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SERS Immunoassay Using Microcontact Printing for Application of Sensitive Biosensors

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We introduced a promising patterned substrate by using a microcontact printing method that can be used for SERS immunoassays based on antigen-antibody binding. SERS spectrum of the Raman reporter with antibody, which is rhodamine 6G (R6G) adsorbed on colloidal gold nanoparticles, was observed only for the surfaces in which prostate-specific antigen (PSA) is present on the substrate that is attached to an immobilized layer of antibody on the gold nanoparticles layer of the patterned substrate. Raman mapping images clearly showed that the antibodies on the Raman reporter were successfully and selectively conjugated with the antigen on the patterned substrate. This method could be potentially extended to multi-protein detections and ultrasensitive biosensors.

Key Words : Surface-enhanced raman scattering (SERS), Immunoassay, Microcontact printing, Gold nanopaticles, Biosensors

Introduction

Surface-enhanced Raman scattering (SERS) spectroscopy, in which the scattering cross-sections are dramatically enhanced for molecules adsorbed on metallic nanostructures, has recently received considerable attention as a powerful analytical tool in ultrasensitive chemical analysis, biological imaging, and material studies.[1-32] Metallic nanomaterials and metallic nanostructures for the successful application of SERS have been reported.[10,11,23,24,27] Particularly, patterned metallic surfaces separated by small distances, which create regions where the electromagnetic field is enhanced, have shown great promise. The microcontact printing method has been widely used as a simple and efficient method for surface patterning of molecules that can be applied like ink to the surface of an elastomeric stamp and transferred to the substrate by printing.[33-35]

Applications of molecular sensors operating *via* SERS have been extensively studied for use in biochemical research, clinical diagnoses, and environmental monitoring.[30] Among these applications, SERS applications in biological systems have especially increased. The potential of SERS immunoassays based on antigen-antibody binding has been reported for biomarkers used in early cancer diagnosis.[12,15-19,26,28,32] Immunoassays, with high selectivity and affinity for the antibody molecules to their corresponding antigens, have been widely exploited for analytical purposes in the field of clinical diagnosis.[36-41] Fluorescence spectroscopy has been one of the most widely used readout methods for immunoassay due to its high sensitivity. Immunoassay based on SERS provides a high sensitivity that is comparable to that of fluorescence spectroscopy.[42]

We have recently reported a sandwich type SERS immunoassay for the detection of the prostate-specific antigen (PSA), which has been used as a serological marker for detecting prostate cancer, with high sensitivity.[12] It showed the great potential of the SERS immunoassay to detect PSA successfully at very low levels that is sensitive enough for analysis of PSA in human blood samples or cancer cells.

In this study, we applied the microcontact printing method to prepare a patterned substrate of a sandwich type SERS immunoassay in which antigens on the substrate selectively capture antibodies on a Raman reporter. This strategy can be successfully applied to biosensors for cancer detection and opens a way for multi protein detections with high sensitivity.

Experimental Section

Gold (III) chloride hydrate ($HAuCl_4$, 99%), sodium citrate tribasic dehydrate (Na_3 citrate, 99%), 3-aminopropyl trimethoxysilane (APTMS, $H_2N(CH_2)_3Si(OCH_3)_3$, 97%), octadecyltrichlorosilane (OTS), poly-dimethlylsiloxane (PDMS), and prostate-specific antigen (PSA) were purchased from Sigma Aldrich at the highest purity available and were used as received without further purification. Prostate-specific monoclonal antibody (*anti*-PSA-mAb) was obtained from Boditech Co. (Korea). *N*-hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiimide (DCCD), and 5,5'-dithiobis(2-nitrobenzoic acid) (DNBA) were purchased from Alfa Aesar at the highest purity available and were used as received without further purification. Sylard A and Sylard B solutions for the preparation of the PDMS stamp were purchased from Dow Corning at the highest purity available and were used as received without further purification.

Gold colloidal nanoparticles were prepared by the aqueous reduction of $HAuCl_4$ solution (100 mL, 10^{-2}%) with trisodium citrate solution (10 mL of 1%) using a process

developed by Frens.[43] The particle size of the gold colloidal nanoparticles can be controlled by adjusting the amount of the dropping solution. The gold colloidal nanoparticle solution showed an absorption maximum near 523 nm. The size of the gold colloidal nanoparticles was 13-20 nm as analyzed by the high-resolution transmission electron microscopy (TEM) images. The high-resolution TEM images were obtained with a JEOL JEM 2010 transmission electron microscope operated at 200 kV. Field emission scanning electron microscope (FE-SEM) images were obtained with a Hitachi S-4300 field emission scanning electron microscope operated at 15 kV. UV-vis absorption spectra were obtained with a Sinco S-3100 UV-vis spectrometer.

