Gold Nanoparticle-Hybridized "Nano-Sponge" Polymer Coatings to Enhance the Reliability and Sensitivity of Biosensors[a]

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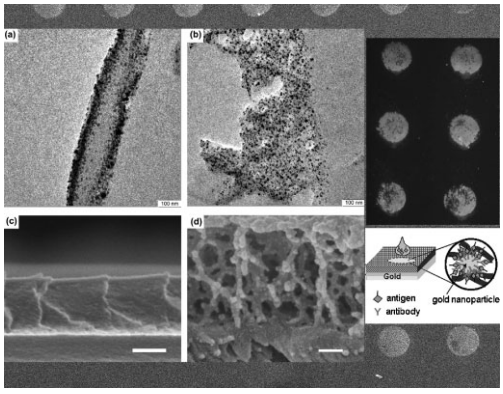
We have created a new functional biosensor coating composed of polyelectrolyte multilayers containing gold nanoparticles. This gold-hybridized polyelectrolyte multilayer film possesses a stable nanoporous structure under physiological conditions. Antibody molecules were successfully conjugated onto the gold nanoparticles within the film. This functional coating successfully extinguished false signals from non-specific binding of proteins and cells and also provided highly enhanced detection sensitivity. Furthermore, the drastic differences in protein and cellular adhesion properties between a chip coated with the nanoporous PEM film and a bare chip demonstrate that morphological control of biological interactions on chip surfaces is possible.



Introduction

Protein-based biosensors have attracted much interest because they may provide direct information on disease state, a feature not possible with other biosensors. Among them, surface plasmon resonance (SPR)-based biosensors have recently received great attention in use for antigen detection due to the fast and *label-free* detection mechanism and real-time detection of antigen without the need for a long labeling process.[1,2] In spite of all these advantages, there still remain challenges for SPR sensors, which relate to surface modification of the biosensor to assure their proper functions. Proteins need to be kept in a humid environment, as found *in vivo*, to maintain their native states. Effective methods to immobilize proteins onto various substrates require further development. Most importantly, non-specific interactions of the proteins with other materials must be controlled so that reliable data may be obtained. Many proteins, for example, albumin and immunoglobulin G (IgG), are well known to non-specifically adsorb onto various hydrophobic surfaces[3] and metal substrates including silver, gold and silica.[4,5] Therefore, the SPR gold sensor chip is extremely vulnerable to the non-specific adsorption of biomolecules.

In efforts to block non-specific adsorption, passive coatings which have hydrophilic groups such as ethylene glycol (EG)-based monolayers[6,7] or hydrogels[8] have been used. A more recent approach using an active coating like a layer of protein G[9] has been employed to enhance a specific interaction of a protein.[10] These approaches, however, still have problems with regard to surface coverage, long term stability and film thickness control. It is also difficult to

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create ultra-thin coatings with hydrogels onto the metal substrates that are frequently used for biosensor applications. In particular, the further chemical modification of the monolayer coating or hydrogel is very limited, but such modifications can be extremely important in biosensor applications.[11] Therefore, a new surface coating to meet the requirements for protein sensors needs to be developed.

Here we present a novel surface coating suitable for protein biosensors; our coating exhibits both anti-fouling property and chemical reactivity for bio-conjugation. These attributes were achieved by control of the surface morphology, and not only by attention to the chemical structure of the film. We created a nanoporous film, which could be incorporated with gold nanoparticles to increase the surface area for bio-conjugation. Gold colloidal particles[12,13] have recently received a great deal of attention due to their functions as components of novel nano-scale optical and electric devices including biosensors.[14–17] These studies have successfully demonstrated that the gold colloids may enhance SPR sensitivity, even better for the commercially modified dextran surface; however, the surface properties required for biosensors, such as anti-fouling or reserving the activity of biological molecules, cannot be achieved solely by the addition of gold colloids. Our porous functional coating combined with gold nanoparticles provided greatly improved biosensor sensitivity compared to that of uncoated sensors. Moreover, we observed an *anti-fouling* effect of the nanoporous film, which was not shown by the non-porous film. This phenomenon may relate to the antifouling property from hydrogels which have networked porous structure; however, the nature of the hydrogel has not been studied microscopically, but rather has been observed as a bulk property. Our porous polymer coating is only about a few tens of nanometers thick, but it maintains the integrity of a three-dimensional networked structure and controls the distribution of gold colloids on its surface. This study may provide insight into biomaterial research by showing that biological interaction can be controlled purely by the nanoscopic morphological structure of the interface.

