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Oriented immobilization of IgG on hydroxylated Si(001) surfaces via protein-A by a multiple-step process based on a self-assembly approach

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Abstract The aim of this study was the oriented immobilization of IgG molecules on the silicon surfaces. A multiple-step procedure was applied for oriented immobilization of IgG in this study. After hydroxylation of the Si(001) surfaces, 3-glycidoxypropyltrimethoxysilane (GPTS) molecules were self-assembled onto these substrates. Dipping time and GPTS concentration were found to be effected by on both layer thicknesses and water-contact angles. 2,2′-(ethylenedioxy)diethylamine (EDA) molecules were then covalently attached to the silicon surface with GPTS molecules. There was no effect of concentration on the formation of EDA molecules on the surfaces, while EDA deposition increased with the dipping time significantly. Imaging ellipsometry and atomic force microscopy (AFM) images exhibited aggregate formation at this step. Protein-A molecules were bound to the free amino groups of EDA molecules on the substrate surface, especially onto the aggregates by using a carbodiimide (i.e., EDAC) as the activating agent. We were able to immobilize IgG molecules in an oriented form onto the protein-A attached surfaces, especially in the regions, where EDA aggregates are located.

Introduction

Antibody-based immunosensors have been the subject of an increasing interest during the past decade due to their potential applications as an alternative immunoassay technique in many areas such as clinical diagnostic, biological research and environmental control [1]. Immunosensors are generally consisted of an immobilized antibody layer and a physical transducer. However, the control of immobilized antibodies has become more important as requirements for sensitive assays. Assay sensitivity and reproducibility are affected by the surface area available for a binding antibody. Therefore, one of the most important steps in the design of an immunosensor is the selection of the immobilization method, in terms of retaining the activity of the antibody. Well-defined antibody molecular layers on the solid surfaces are needed to attain final selectivity as well as sensitivity for immunosensing [2]. At the same time, immobilization of immunoglobulin G (IgG) molecules on the solid substrate is also more important for the development of an immunosensor. Several techniques including physical and chemical adsorption for the immobilization of IgG have been reported in the literature [3–6]. Recently, the technique of physical immobilization of proteins has been little used because of denaturation of the protein molecules and poor reproducibility, in spite of its simplicity. The chemical immobilization technique has been also found to show good reproducibility and coverage, because the proteins are covalently immobilized onto the solid surface [7]. On the other hand, to improve the sensitivity of the immunosensors, there is a problem that remains to be solved. Immunoglobulin molecule has a Y-shape, which is composed of two Fab fragments used for binding specific antigens and a Fc fragment containing antibody-effector functions [7, 8]. When IgG molecules are chemically bonded on a solid surface with disordered orientation, their Fab fragments might be hidden, which results in the antibody–antigen binding being hindered. Moreover, a random orientation of IgG molecules can

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result in the loss of biological activity [7, 8]. Therefore, when IgG molecules are bonded on a surface, it should be oriented in a manner, and results in Fab fragments pointing away from the surface, in order to allow for easy antigen–antibody binding, which is a very important issue in the development of an immunosensor.

In the biosensor studies, the process of self-assembled monolayers (SAMs) and more recently, self-assembly of multilayer systems have attracted a great deal of interest. SAMs technology provides a powerful tool for generating monolayers of biological molecules on various solid substrates. The orientation of monolayers offers great versatility in terms of the complex bio-recognition, which might provide a method for the in vitro development of biosurfaces that are able to mimic naturally occurring molecular recognition process [2]. For the construction of a highly ordered antibody surface, protein-A molecules are commonly used as a binding material. It is well-known that the IgG molecules are immobilized onto the protein-A bonded surface mainly via the specific binding activity between protein-A and F_c fragment of the IgG. Therefore, protein-A mediated antibody immobilization leads to highly efficient immunoreactions and enhances detection system performance [9].

In this study, we have reported that the preparation of a novel multilayer system consisting of 3-glycidoxypropyltrimethoxysilane (GPTS); 2,2′-(ethylenedioxy)diethylamine (EDA) and protein-A which are covalently attached on the Si(100) surface and its use for the oriented immobilization of IgG molecules. GPTS and GPTS-EDA overlayers were characterized by imaging ellipsometry, AFM and water-contact angle measurements. The images of fluorescein-labeled IgG immobilized on the designed surface were also determined by fluorescence microscopy.

