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ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 2009

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Highly Sensitive Immunoassay of Lung Cancer Marker Carcinoembryonic Antigen Using Surface-Enhanced Raman Scattering of Hollow Gold Nanospheres

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A quick and reproducible surface-enhanced Raman scattering (SERS)-based immunoassay technique, using hollow gold nanospheres (HGNs) and magnetic beads, has been developed. Here, HGNs show strong enhancement effects from individual particles because hot spots can be localized on the pinholes in the hollow particle structure. Thus, HGNs can be used for highly reproducible immunoanalysis of cancer markers. Magnetic beads were used as supporting substrates for the formation of the immunocomplex. This SERS-based immunoassay technique overcomes the problem of slow immunoreaction caused by the diffusion-limited kinetics on a solid substrate because all of the reactions occur in solution. For the validation of our SERS immunoassay, a well-known lung cancer marker, carcinoembryonic antigen (CEA), was used as a target marker. According to our experimental results, the limit of detection (LOD) was determined to be 1−10 pg/mL, this value being about 100−1000 times more sensitive than the LOD of enzyme-linked immunosorbent assay. Furthermore, the assay time took less than 1 h, including washing and optical detection steps.

Immunoassay is a fast and cost-effective method that can be applied to clinical diagnostics, biological sensors, and food safety tests.[1−3] At present, enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) spectroscopy are the most widely used technical tools to detect the presence of antigen or its complementary antibody. To identify a specific antigen−antibody interaction in immunoassays, molecular labels such as fluorescence dyes are commonly used. Recently, however, metal nanoparticles have been widely used as detection tags because of their excellent optical properties. For example, fluorescent quantum dots, which provide tunable and narrow emission bands with high photostability, are used as efficient fluorescent labels for biological immunoassays.[4−7] More recently, the surface-enhanced Raman scattering (SERS)-based immunoassay technique using antibody-conjugated metal nanoparticles has attracted great interest from many research scientists because of its rapid and sensitive sensing capability.

One of the most popular platform technologies for the SERS-based immunoassay is the detection of a sandwich immunocomplex immobilized on a solid substrate. Here, polyclonal antibodies (PAb) are immobilized on a solid substrate and then antigen solutions and monoclonal antibody (mAb)-conjugated metal nanoparticles are sequentially added. After formation of the sandwich immunocomplex on a solid substrate, nonspecific binding antigens and mAb-conjugated metal nanoparticles are washed out using buffer solution and then the SERS spectra are measured. Many SERS-based immunoassay results for the detection of human immunoglobin (IgG) antigens,[8,9] rabbit IgG antigens,[10] feline calicivirus (FCV),[11] hepatitis B virus,[12] prostate-specific antigens (PSA),[13] and protein A[14] have been reported using this surface immobilization technique. In addition, a duplex analyte immunoassay, based on SERS detection for two different types of IgGs immobilized on a solid substrate, has also been reported.[15]

Although this SERS-based detection technique provides a sensitive method for immobilized immunocomplexes, it has several drawbacks. First, an extended incubation time was required for each binding step because the restriction on molec-

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10.1021/ac802722c CCC: $40.75
Published on Web 03/20/2009

ular diffusion near the surface makes the kinetics of protein−protein assays much slower. Second, the repetition of washing steps to remove nonspecific binding proteins also makes this immobilization-based technique inconvenient. Third, all of the immunoreagent components should be immobilized on the surface of a solid substrate in air. In many cases, however, the exposure of proteins to air seriously reduces their biological activity. Finally, in the immobilization-based SERS chip, it is very difficult to fabricate surfaces with highly reproducible enhancements. As a result, reproducible SERS signals could not be obtained for different laser spots.