To prepare thin films with an interconnected porous structure, we used polyelectrolyte multilayers (PEMs). PEM films are usually fabricated by the repetitive, sequential dipping of a substrate into aqueous solutions of oppositely charged polyelectrolytes[18] or of polymers containing hydrogen bonding donor and acceptor groups.[19–21] In this layer-by-layer manner, the PEM film can be deposited with nano-scale control over the film thickness. The functional groups in PEM films usually remain reactive for further chemical reactions including micro-patterning,[22–23] and thus have been intensively studied to functionalize various types of substrate surfaces for chemical, electrical, optical and biomedical applications.[24–30] Previous studies to modify the surface of SPR biochip have been mostly conducted by using a self-assembled monolayer of small organic molecules.[31–33] Compared to the SAM coating, where its surface functionality mostly relies on the end functional group, the PEM film can provide more versatile surface modification by utilizing the multi-functionality of the polyelectrolytes.

Experimental Part

Materials

Weak polyelectrolytes including poly(allylamine hydrochloride) (PAH) ($\overline{M}_w = 60\,000$), poly(acrylic acid) (PAA) ($\overline{M}_w = 90\,000$), poly (methacrylic acid) (PMA) ($\overline{M}_w = 100\,000$) and polyacrylamide (PAAm) ($\overline{M}_w = 5\,000\,000$) were purchased from Polysciences. Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4 \cdot 3H_2O$) (Aldrich) as a source for $AuCl_4^-$ and dimethylamine-borane complex (Sigma Aldrich) (DMAB) as a reducing agent were used to synthesis gold nanoparticles in the film. Dulbecco's Modified Eagle Medium (DMEM) and trypan blue were purchased from Sigma. Immunoglobulin G from human serum was purchased from Fluka and Anti-Rabbit IgG (whole molecule), antibody produced in goat was purchased from Sigma. Sulfo-LC-SPDP (sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido] hexanoate) and DTT (Dithiothreitol) were purchased from PIERCE.

Deposition of Polyelectrolyte Multilayer (PEM) Films

PAH/PAA multilayers were assembled on SPR gold substrates by the sequential adsorption of oppositely charged polyelectrolytes from dilute aqueous solution. Slide glasses and silicon substrates were used for the UV-vis measurements, absorbance measurements for gold synthesis and FT-IR experiments. SPR substrates were cleaned with piranha solution for a minute followed by rinsing with de-ionized water using ultrasonic cleaner. Other substrates were washed with a detergent (MICRO®-sol) and de-ionized water with sonication. After cleaning, the substrates were blown dried with N_2 gas and plasma treated for 1 min prior to dipping. Polymer solutions were prepared with a concentration of 0.01 M based on the molecular weight of the repeating unit. Hydrochloric acid (HCl) and potassium hydroxide (KOH) aqueous solutions were used to adjust the pH of the dipping solution. pHs of the PAH and PAA solution were adjusted as 8.5 and 3.5, respectively. De-ionized water ($\approx$18 M$\Omega \cdot$cm, Milli-Q) was used for the preparation of all aqueous solutions and rinsing water. The substrate was immersed into the polycation (PAH) aqueous solution for 15 min followed by 2 min rinsing with water with agitation. The rinsing step was repeated at least twice. PAH adsorbed substrate was then dipped into PAA solution for 15 min and then rinsed with water. This process was repeated until we obtained the desired film thickness.

Pore Formation of PEM Films and Crosslinking

Nano-scale pores were formed within the PAH/PAA films with a simple acid treatment. The (PAH/PAA) multilayer films were

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immersed into the pH 2.0-adjusted aqueous solution for 2 min followed by two step (2 min, 1 min) rinsing with de-ionized water. Crosslinking of the porous film was taken at 120 °C for 3 h in a vacuum oven. The crosslinking reaction was examined by FT-IR (Thermo Nicolet) spectroscopy.