Experimental

The substrates used in this study were Si(001) wafers (n-type, obtained from Shin-etsu, Handoutai, Japan), and were cut into 5 × 5 mm pieces for further modifications. They were cleaned very carefully by applying the following steps: (i) they were rinsed with deionized water and ethanol, several times; (ii) then were treated in a mixture of NH_3 (25% v/v), H_2O_2 (30% v/v), and deionized water having a volume ratio of 1:1:5 at the temperature of 70 °C for 20 min; (iii) were washed with ethanol and dried under nitrogen stream; and (iv) finally were exposed in UV/ozone chamber (Irvine, CA: Model 42, Jelight Company Inc., USA) for 15 min prior to modification in order to remove hydrocarbon and to produce a hydrophilic surface. Note that the water-contact angles of these cleaned substrate surfaces were around 3°, which was obtained with an automatic contact angle goniometer.

Freshly prepared 3-glycidoxypropyltrimethoxysilane (GPTS) (Aldrich, USA) solutions, in absolute ethanol (Aldrich, USA) with different concentrations of GPTS (0.5–10%, v/v) were used for the monolayer formation. The cleaned silicon wafers were incubated in these GPTS solutions for different periods of time ("dipping time") (0–24 h) at room temperature.

In order to convert the epoxy end groups to the amino groups, the GPTS-modified surfaces were immersed in ethanolic solutions of 2,2′-(ethylenedioxy)diethylamine (EDA) (Aldrich, USA) with different concentrations (1.0, 2.0 and 4.0%, v/v) for a range of time periods (1–24 h). After this treatment, amino-terminated substrates were removed from the solution, rinsed rigorously with ethanol, sonicated in ethanol for 10 min, and finally dried under the nitrogen stream.

The amino-terminated surfaces were first treated in a protein-A (Sigma, USA) solution consisting of 1 mg protein-A + 10 mL pH 7.5 phosphate buffer + 0.1 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) (Aldrich, USA) at 4 °C for 24 h. The protein-A immobilized surfaces were then washed with deionized water several times and dried under the nitrogen stream. Then, these protein-A immobilized substrates were incubated in fluorescein-labeled IgG solution (1 mL fluorescein-labeled IgG (Sigma, USA) + 10 mL pH 7.5 phosphate buffer) at 4 °C for 24 h, washed with deionized water several times and dried under a nitrogen stream. These experiments were all done in a dark room.

Static water-contact angles of the sample surfaces were measured at 25 °C in ambient air using an automatic contact angle goniometer equipped with a flash camera (Model DSA 100, Krüss, Germany) applying a sessile drop method. The volume of the drop was 1 μL in all measurements. The contact angles were calculated by using the software of instrument. Note that the values reported here are the averages of at least three measurements taken at three different locations on each sample surface and have a maximum standard deviation of ±1°.

The vertical structures of the samples, as the "optical" thickness of layers, were measured by an auto-nulling imaging ellipsometer (Nanofilm EP3, Germany). All thickness measurements were done at a wavelength of 532 nm with an angle of incidence of 72°. In the layer thickness analysis, a four-zone auto-nulling procedure, integrating over a sample area of approximately 50 × 50 μm followed by a fitting algorithm was applied. In the analysis of the hydroxylated Si(001) substrate and formed the SAMs, a four-phase model consisting of silicon substrate/SiO_2/overlayer/air was assumed. The designed overlayers were also assumed which is a well-accepted

approximation for organic layers with C-chains on the substrate surfaces [10]. Note that, since the thickness and refractive index are highly correlated for very thin films (<10 nm), refractive index of the overlayer can be reasonably assumed and then thickness of the overlayer can be determined. In the optical model applied, the refractive indices were assumed as 3.8650, 1.4605 and 1.46 for the Si substrate, the SiO_2 layer and the organic layers, respectively.

Fluorescence microscope measurements were also performed with a reflected fluorescence system (Nikon Eclipse E600, Japan) equipped with a mercury lamp to detect the fluorescein-labeled IgG on the modified surfaces.