To resolve these problems, a quick and reproducible SERS-based immunoanalysis technique using magnetic beads has been developed. This method does not use an immobilization procedure on a solid substrate; instead, it uses magnetic beads as antibody-supporting materials. In this system, the sandwich immunocomplexes are immobilized on the wall of a microtube using a small magnetic bar. This technique overcomes the slow immunoreaction problems caused by the diffusion-limited kinetics on a solid substrate because the reaction occurs in solution. As a result, the assay time is greatly reduced to less than 1 h if properly designed metal nanotags are prepared. Furthermore, it is possible to obtain more reproducible results because the SERS signals are measured for the average nanoparticle ensembles in solution. Gong et al.[16] recently demonstrated a SERS immunoassay method combining a glass-encapsulated silver nanotag as a detection probe and glass-encapsulated magnetic nanoparticles as an immobilization matrix and separation tool for immunoassay of α-fetoprotein (AFP). When silver nanoparticles are used for quantitative measurements, optimal aggregation conditions are required for signal enhancement. In many cases, however, the overaggregation of silver nanoparticles leads to a precipitation of the particles with time, and this reduces the reproducibility of the SERS signals.[17,18] Hence, to resolve this problem, hollow gold nanospheres (HGNs) were fabricated and used as single nanoparticle SERS agents in this work. These particles show strong enhancement effects from individual particles because of their ability to localize the surface electromagnetic fields through the pinholes in the hollow particle structures.[19,20] As a result, they can be used as highly reproducible sensing probes for the quantitative immunoanalysis of specific markers.

In the present study, we explore the feasibility of the SERS-based detection method using HGNs and magnetic beads for fast and reproducible immunoassays. To investigate its potential applicability as an optical immunosensor, this template was applied to a well-known lung cancer marker, carcinoembryonic antigen (CEA). CEA is not usually present in the blood of healthy adults; however, its level is raised for heavy smokers. It has been reported that serum from individuals with colorectal, gastric, pancreatic, lung, and breast carcinomas, as well as individuals with medullary thyroid carcinoma had higher levels of CEA than healthy

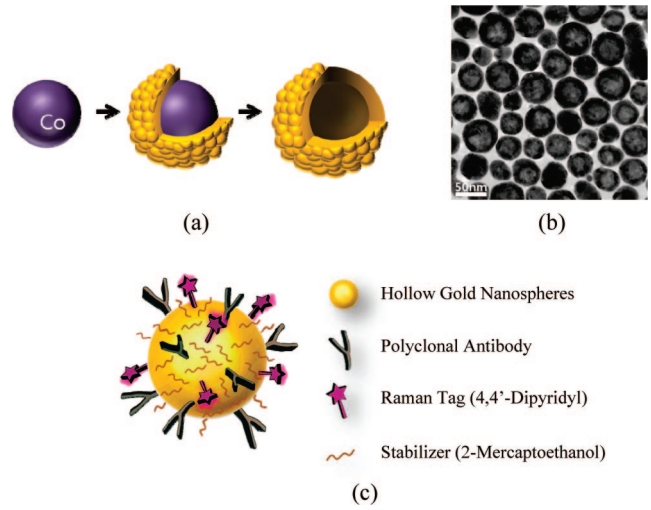

Figure 1. Schematic illustration of HGNs for SERS immunoassay: (a) experimental procedure that generates HGNs by templating against a cobalt nanoparticle, (b) TEM image of HGNs prepared using the template of cobalt nanoparticles, and (c) conjugation of Raman tag (4,4′-dipyridyl) and CEA antibodies onto HGNs.

individuals.[21,22] CEA is mainly used as a tumor marker to identify recurrences after surgical resection. Cutoff values of CEA are given as 2.5 ng/mL for nonsmokers and 5.0 ng/mL for smokers, respectively. Thus, a sensitive immunoassay technique to detect a subnanogram per milliliter level is required to satisfy detection of this cutoff level. For the validation of this SERS-based sensing technique, its sensitivity and limit of detection (LOD) for the CEA markers were compared with the experimental results using ELISA.