Gold Nanoparticle Synthesis

The deposited multilayer film was immersed into the $HAuCl_4$ (5×10^{-3} M) solution for 5 min, followed by rinsing two times with de-ionized water for 2 min each. The gold ion absorbed film was then immersed into the reduction bath (5×10^{-3} M, borane-dimethylamine aqueous solution) for 5 min and the film was rinsed with de-ionized water twice. Characterizations of gold-embedded multilayer films were examined with a UV-vis spectrometer and scanning and transmission electron microscopy.

Morphological Characterization of PEM Films

Film morphology was examined by atomic force microscopy (AFM) using Park System XE200 and scanning electron microscopy (SEM) from JEOL (model. JSM-7000F). To check gold nanoparticle distributions in metal hybrid multilayer films, cross-sectional transmission electron microscopy (TEM) images were taken with a Tecnai G220 S-Twin TEM (FEI Phillips) at Korea Research Institute of Chemistry and Technology. For cross-sectional TEM imaging, polyelectrolyte multilayer films assembled on PS substrates were cut along a direction normal to the film plane using an ultra-microtome with a diamond knife at room temperature. Cut sections were placed on copper TEM grids and TEM was performed at 200 kV.

Cell Study

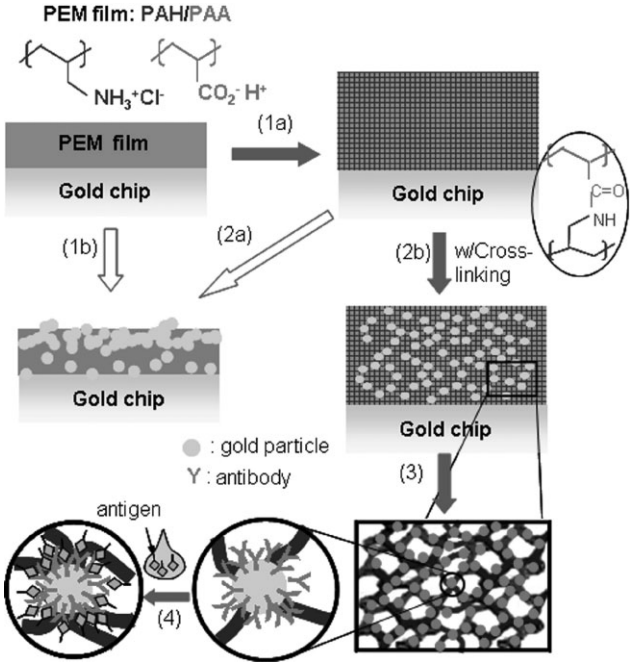
293 Human embryonic kidney cells were cultured on tissue culture polystyrene (TCPS) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The pH of the medium was adjusted to 7.4. For attachment and proliferation experiments, cells were removed from their growth surface by trypsin and then spun down in a centrifuge at ≈1 000 rpm for 5 min. The cells were then responded in fresh media, mixed in a 1:1 ratio with 0.4% trypan blue and counted with a hemocytometer with trypan blue exclusion to determine cell viability prior to seeding. The 293 cells were seeded onto sterilized multilayer-coated glass and gold-coated silicon slides. Cellular interaction on the films was monitored by inverse phase optical microscopy for at least 5 d. Live cell images were taken with an Olympus optical microscope (IX71) equipped with a high resolution digital camera (Roper Scientific Photometrics) and QED and RS image programs.

Antibody Immobilization and SPR Measurement

Immunoglobulin G (IgG) was immobilized in the polymer/gold nanoparticle-hybridized porous coating on SPR chips for detecting antigen IgG. Antigen binding was monitored by a SPR instrument (model: SPR Lab, *K-MAC*). Thiol-activated crosslinker for IgG immobilization onto gold surfaces was prepared by the conjugation of sulfo-LC-SPDP (sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido] hexanoate) and DTT (dithiothreitol) with concentrations of 5 μm. For the antigen detection experiment, anti-rabbit IgG was flowed on the modified SPR chip. When the real-time experiments were taken, firstly PBS buffer solution was injected in the channel with a flow rate with 20 μL per minute until the baseline was stabilized. After the signal was stable, antigen was injected. The amount of injected antigen was fixed at 300 μL for 6 min. A PBS buffer solution was delivered into the chamber again, when the antigen injection was finished, in order to rinse-off the unbound antigen from the chip.