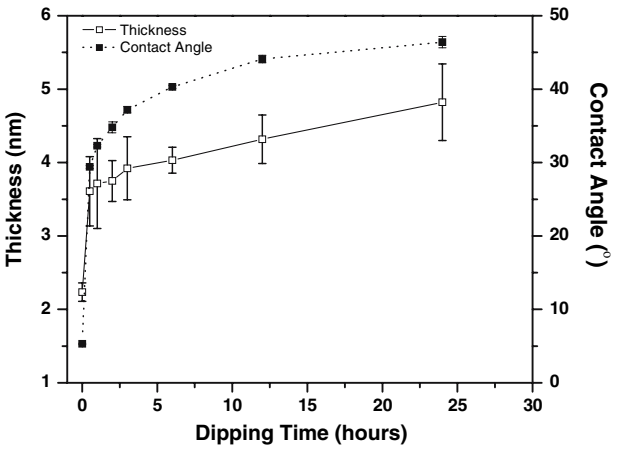

Fig. 1 Effects of dipping time on GPTS-layer thickness and contact angle. GPTS concentration: 4% (v/v)

Results and discussion

Effects of dipping time on the GPTS-monolayer formation

The effect of dipping time on the formation of GPTS-monolayers on the Si(001) surfaces was investigated by using 4% (v/v) GPTS solution in absolute ethanol within various time intervals (0–24 h) at room temperature. Note that, in our previous study, we have done some preliminary studies with a similar alkoxysilane, namely *N*-(3-trimethoxysilylpropyl) diethylenetriamine (TPDA), and investigate the effects of solvent type on the mono- or multi-layer formations on the silicon substrates by using four different solvents: ethanol, acetone, THF and benzene [11]. These initial studies showed that ethanol was found as the most suitable solvent for monolayer formation of alkoxysilanes, and we have decided to use ethanol as the solvent for GPTS in this study.

Figure 1 shows the changes in the thicknesses and water-contact angles of the GPTS treated silicon surfaces versus dipping time. As shown in Fig. 1, thickness of the hydroxylated silicon surface (the original Si(001) surface without GPTS) is about 2.24 nm. This high thickness value may be an indication of the surface roughness, note that the surface roughness was not included in the optical model that we have applied to obtain the thicknesses with the ellipsometer. Therefore, the thickness values reported here can be considered as "effective thickness" not an "absolute thickness." In the immersion steps of the cleaned silicon wafers in to the treatment medium, we paid attention to prevent contamination of the surfaces, and all these steps were done immediately. However, the hydroxylated surfaces are highly attractive for oxidation and for adsorption of contaminants, which are the possible reasons for the thickness values that we observed for the bare wafer surfaces.

The thickness of the GPTS overlayer formed after 1 h was about 3.72 nm, which shows almost a monolayer formation (theoretical molecular length of GPTS molecule is calculated by VASP [Viena Ab initio Simulation Package (VASP)], and found as 9.81 Å. There was an increase in the thickness up to about 5 nm in long incubation time (24 h). This increase may be due to reorientation of the surface attached molecules for more closely packed morphology for full coverage, or even some are not oriented but randomly adsorbed GPTS molecules in some part of the surfaces as a second layer forming somewhat agglomerates. After these studies, 1 h dipping time is preferred as appropriate for forming GPTS monolayer on the silicon surfaces and this time interval applied in the following studies. It should be noted that surface coverage was most probably a loose replacement and there were even some unoccupied regions, but we accepted that the surface coverage was satisfactory enough for further modifications as described in the later parts of this text.

The change in the contact angle roughly indicates the variation in the surface chemical composition of the substrate as well as the extent of the surface coverage [11, 12]. Figure 1 also shows the changes of the contact angle of the hydroxylated Si(001) surfaces after treatment by GPTS molecules for various time intervals. Notice that the hydroxylated silicone surface without GPTS molecules was a very hydrophilic surface with a contact angle of 3°±1°, while attachment of GPTS molecules made this surface more hydrophobic as expected and the contact angle values increased to 32° after 1 h treatment. In parallel to the thickness data discussed above, longer dipping times did not affect significantly the contact angles but considerable increases it up to 46° in 24 h, which probably corresponds to a closely packed full coverage of the surface, with a GPTS monolayer [13].

Effects of GPTS concentration on the GPTS monolayer formation

The effect of GPTS concentration on the layer-thicknesses and water-contact angles of the GPTS overlayers formed on the hydroxylated silicon surfaces were investigated by using different concentration of GPTS solutions (0.5–10%, v/v, in absolute ethanol) at room temperature, in which the dipping time was kept constant at 1 h. As seen in Fig. 2, the thicknesses of the GPTS overlayers were between 3.47 and 4.46 nm, in this wide range of concentration change, and slightly increased with the solution concentration. As discussed in the previous section, almost full coverage, most probably as closely packed monolayers were achieved even in 1 h, at high GPTS concentrations.