EXPERIMENTAL SECTION

Preparation of Hollow Gold Nanospheres. HGNs were prepared by the method previously reported by Schwartzberg et al.[19] Cobalt nanoparticles were synthesized by reducing $CoCl_2$ with $NaBH_4$ under an N_2 purging condition and were used as templates for the HGNs. The 0.1 M $HAuCl_4$ was added 10 times in 50 μL aliquots. Here, gold atoms were nucleated and grown up to small shells around the cobalt template. When the solution was exposed to ambient conditions by stopping the N_2 purging, the cobalt completely dissolved and a hollow interior was formed. At this stage, the color of the solution changed from dark brown to deep blue. The wall thickness could be controlled by changing the concentration of $HAuCl_4$. Figure 1a shows a schematic illustration for the synthetic production of HGNs. Figure 1b shows their transmission electron microscopy (TEM) images. According to our measurements, the diameter of the HGNs and the wall thickness were estimated to be 45 ± 12 and 15 ± 5 nm, respectively.

Antibody Conjugations onto Metal Nanoparticles. To use these HGNs as SERS-active probes, 4,4′-dipyridyl (DP) was adsorbed onto the surface of the HGNs. Here, DP was used as a Raman reporter. An amount of 1.0 μL of 10^{-4} M DP was added to 1.0 mL of 0.7 nM HGNs, and the mixture was reacted for 1 h under stirring. The number of adsorbed DP molecules per

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particle is estimated to be approximately 140. Then, dihydrolipoic acid (DHLA) was used for the antibody conjugation. The two −SH terminal groups of DHLA were cleaved and chemically bonded to the HGN surface. An amount of 2.0 μL of 5.0 mM DHLA was added to 1 mL of 0.7 nM dye-adsorbed HGN and allowed to react for 1 h. The nonspecific sites remaining on the surface of the nanoparticle were coated with 2.0 μL of 2.5 mM mercaptoethanol. Excess nonspecific binding mercaptoethanol in solution was removed by centrifuging the solution, and the precipitate was washed twice with phosphate-buffered saline (PBS) buffer and resuspended using an ultrasonicator. As a result, nonspecific interactions of HGNs with other proteins as well as nanoparticle aggregations could be effectively prevented. For the activation of other −COOH terminal groups, 1.0 μL of 1.0 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1.0 μL of 1.0 mM *N*-hydroxysuccinimide (NHS) were added and allowed to react for 1 h. Carboxylate-terminated HGNs were used to achieve a stable immobilization of proteins by covalent bonding in the esterification of NHS with EDC. Finally, 1.0 μL of 2.5 mg/mL polyclonal CEA antibody (excess amount) was added to NHS-activated HGNs and reacted for 2 h. Here, antibody immobilization onto the HGNs occurs by displacement of the NHS group by the lysine residues of the antibody. Unreacted NHS groups on the surface of the HGNs were deactivated with 1.0 μL of 1 mM ethanolamine for 2 h. Nonspecific binding chemicals and antibodies were removed by centrifuging, and the final nanoprobes were washed twice with PBS buffer. Figure 1c shows polyclonal CEA antibody-conjugated HGN nanoprobe for the CEA marker detection.

For the activation of other −COOH terminal groups on the magnetic beads, 10 μL of 3 mM NHS and 10 μL of 3.0 mM NHS were added to 1.0 mL of 0.5 mg/mL magnetic beads and allowed to react for 1 h. Then, 6.0 μL of 1.0 mg/mL monoclonal CEA antibody (excess amount) was added to NHS-activated magnetic beads and reacted overnight. Here, unreacted antibodies were washed out using a micropipette after immobilization of the magnetic beads by use of a magnetic bar. Unreacted NHS groups on the surface of the HGNs were also deactivated with 10 μL of 3.0 mM ethanolamine for 2 h. Nonspecific binding chemicals and antibodies were removed by centrifuging, and the final nanoprobes were washed twice with PBS buffer.