Results and Discussion

Scheme 1 illustrates the overall procedure used in our surface modification of SPR biosensors. Microstructures of the films prepared during each step of this procedure were examined by atomic force, scanning and transmission electron microscopy. Poly(allylamine hydrochloride) (PAH)/poly(acrylic acid) (PAA) multilayers were used as the PEM coating. We have studied various PEM films comprised of PAH/PAA assembled at different pH conditions[34] and found the most inter-connected porosity with



Scheme 1. The procedure used to create a gold nanoparticle-hybridized polymer film on a biosensor chip: (1a) pore formation; (2b) gold nanoparticle synthesis in porous film after crosslinking of the film; (3) antibody immobilization; (4) antigen detection. Note, a gold synthesis without crosslinking of the porous film, process (2a), produces the same result obtained from non-porous film (1b). The total processes to create a functional SPR coating follow the filled arrows.

a high yield in gold nanoparticle synthesis from the PEM film assembled at pH 8.5/3.5. At first, the PEM film comprised of PAH and PAA was coated onto SPR chips. Due to the limitation of SPR measurement in film thickness, we had to determine the optimum thickness condition of the PEM film for a SPR chip. In the experiment of the spectral changes of the PEM-coated SPR chips upon the increase of the multilayer thickness, SPR angles were shifted from 52° (bare chip) to the higher angle as there was an increase of the number of bilayers, (*n*), (Supporting Information, Figure S1). The 3.5 bilayers of PEM coating (about 40 nm thick), presenting a 63° SPR peak angle, were chosen for the further processes.

The assembled PEM film was then treated with an acidic aqueous solution (pH 2.0). With this treatment, interconnected nanopores appeared in the film, as shown in AFM measurements (Figure 1(b)). We used this porous film to synthesize gold nanoparticles. Initially, the PEM coated chip was immersed in a gold precursor solution ($HAuCl_4$) and then treated with a reduction reagent, dimethylamine-borane complex (DMAB) solution. In this way, gold ions were bound to the amine functional groups in the PEM film and then reduced to Au (0). As the reduction was completed, a reddish color appeared in the PEM film, indicating the presence of gold nanoparticles. The amount of gold nanoparticles in the film could be controlled by the number of the reaction cycle proceeded. This gold synthesis method, step **(2a),** turned out to be improper to maintain the nanoporous structure of the film. The surface morphology of the porous film after gold synthesis appeared more similar to a non-porous film than a porous film (Figure 1). The pHs of the solutions in gold synthesis were around pH 5. Even at this pH, the porous film changed the morphology close to the initial non-porous film. It is essential for the biochip coating to be exposed under buffered saline solution (pH 7.4). These results led us seek to create a new nanoporous film whose structure is morphologically stable under physiological conditions. We solved the problem using partial crosslinking of the PEM film. In step **(2b)** of Scheme 1, the porous PEM film was heated at 120 °C for 3 h under a vacuum, and amide linkages[35] were formed between amine and carboxyl groups in the PAH/PAA film. In this reaction, about 4∼5% of PEM film functional groups formed crosslinks, as judged by FT-IR analysis (Supporting Information, Figure S2). Figure 1(d) is the film that was treated by this thermal crosslinking prior to the gold nanoparticle synthesis.

In order to investigate the detailed morphologies of gold nanoparticles and polymer films, we conducted TEM and SEM studies of the PEM films prepared at each step. For SEM and TEM measurements, thicker films were used for the photographs shown in Figure 2 in order to obtain clear images. Overall properties, including porous morphology, measured from those of the thicker films, were in good agreement with 3.5 bilayer-films.

The structural changes of PEM films at each step are clearly demonstrated by the SEM images in Figure 2. As most functional groups in the film remained reactive after crosslinking, we utilized these for further chemical reaction, synthesis of gold nanoparticles. After the subsequent crosslinking reaction, the porous structure became stable under any tested pH condition. Figure 2(d) shows a well-maintained porous structure and gold nanoparticles incorporated within the film. If the porous film was not crosslinked, the pores completely disappeared during further chemical treatment as shown in Figure 2(c). As revealed by TEM images, the synthesized gold nanoparticles in the nanoporous coating are well distributed in the film (Figure 2(f)), while non-stabilized film cannot maintain its porous structure and gold nanoparticles are mostly generated on the outer surfaces of the coating (Figure 2(e)).