Figure 2 also shows that the variations of water-contact angle of the GPTS attached silicon surfaces with the GPTS concentration, results are in good agreement with those obtained from the overlayer thicknesses. The highest contact angle value was 44°, which corresponds most probably to the monolayer surface formation, as also obtained in the previous group of experiments.

Attachment of EDA on modified Si(001)

Forming of amino-terminated self-assembled layers are one of the most widely studied approaches to create functional surfaces for diverse nanobiotechnology applications such as immunosensors, biochips, etc. [14–17]. Following this trend, in this study, the Si(001) surfaces covered with GPTS monolayers in the previous steps (by treating 4% v/v solution of GPTS in absolute ethanol, for 1 h, at room temperature) were incubated with a well-known diamine, 2,2′-(ethylenedioxy)diethylamine (EDA) molecule. Here, we were expecting that the EDA molecules would bind covalently to the GPTS via the epoxy groups of the GPTS molecules assembled on the silicon surfaces from one end, leaving the other amino-end group oriented towards the solution. The EDA molecules are quite flexible and behave as a good spacer arm for the further step in which protein-A molecules were immobilized on the silicon surfaces via these free amino groups of EDA molecules as explained in the later parts. The EDA concentration within the incubation medium was 2% (v/v) in absolute ethanol. Incubations were performed at three different dipping times, as 1, 3 and 24 h, at room temperature. As seen in Fig. 3, the thicknesses of the EDA deposited surfaces depends on the dipping time, and for 1, 3 and 24 h treatments, the average thicknesses are 4.54, 13.16 and 19.77 nm, respectively. This significant increase in thickness with dipping time may be explained as follows: the amino groups at the one end of the EDA molecules initially react with the surface epoxy groups coming from GPTS and covalently attached. However, some of the approaching EDA molecules approaching to the surface in time may form hydrogen or ionic bond with other EDA molecules in solution or on the surface and forming aggregates and deposition on the surface results an increase at the average thickness of the overlayer formed at this stage.

The effect of EDA concentration on thicknesses of the EDA overlayers were also investigated by using EDA solutions having different concentrations (1.0, 2.0 and 4.0%, (v/v), in absolute ethanol) at room temperature, with a dipping time of 3 h. The thicknesses of the overlayers formed in these treatments did not change significantly and measured as 12.2 ± 1.3 nm.

Figure 4 gives representative ellipsometric 2-D surface images of the three different surfaces, named as, (a) the GPTS treated Si(001) surface "GPTS-Si(001)" without

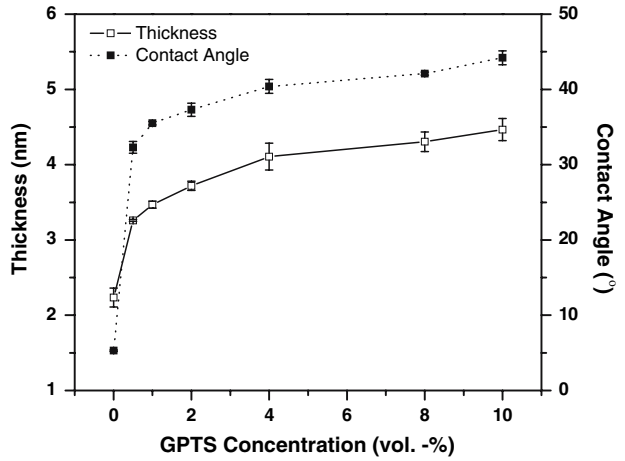

Fig. 2 Effects of GPTS concentration on GPTS-layer thickness and contact angle. Dipping time: 1 h

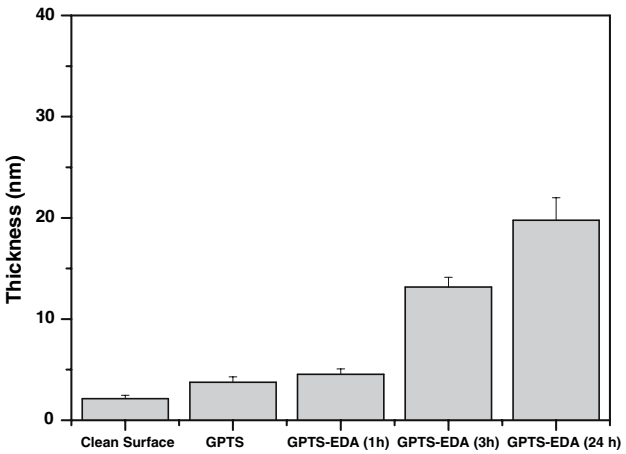

Fig. 3 Effects of dipping time on EDA-GPTS-layer thickness. EDA concentration: 2% (v/v)