Surface-Enhanced Raman Scattering Detection. SERS measurements were performed using a Renishaw 2000 Raman microscope system (Renishaw, U.K.). A Melles Griot He−Ne laser operating at $\lambda = 632.8$ nm was used as the excitation source with a laser power of approximately 30 mW. The Rayleigh line was removed from the collected Raman scattering using a holographic notch filter located in the collection path. Raman scattering was collected using a charge-coupled device (CCD) camera at a spectral resolution of 4 cm^{-1}. An additional CCD camera was fitted to an optical microscope to obtain optical images. Sandwich immunocomplexes in a microtube were collected using a capillary tube, and a 20× objective lens was used to focus a laser spot on the glass tube. All of the Raman spectra reported here were collected for 10 exposure times in the range of 900−1800 cm^{-1}.

Enzyme-Linked Immunosorbent Assay Detection. ELISA was performed using a microplate reader (Power Wave X340, BioTEK, U.S.A.) equipped with a 96-well plate. First, 50 μL of 10 μg/mL CEA monoclonal antibodies was added to the 96-well plate. For stable immobilization of the antibodies on the surface, it was reacted for 24 h at 4 °C. Any nonspecific binding sites on the surface were blocked by adding 50 μL of 3% BSA. Then 50 μL of different concentrations of CEA antigen (10 pg/mL to 10 μg/mL) were added to each well and reacted for 2 h. The plate was washed with 0.1 M PBS buffer solution to remove unbound antigens. Here, 50 μL of 10 μg/mL enzyme (HRP)-linked polyclonal antibodies were added to each well and reacted for 2 h to make a sandwich immunocomplex. The plate was washed again to remove unbound antibody−enzyme conjugates. When 50 μL of ABTS solution was added to each well, the color was changed to green by the enzyme. The absorbance at 405 nm was measured for each plate well to determine the presence and quantity of antigen.

RESULTS AND DISCUSSION

As mentioned above, the SERS technique using antibody-conjugated metal nanoparticles is emerging as a powerful new tool for the detection of specific cancer markers. Silver nanoparticles are the most widely used enhancing agents in SERS. However, aggregation is needed to achieve enhancements large enough for highly sensitive detection in this case. This aggregation process leads to poor homogeneity of SERS signals because aggregation is a random process, and it is almost impossible to control the degree of aggregation for a reproducible analysis. To resolve this problem, the HGNs, conjugated with specific antibodies were used for a highly homogeneous immunoassay of cancer markers. As mentioned in the introduction, HGNs show strong enhancement effects from individual particles because hot spots can be localized on the pinholes in the hollow particle structures. Schwartzberg et al.[19,20] evaluated the homogeneous scattering properties of the HGNs by comparing the SERS spectra of MBA bound to HGNs and silver particles. More recently, we used the HGNs for highly homogeneous imaging of HER2 cancer markers overexpressed in single MCF7 cells.[23] In the present work, antibody-conjugated HGNs were used for the quantitative immunoanalysis of cancer biomarkers.

To test the reproducible SERS enhancement effects of HGNs, the SERS signals of the Raman reporter, crystal violet (CV), adsorbed on HGNs and silver nanoparticles were compared with each other. Figure 2 shows the SERS spectra (Figure 2a) for HGNs and (Figure 2b) for silver nanoparticle aggregates, respectively. With an increase in the salt concentration, the SERS signals of the Raman reporter, CV, adsorbed on silver aggregates rapidly increased. On the other hand, the SERS signals of CV adsorbed on HGNs were relatively constant with the increase in salt concentration. HGNs have a comparable SERS signal intensity without NaCl salts. This means that the SERS signals of HGNs are much less affected by ionic circumstances and they are more suitable than silver aggregates for the quantitative analysis of biological samples. Tally et al.[24] reported similar results with ours