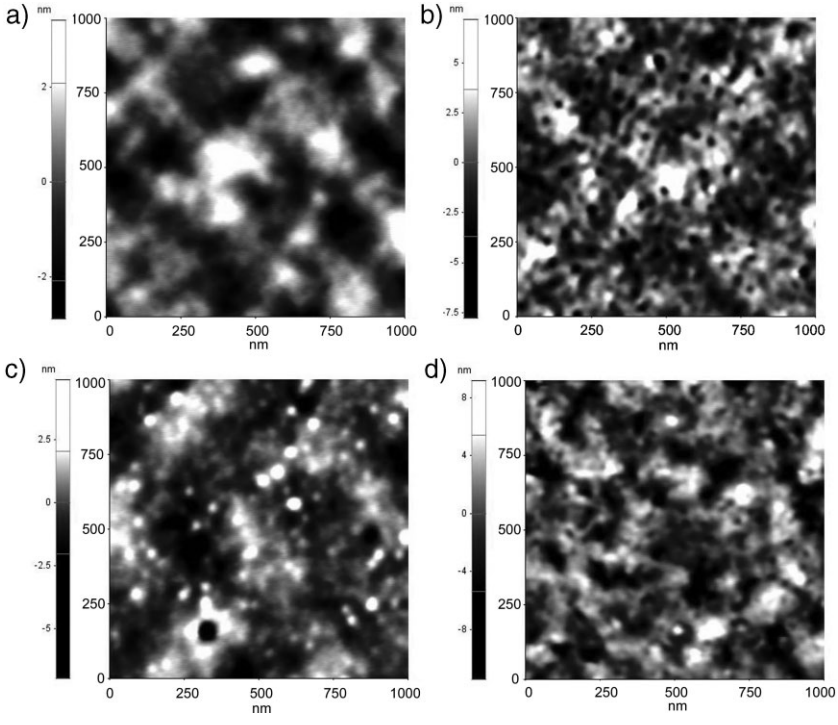


Figure 1. Height contrast AFM images of PEM films on SPR gold chips: 3.5 bilayers of PEM films, (a) as assembled and (b) after acidic solution (pH 2.0) treatment. Film (b) processed for gold nanoparticle synthesis (c) without crosslinking or (d) after a partial crosslinking of the porous film.

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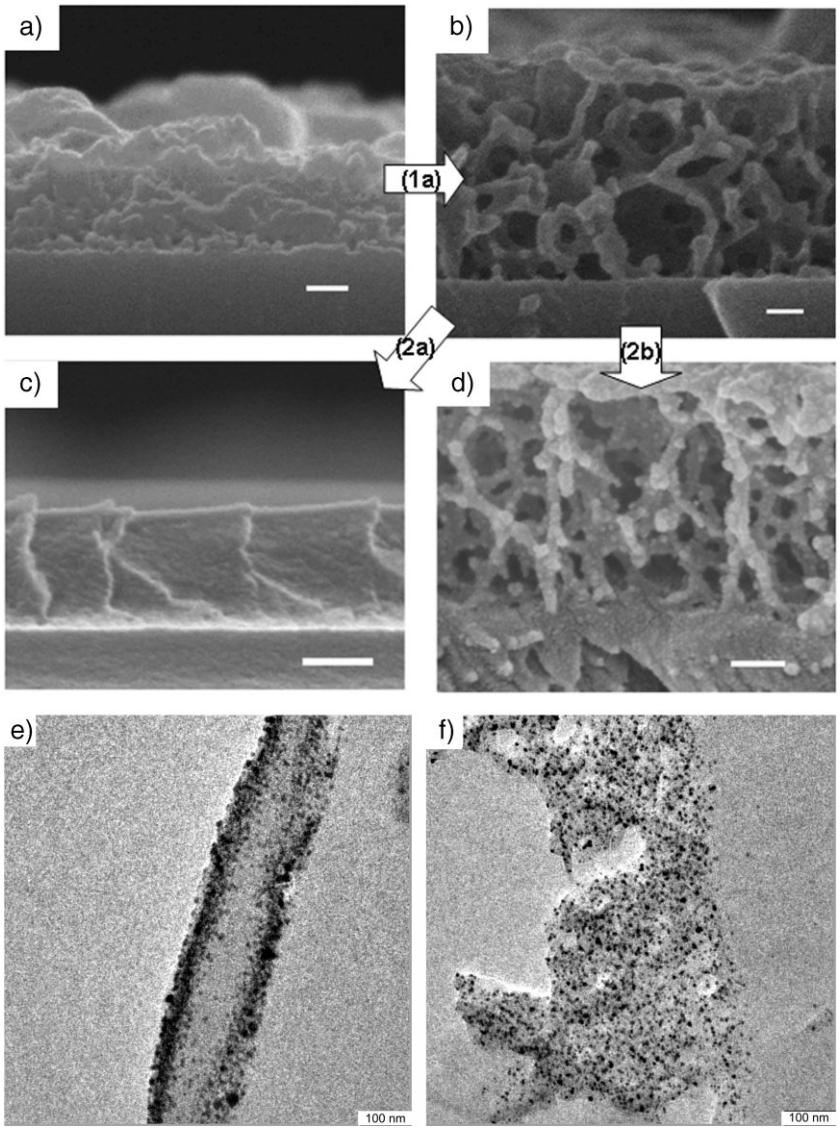