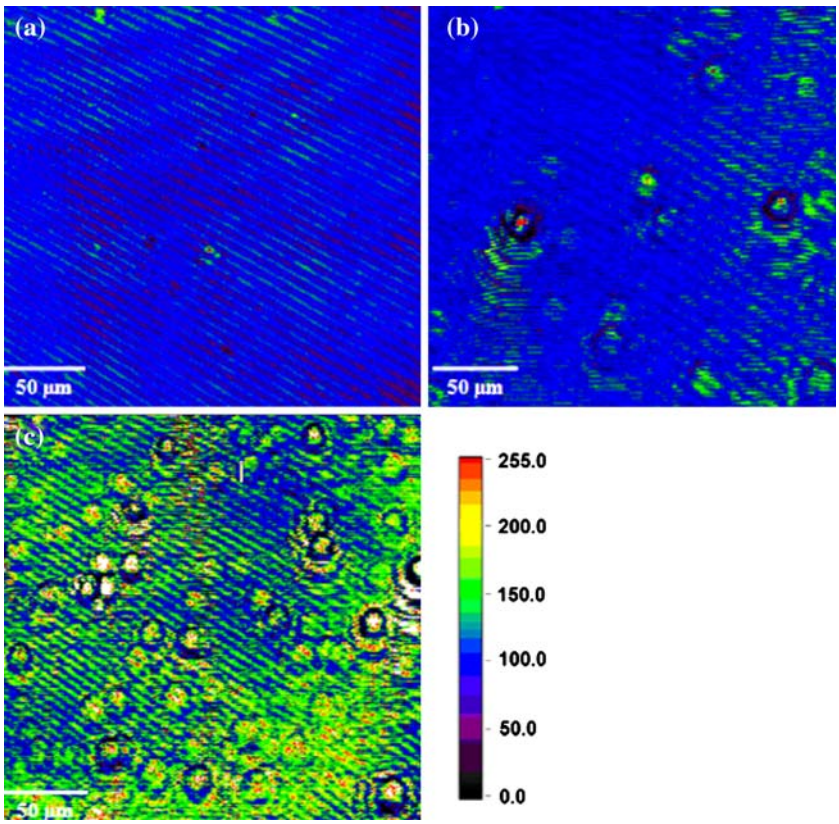

Fig. 4 Representative ellipsometric 2-D surface images of three different surfaces: (**a**) "GPTS-Si(001)" with no EDA; (**b**) "EDA/3h-GPTS-Si(001);" (**c**) "EDA/24h-GPTS-Si(001)"

EDA, (b) the EDA treated GPTS-Si(0001) surface with a dipping time of 3 h "EDA/3h-GPTS-Si(001)" and (c) the EDA treated GPTS-Si(001) surface with a dipping time of 24 h "EDA/24h-GPTS-Si(001)." As seen from Fig. 4, the morphologies of these surfaces are quite different. Treatment of the GPTS-Si(001) surfaces with EDA solution causes some EDA molecules deposition neither evenly nor as a monolayer. The circular structures are most probably