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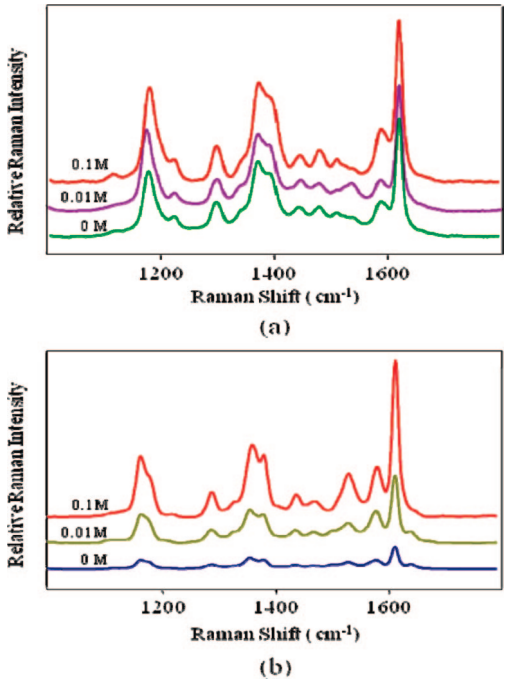

Figure 2. SERS spectra of 10^{-7} M CV (a) for 0.7 nM HGNs and (b) for 0.3 nM silver aggregates. SERS spectra were measured for 10 μL of different NaCl concentrations (0, 0.01, and 0.1 M).

for the SERS effects on Au nanoshells. They measured the SERS intensities for individual Au nanospheres, nanoshells, and nanosphere and nanoshell dimers coated with Raman reporter 4-MBA, respectively. Here, the Au nanoshell has a roughened surface like our HGN. Thus, hot spots could be generated by defects on the Au nanoshell. In this paper, the SERS spectra for a pair of adjacent nanoshells and for an individual nanoshell were measured and compared with each other. To better understand the local electromagnetic properties of each individual nanoshell and nanoshell dimers, theoretical calculations using the FDTD method were also performed. On the basis of both the experimental and computational data, the reasons for the similar SERS intensities for single nanoshell and aggregated nanoshells were explained. For nanoshell dimers, the high-field hot spot is excited only when the polarization of incident light has some components parallel to the interparticle axis. When the polarization is orthogonal to the interparticle axis, the enhancement effect was much weaker. Relatively weak enhancement of the dimers is consistent with the random orientation with respect to the polarization of incident light. The other factor for the low signal enhancement for the aggregated dimer is a distance effect. Because of the strong attractive forces between two nanoshells, the reporter molecules do not have a chance to be located between two nanoshells. It means that adjacent nanoparticles are touching and the interparticle gap was closed. Due to both effects, nanoshells can provide consistent and strong SERS enhancements as an individual nanoparticle and their SERS intensity is less affected by aggregation. Just like nanoshell dimers, the SERS from HGNs are also expected to be much less affected by the salt concentration.

Figure 3a shows an animation picture for the formation of the sandwich-type immunocomplex using two different metal particles, PcAb-conjugated HGNs and McAb-conjugated magnetic beads. When CEA antigens are present, PcAb-conjugated HGNs and McAb-conjugated magnetic beads form a sandwich complex by antibody−antigen−antibody interactions; however, they do not form a sandwich complex in the absence of antigen. Figure 3b shows the TEM images for single magnetic beads. In the left image, HGNs are bound on the surface of the magnetic bead by the antibody−antigen interactions when antigens are present. On the other hand, the right image shows that HGNs are not bound on the surface when antigens are not present.