SERS spectra were recorded using a Jobin Yvon/HORIBA LabRam ARAMIS Raman spectrometer equipped with an integral BX 41 confocal microscope. The radiation from an air cooled HeNe laser (632.8 nm) was used as the excitation source. Raman scattering was detected with 180° geometry using a multi channel air-cooled (–70 °C) charge-coupled device (CCD) camera (1024 × 256 pixels). Data for Raman maps were collected over a 60 × 60 μm area using a step size of 1 μm with a 100x objective lens, controlled by associated software. The intensity of Raman bands at 2883 cm^{-1} (OTS) and 1505 cm^{-1} (R6G) were mapped.

For the SERS immunoassay, we prepared a patterned substrate by using the microcontact printing method that makes the microscale pattern. The elastomeric PDMS stamp was prepared by the same method described earlier.[33-35] OTS, as ink molecules on the PDMS stamp, are transferred directly to the silicon substrate, which makes the patterns to prevent the gold nanoparticles layer. The thin layer of OTS on the PDMS stamp surface was prepared by using the spin coating method. The PDMS stamp was pressed into the silicon substrate surface, leaving the OTS only on the regions defined by the raised structure of the stamp. The stamp was then removed from the silicon substrate surface.[33-35] For patterned gold nanoparticles, the substrate was prepared by using the self-assembled monolayer (SAM) technique. An APTMS-derivatized surface on the OTS patterned substrate was also prepared by using the SAM technique.[44,45] A SAM of APTMS was fabricated by immersing the OTS patterned substrate in APTMS solution for 24 hours, which was only deposited on the silicon surface of OTS patterned substrate, and was then washed several times with ethanol and ultrapure water. A successive gold nanoparticle layer was then prepared by immersing the APTMS-derivatized substrate in a colloidal gold nanoparitcles solution for 24 hours and washed several times with ultrapure water. Gold nanoparticles were only deposited on the patterned APTMS surface.

Rhodamine 6G (R6G) adsorbed on colloidal gold nanoparticles was used as a Raman reporter in this study. 5,5'-dithiobis (succinimidyl-2-nitrobenzoate) (DSNB) was used for antibody conjugation onto gold nanoparticles. DSNB was synthesized, and the DSNB-coated Raman reporter was prepared by the method reported earlier.[12,16,28] The antibody was coupled to R6G adsorbed on gold nanoparticles *via* the succinimidyl terminus of the DSNB-derived coating.

Antibodies were also adsorbed on the gold nanoparticles layer *via* the succinimidyl terminus of the DSNB-derived coating by immersing the patterned gold nanoparticles layer substrate in an antibody solution (in borate buffer) for 1 hour and was then washed several times with borate buffer solution. The PSA was conjugated onto the immobilized layer of antibody on the gold nanoparticles layer by immersing antibody-derivatized substrate in PSA solution (in Tris buffer) for 1 hour and then was washed several times with Tris buffer solution.

Results and Discussion

The microcontact printing method was used to fabricate the micropatterned substrate of a sandwich type SERS immunoassay for applications in biosensing is shown in the schematic in Figure 1. The scheme of the sandwich type SERS immunoassay process was previously reported.[12] As shown in Figure 1, this sandwich type SERS immunoassay involves the immobilization of captured antibodies on a Raman reporter (DSNB-coated gold nanoparticles with R6G), the conjugation of antigens onto the immobilized layer of antibodies on the gold nanoparticles layer of the patterned surface, the immobilized antibodies capturing antigens on the patterned surface, and SERS detection of the Raman reporter on which antigens are selectively conjugated with antibodies. The SAM of APTMS was only fabricated on the silicon surface of the OTS patterned substrate and then the gold nanoparticles were only deposited on the patterned APTMS surface. Figure 2(a), (b), and (c) show the optical microscope image of the PDMS stamp surface, the FE-SEM image of the OTS linked silicon substrate, and the FE-SEM image of the gold nanoparticles layer on the APTMS-derivatized SAM surface of the patterned substrate, respectively. It can be seen that the gold nanoparticles were homogeneously assembled on only the APTMS-derivatized SAM surface of the patterned substrate. This gold nanoparticles monolayer is a good substrate to