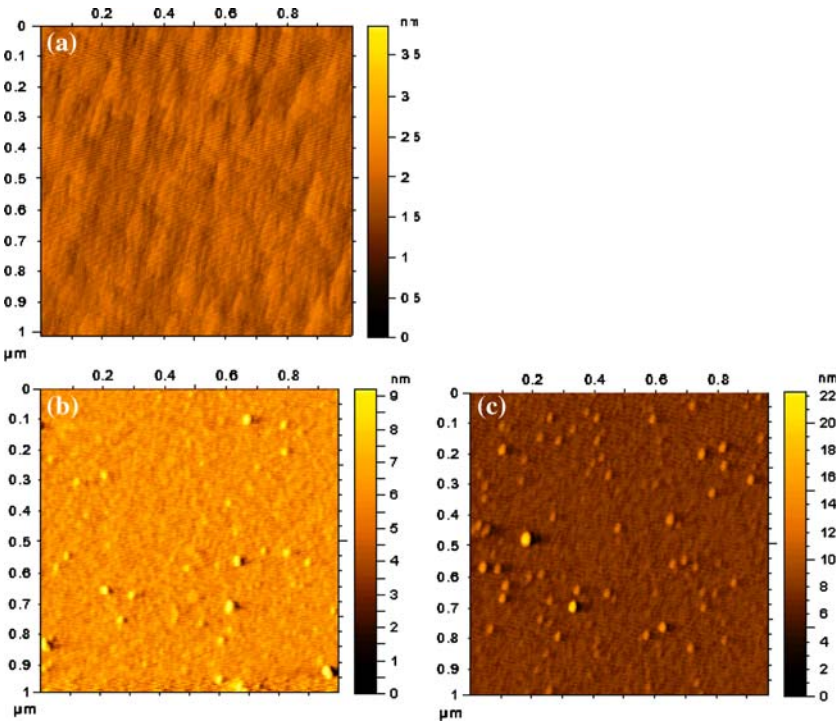

Fig. 5 Representative AFM images of three different surfaces: (**a**) "GPTS-Si(001)" with no EDA; (**b**) "EDA/3h-GPTS-Si(001);" (**c**) "EDA/24h-GPTS-Si(001)"

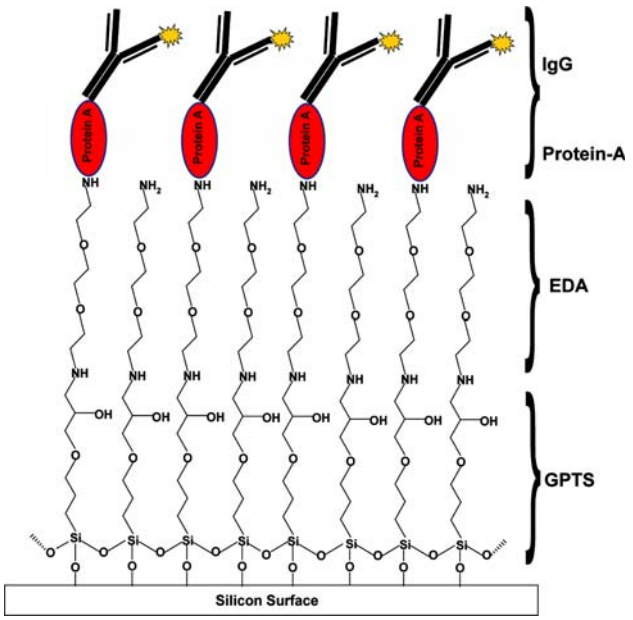

Fig. 6 Schematic representation of multilayer formation

because of formation of the EDA aggregates, which were quite significant and clear especially in the image given in Fig. 3c obtained after 24 h treatment.

Figure 5 gives the AFM images of the same surfaces discussed above. The GPTS-Si(001) surfaces is quite homogeneous, while EDA treatment causes some changes on the surface morphology, and formation of circular structures observed in the ellipsometric 2-D surface images given in Fig. 4 are also clearly visible. The root-mean-square (RMS) roughness of these surfaces were 0.303, 0.629, 0.962 nm for the GPTS-Si(001), EDA/3h-GPTS-Si(001), and EDA/24h-GPTS-Si(001), respectively.

Protein-A and oriented IgG immobilization

In immunoaffinity systems, one of the most important steps is immobilization of the bioligand (which is usually an antibody) on the substrate surface which may be then used, for bioaffinity separation or biorecognition. In this study, we have attempted to use self-assembly approach for oriented immobilization of IgG molecules via protein-A molecules. It is well-known that protein-A molecules are interacted with the F_c fragment of the IgG molecules, leaving free Fab fragments which will be available for further interaction of these correctly oriented IgG molecules with their complementary molecules [7].

First, the EDA/3h-GPTS-Si(001) and EDA/24h-GPTS-Si(001) surfaces prepared in the previous steps were treated with protein-A solution in phosphate buffer (pH 7.5), which also contains an activating agent (EDAC) for the reaction of the surface amine groups (from EDA molecules) with protein-A molecules, at 4 °C for 24 h. Non-covalently (or physically) adsorbed protein-A molecules were removed by washing with deionized water several times and the substrates were dried in nitrogen stream, and then were incubated in the fluorescein-labeled IgG solution in phosphate buffer (pH 7.5) at 4 °C for 24 h in dark room. Non-specifically adsorbed IgG molecules were removed by washing with deionized water several times.