Figure 4 shows a schematic diagram for the formation of sandwich immunocomplex and its SERS immunoassays using HGNs and magnetic beads. Here, HGNs were employed as a Raman-active probe and magnetic beads were used as separation agents. As shown in Figure 4a, sandwich immunocomplexes were generated via a two-step process. In the first step, 10 μL of 0.5 mg/mL McAb-conjugated magnetic beads was added in 10 μL of PBS buffer solution containing 50 ng/mL of CEA antigen for 20 min under shaking. Next, the CEA-captured magnetic beads were isolated by a magnetic bar and then the solution was washed twice with PBS buffer using a micropipette. In the second step, the obtained particles were further reacted with 10 μL of 0.7 nM McAb-conjugated HGNs for 20 min under shaking. The obtained sandwich immunocomplexes were isolated by a magnetic bar on the wall of the microtube. Here, the color of the residual solution changed to colorless ("On"), as shown in the left picture of Figure 4c. This is because all of the HGNs were bound to the surface of the magnetic beads and they are collected together with the magnetic beads on the wall. As a result, the color was changed to colorless because the HGNs, which show a characteristic purple color, are attached to the surface of the magnetic beads by forming sandwich immunocomplexes and collected on the wall. The residual solution was washed twice with PBS buffer using a micropipette. Then the magnetic bar was removed and the immunocomplexes were redispersed in the PBS solution. The immunocomplex particles were collected using a capillary tube for SERS measurements. In this case, the SERS signals from the HGNs were observed because a sandwich immunocomplex was formed in the microtube. On the other hand, 10 μL of 0.5 mg/mL McAb-conjugated magnetic beads and 10 μL of 0.7 nM McAb-conjugated HGNs were mixed together without CEA antigen, as shown in Figure 4b. The samples were isolated by a magnetic bar, washed, and redispersed by the same process as in Figure 4a. In this case, however, the color of the residual solution remained purple ("Off"), as shown in the right picture of Figure 4c. This is because all of the HGNs remained in the residual solution without forming immunocomplexes. The corresponding Raman spectrum does not show any reporter SERS signal in Figure 4b because all of the HGNs were washed out and only magnetic beads are left in the microtube. As a result, this technique can be used for the fast identification of specific antigen markers in solution.

For the evaluation of the SERS immunoassay technique using HGNs and magnetic beads, an ELISA measurement was performed for CEA antigens. First, capture antibodies were immobilized on the surfaces of a 96-well plate and the remaining sites were treated with BSA to prevent nonspecific binding. Then CEA antigens were added and bound to the capture antibodies. After washing three times with a micropipette, detecting antibodies were added and bound to antigens. Enzyme-linked secondary

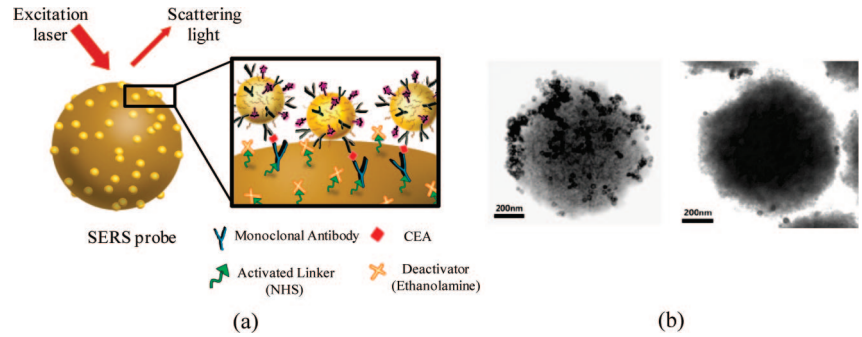

Figure 3. Illustration of SERS immunoassay using HGNs and magnetic beads: (a) formation of the sandwich immunocomplex between antibody-conjugated HGNs and magnetic beads and (b) TEM images of a single magnetic bead with CEA antigens (left) and without CEA antigens (right).