Figure 2. Electron microscopic images of PEM films; a∼d: SEM images of PEM films at each step described in Scheme 1. (a) As prepared, (b) after pore generation (c) Au particles in nonporous film and (d) Au particles in porous, crosslinked film. e∼f: TEM images of Au nanoparticles synthesized in (e) non-porous and (f) porous PEM Films. All samples were prepared from 9.5 bilayers. (Scale bar, 100 nm).

The porous morphology of the film unprecedentedly affected the surface properties of the PEM film. As we mentioned in the Introduction, the SPR sensor is extremely vulnerable to the non-specific adsorption of biomolecules. We initially designed experiments to coat the some anti-fouling moieties in the last step of the process. However, when cellular interactions with the PEM films were explored, surprising results were obtained. 293 HEK cells, which are well-known adhesive cells, adhered to the film surface when they were seeded onto the non-porous PEM film, as was the case with the bare substrate; however, cells exposed to the same PEM film but with a porous structure were floating in culture medium instead of adhering to the film surface (Figure 3). Furthermore, this porous PEM film seemed to only block non-specific adhesion of cells, but not kill the cells, because the MTT cell viability test confirmed that the porous multilayer coating was not cyto-toxic. This non-toxic nature of the coating also appeared in Figure 3(d), as when we collected the floating cells after 24 h post seeding and re-seeded onto a new bare culture dish, they adhered and proliferated similarly to the freshly seeded cells on the culture dish. The water contact angles of PEM film significantly dropped after pore formation. The static and receding contact angles for the non-porous film, 60° and 25°, changed to 15° and 3°, respectively for nanoporous film. This low contact angle of the porous film was kept throughout the following processes. The porous film probably soaked up water through the interconnected network, even though it was still a very thin film, thus acting like a "nano-sponge", and the hydrated surface weakens cellular adhesion.[19,23] Some degrees of the swelling of the coating under the buffered condition, indicated as the change of refractive index of the film, might happen. Further investigation is ongoing.

This blocking ability of the nanoporous PEM film was also evident when protein-film interactions were studied (Figure 4). We injected IgG antigen solution onto un-coated and porous film-coated SPR chips. These films had no bound antibody; hence, no signal should be found if non-specific binding was blocked. Figure 4(a) shows that no signal was obtained from the nanoporous coating whereas a certain amount of antigen binding to the bare chip was noted.

As a final step, we immobilized antibodies, for example, IgG, onto the nanoporous PEM film using gold-reactive cross-linkers. Because of the increase in the surface/volume ratio of gold nanoparticles in the film, compared with a flat gold surface, we could increase the amount of antibody immobilized. Therefore, we expected increased detection sensitivity from gold particle bounded porous film coated chips. The result gave much greater detection sensitivity compared to the ratio of the increased amount of bound antibody.