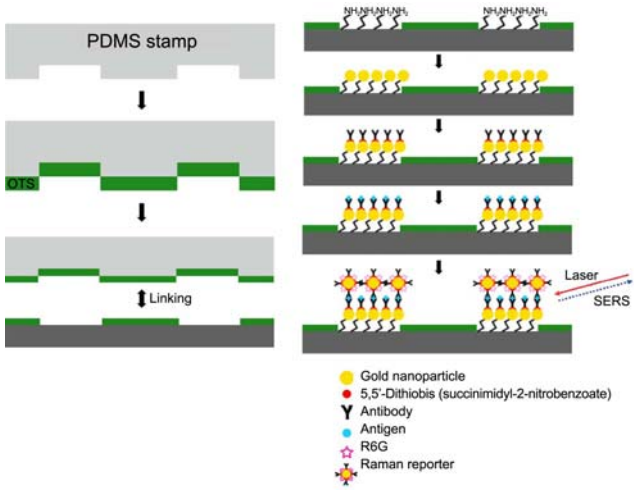

Figure 1. Scheme showing the fabrication of the micropatterned substrate for the sandwich type SERS immunoassay process.

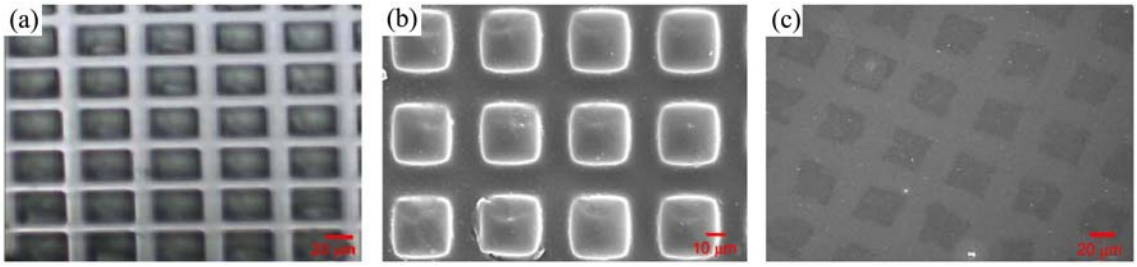

Figure 2. The optical microscope image of the PDMS stamp surface (a), the FE-SEM image of the OTS inked silicon substrate (b), and the FE-SEM image of the gold nanoparticles layer on the APTMS-derivatized SAM surface of the patterned substrate (c).

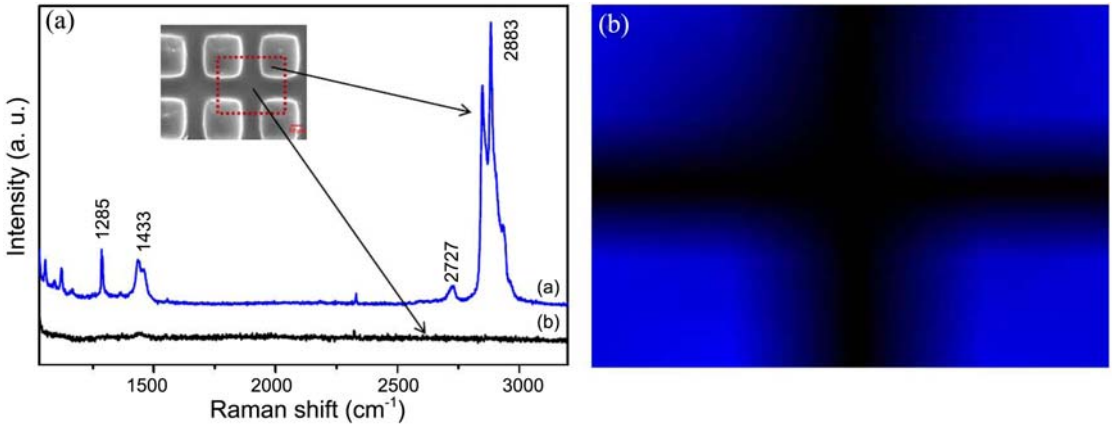

Figure 3. (A) Raman spectra of OTS in the regions of the OTS linked silicon substrate (a) and silicon substrate without OTS (b), and (B) Raman mapping image for the band at 2883 cm^{-1} .