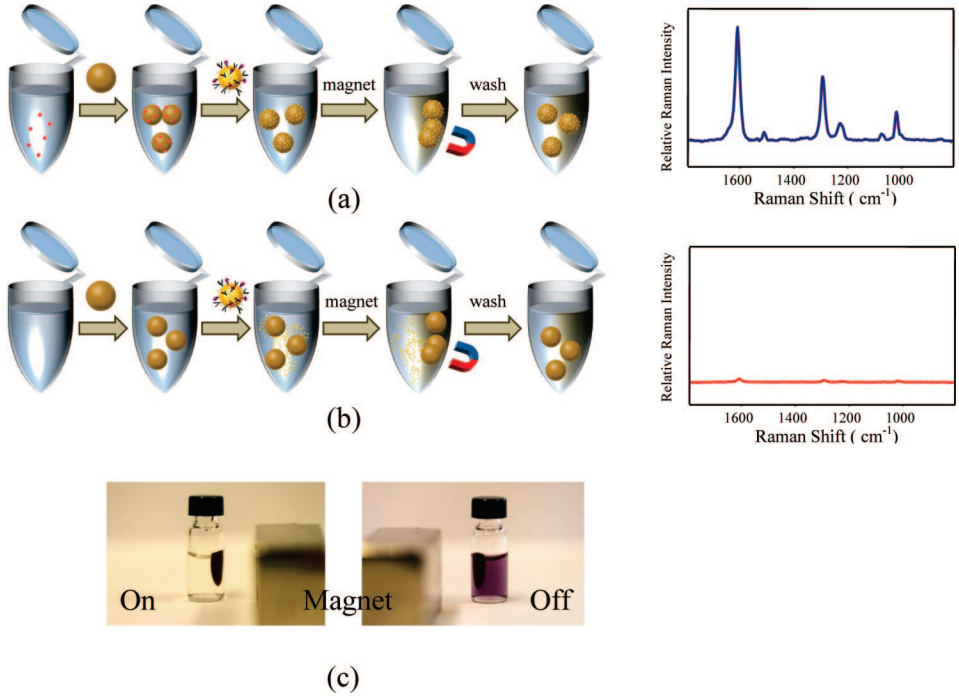

Figure 4. Schematic illustration of immunoassay processes and corresponding Raman spectra: (a) with CEA antigens and (b) without CEA antigens. (c) Photographs showing suspended magnetic beads attracted to the wall of a microtube by a bar magnet: with CEA (left) and without CEA (right).

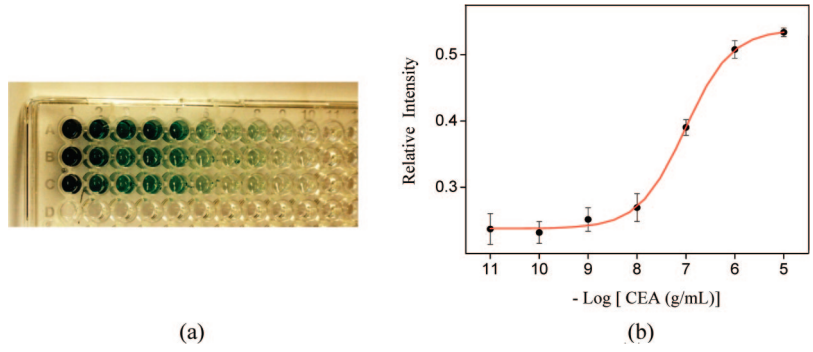

Figure 5. (a) Colorimetric analysis for various concentrations of CEA antigen in a 96-well plate and (b) corresponding calibration curve for their immunoassay. Error bars indicate standard deviations from three measurements.

antibodies were added and bound to detecting antibodies. Finally, a substrate was added and converted by the enzyme to a detectable form. Figure 5 shows the ELISA experimental results. With a decrease in CEA concentration, the color changed from dark green to colorless, as shown in Figure 5a. Figure 5b displays the ELISA standard curve for the formation of sandwich immunocomplex at different CEA concentrations. The related standard deviations from three measurements are indicated by error bars.