Figure 6 exhibits the expected (attempted) substrate surfaces carrying oriented IgG molecules on the

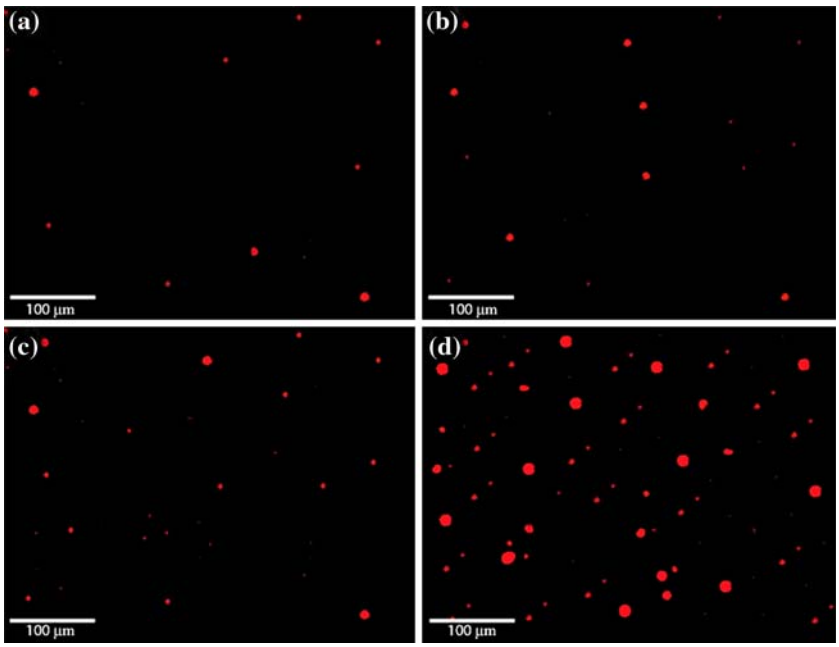

Fig. 7 Representative fluorescence images of four different surfaces: (**a**) "IgG-EDA/3h-GPTS-Si(001);" (**b**) "IgG-EDA/24h-GPTS-Si(001);" (**c**) "IgG-Protein-A-EDA/3h-GPTS-Si(001);" (**d**) "IgG-Protein-A-EDA/24h-GPTS-Si(001)"

self-assembled overlayers. Figure 7a–d shows the representative images which were taken with a fluorescence microscope. As expected, there were very few IgG molecules (non-covalently adsorbed, most probably left even after several washing steps) were detected on the EDA/3h-GPTS-Si(001) and EDA/24h-GPTS-Si(001) surfaces carrying no protein-A molecules. However, after protein immobilization, especially the long dipping time (treated with EDA for 24, Fig. 7d) significant fluorescence signal was observed. Notice that these visible areas were as dots or spherical structures, very similar to those observed in Figs. 4, 5. It seems that we were able to immobilize more protein-A molecules, and therefore, IgG on these spherical structures which were assumed as the EDA aggregates, mentioned in the previous section. In the dark parts, most probably the concentration of the IgG molecules were not enough to be detected with the microscope or the orientation of the protein-A molecules was not correct to interact with the IgG molecules.

Conclusion

In this present work, oriented immobilization of the IgG molecules on the protein A terminated surface was investigated. First of all, effects of dipping time and solution concentration for the GPTS sub-layer formation on the silicon surface were examined. Studies performed with ellipsometer showed that monolayers with a thickness of about 3.72 nm were formed when the dipping time is about 1 h. The effect of the GPTS concentration on the thickness of the deposited layer was not very profound. However, the contact angle data exhibited the importance of concentration on the surface coverage. After obtaining well-ordered GPTS monolayer on the substrate surface, amino-terminated second layer was formed by using EDA molecules. The thicknesses of the EDA layers were highly dependent on the dipping time, and for 1, 3 and 24 h treatments, the average thicknesses were found to be 4.54, 13.16 and 19.77 nm, respectively. Finally, based on the fluorescence microscope analyses, it was concluded that the IgG is bonded on the protein-A terminated layer in the manner that its F_c domain is bound to the protein A layer and the Fab domains face away from the surface.

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