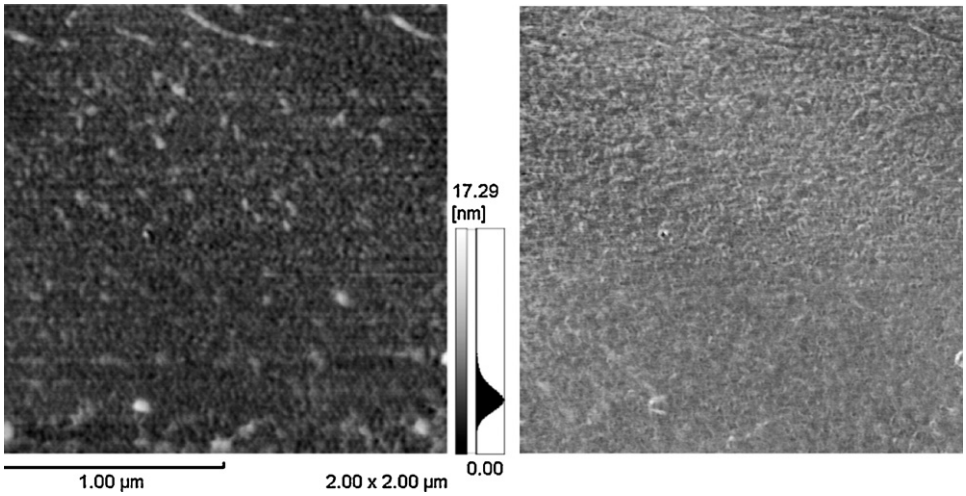

Fig. 5. AFM topographical and phase images of the mixed SAM after the immobilization of antibody. Left is topographical image and right is phase image. The white dots were antibody immobilized on the surface.

detectable non-specific binding with 50 μg mL^{-1} of HSA. It also indicated that the chip surface had a high anti-fouling ability and improved the sensing signal based upon the signal to noise ratio. After introducing the troponin T solution on the chip surfaces, rapid increases in the angle shift were detected, which plateaued after 2 min. It indicates that the mixed SAM fabricated is useful for a high throughput detection. The correlation between the concentration of troponin T and angle shift fit the linear equation $y = 0.0054x + 0.0004$. The linear regression of the angle shift exhibited a good linear correlation ($R = 0.991$, $P < 0.0009$) which suggested that the mixed SAMs can produce highly accurate and precise detections. The limit of detection (LOD) of cardiac troponin T was 100 ng mL^{-1}, which is not yet sufficient for clinical diagnostics. Further investigation and simulation is needed in order to improve the LOD, which might be possible by the increasing the carboxyl group content on the chip while still retaining its anti-fouling ability.

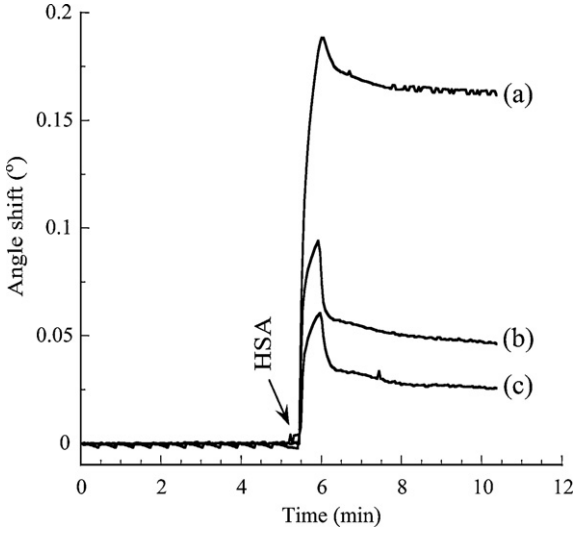

Fig. 6. Sensorgrams of HSA adsorption (concentration: 1 mg mL^{-1} and solvent: D-PBS) on (a) bare Au, (b) the mixed SAM, and (c) antibody immobilized surface for the mixed SAM. The adsorption mass of HSA decreased, particularly on the antibody immobilized surface.

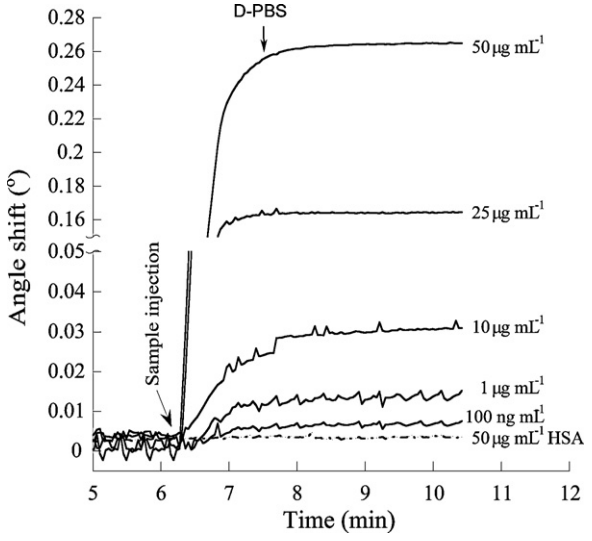

Fig. 7. Specific binding of troponin T on antibody immobilized surface. Sensorgrams of troponin T used different concentrations from 50 μg mL^{-1} to 100 ng mL^{-1}. The protein was injected at 6.5 min for 1 min then the residual was washed away starting at 7.5 min with the D-PBS.

4. Conclusion

The mixed SAM of OEG and MHDA with a chemical composition of 0.69 on Au had an atomically smooth surface, no phase segregation, and homogeneous/dense structure. The HSA adsorption tests indicated that the mixed SAM with the packing density of OEG at 82% had high anti-fouling ability which can be applicable for bio-recognized molecules even after immobilization of antibody. The SPR experiments successfully detected and quantified troponin T within 2 min after injection with the linear detection range lower than 50 μg mL^{-1}. The LOD value was insufficient to detect concentrations below 100 ng mL^{-1}. These results exhibited the mixed SAM immobilized antibody combined with SPR is a promising surface for rapid clinical diagnosis.

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