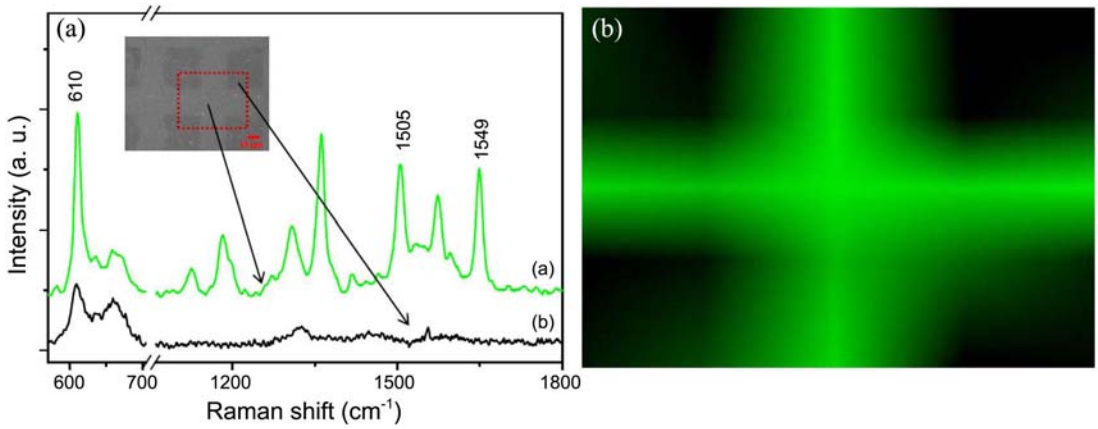

Figure 4. (A) SERS spectra of the immobilized Raman reporter with antibody, which is R6G adsorbed on colloidal gold nanoparticles, on the patterned substrate in the region of with (a) and without (b) PSA, and (B) the Raman mapping image for the band at 1505 cm^{-1}.

capture antibodies in this sandwich type SERS immunoassay. By the specific antigen-antibody interaction, PSA was thus conjugated successfully with antibodies on the gold nanoparticle monolayer *via* the succinimidyl terminus of the DSNB.

Figure 3(a) shows Raman spectra for OTS in the region of the OTS linked silicon substrate and in the silicon substrate without OTS. The Raman mapping image for the band at 2883 cm^{-1}, which is indicative of OTS, is shown in the Figure 3(b). The Raman spectrum of OTS was only observed on the OTS linked surface of the OTS patterned silicon substrate. The Raman mapping image indicates that the OTS patterned substrate was successfully fabricated by using the microcontact printing method.

The SERS immunoassay using antigen-antibody binding is a powerful method for the detection of specific cancer.[12,15-19,26,31] Figure 4(a) displays the SERS spectra of the immobilized Raman reporter of the antibody on the patterned substrate in the region of with and without PSA. PSA was captured with the immobilized antibody on the

gold nanoparticles layer of the patterned substrate. As shown in Figure 4(a), the SERS spectrum of the Raman reporter with antibody, which is R6G adsorbed on colloidal gold nanoparticles, was observed only for the surfaces in which PSA is present on the substrate that is attached to an immobilized layer of antibody on the gold nanoparticles layer of the patterned substrate. While the SERS spectrum of the Raman reporter of the antibody on the substrate of the immobilized antibody on a gold nanoparticles layer in which PSA is not present was not observed. The corresponding Raman mapping image for the band at 1505 cm^{-1}, which is indicative of the Raman reporter, is shown in Figure 4(b). Raman mapping visualizes the selective surface distribution of the PSA that was selectively conjugated with the antibody. It clearly indicates that Raman reporter of the antibody readily binds to the antigen on the gold nanoparticles layer of the patterned substrate and is not just adsorbed onto the surrounding surface area. The immobilized Raman reporter with the antibody specifically extracts the available PSA when PSA is present on a substrate that is interacting with an immobilized layer of the antibody on the gold nanoparticles layer on the patterned substrate. This means that antibodies on the Raman reporter are successfully and selectively conjugated with the antigen (PSA) on the immobilized antibody on the gold nanoparticles layer of the patterned substrate. We could finally detect PSA at very low levels (~1 pg/mL) by using this SERS immunoassay method, which is within PSA concentration in human blood samples. This SERS immunoassay method on the patterned substrate created by microcontact printing can be applied for the sensitive detection of biomolecules.

Conclusion

A promising substrate for SERS immunoassays, which is micropatterned by the microcontact printing method, has been introduced. For this effective detection method for antigen (PSA) in cancer cells, we performed a sandwich type SERS immunoassay process using antigen-antibody binding on the particularly patterned surface. SERS spectra and Raman mapping images clearly showed that the antibodies on the Raman reporter are successfully and selectively conjugated to the antigen (PSA) on the immobilized antibody on the gold nanoparticles layer of the patterned substrate.

It would be possible to analyze many antibodies rapidly and simultaneously if various antigens with different Raman reporters are selectively captured on each patterned surface. The results of this study could be potentially extended to multi-protein detections and ultrasensitive biosensors.

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