The level of IgG immobilized in the porous film used in Figure 4(b) was about twice that of the bare gold chip. However, when we measured antigen (anti-IgG) binding

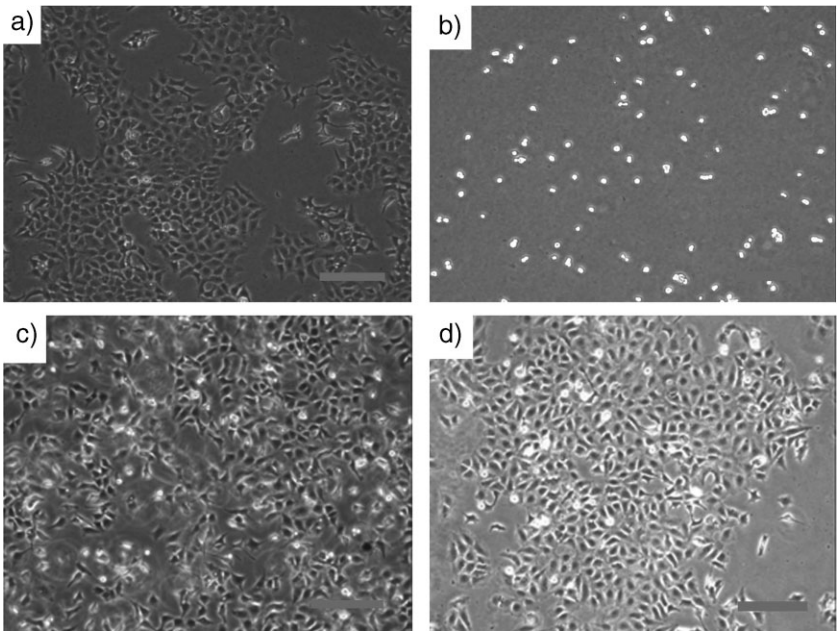

Figure 3. Optical microscopic images of human embryonic kidney (HEK) cells that were cultured on PEM film coated surfaces compared to the bare substrates: cells on (a) non-porous film, (b) porous PEM film and (c) uncoated substrate. (d) Surface-attached cells, which were originally exposed to the porous film-coated surface for 24 h, and then transferred to a new bare substrate. (Scale bar, 200 μm)

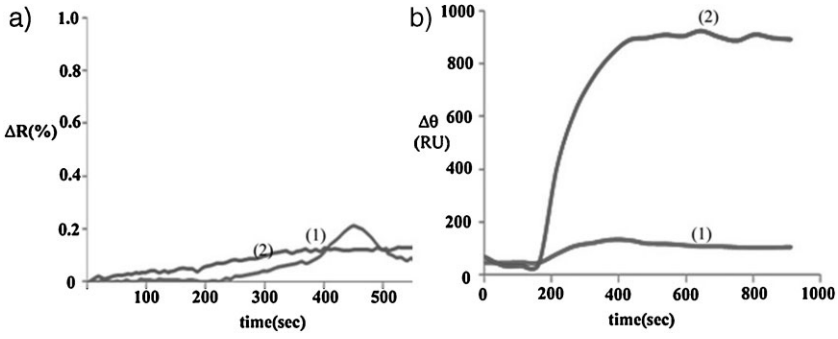

Figure 4. Time-resolved SPR spectra of a regular bare chip and a gold-hybrid porous film coated chip on (a) testing non-specific adsorption and (b) detecting specific binding of an antigen, anti-IgG. Spectrum (1) and (2) are obtained from bare and film-coated chips, respectively.

onto the nanoporous film with bound IgG, we obtained at least 10-fold greater signal than that delivered by anti-IgG on an un-modified chip (Figure 4(b)). It seems that the nanoporous film assists in the preservation of antibody function, to an extent much greater than is afforded by a bare surface, by providing a humid environment for protein activity.

Conclusion

In conclusion, we have created a functional biosensor coating composed of a nanoporous polyelectrolyte multilayer film, hybridized with gold nanoparticles, that was stable under physiological conditions. Antibody molecules were successfully conjugated onto the gold nanoparticles within the film. This functional coating successfully extinguished false signals from non-specific binding of proteins and cells and also provided highly enhanced sensitivity of the biosensor signal. Furthermore, the protein and cellular adhesion properties with the nanoporous film demonstrate that morphological control of biological interactions on a biochip surface is possible. These results will be valuable in biosensor and other biological applications.

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