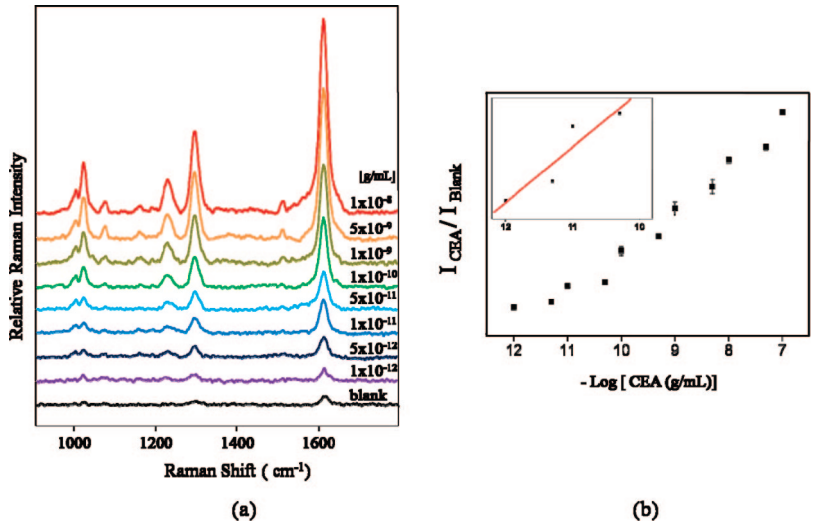

Figure 6. (a) SERS spectra for decreasing concentrations of CEA antigen and (b) corresponding intensity ratio (I_{CEA}/I_{Blank}) of the SERS signal at 1612 cm^{-1} for the logarithmic concentration of CEA. Inset shows a linear relationship in the lower concentration range from 1 to 100 pg/mL (coefficient of determination, $R^2 = 0.93$). Error bars indicate standard deviations from four measurements.

The LOD determined by the ELISA immunoassay method was in the 1−10 ng/mL range. However, this LOD value does not satisfy the cutoff level of CEA.

Figure 6a illustrates the SERS spectra of the sandwich immunocomplex for various concentrations of CEA antigen. The concentration of CEA was varied from 1 pg/mL to 10 ng/mL. The Raman peak of DP centered at 1612 cm^{-1} was used for the quantitative evaluation of CEA antigen. In the absence of CEA antigen, a weak SERS signal was obtained (Blank). This indicates that a small amount of HGNs still remained in the solution, even though most of the HGNs were removed from the solution by the washing process. When CEA antigen was added, the SERS signals were obviously enhanced with the increase in its concentration. Here, the LOD was estimated to be 1 pg/mL because the Raman peak at 1612 cm^{-1} for this concentration is difficult to distinguish from that in the blank spectrum. The calibration curve, constructed from the ratio of peak intensities (I_{CEA}/I_{Blank} at 1612 cm^{-1}) is shown in Figure 6b, where the error bars indicate standard deviations from four measurements. I_{CEA}/I_{Blank} gradually increases logarithmically with increasing concentration of CEA in the range from 1 to 100 ng/mL. The inset figure shows a very good linear response in the lower concentration range from 0 to 100 pg/mL. Our experimental data demonstrate that the HGN-based SERS immunoassay is a very useful analytical technique for the highly sensitive detection of CEA markers. In particular, its detection sensitivity is increased by 2 or 3 orders of magnitude over the conventional ELISA method. Furthermore, the assay time was less than 1 h, including washing and optical detection steps.

CONCLUSION

In the present study, a conceptually new SERS-based immunoassay technique using HGNs and magnetic beads has been developed. For this purpose, HGNs have been fabricated for sensitive and reproducible immunoanalysis. Commercially available 1 μm diameter magnetic beads were used as supporting substrates. For specific targeting, the surfaces of HGNs and magnetic beads were conjugated with polyclonal and monoclonal anti-CEA antibodies, respectively. Then, they were simply mixed with CEA antigens for the formation of sandwich immunocomplexes. Nonspecific binding HGNs were removed by washing with a micropipette. Quantitative analysis was performed by measuring the SERS signal at 1612 cm^{-1}. The LOD was determined to be 1−10 pg/mL from three standard deviations above the background. As a result, the SERS immunoassay technique using HGNs and magnetic beads is about 100−1000 times more sensitive than that of ELISA. Our proposed SERS-based immunoassay technique, with antibody-conjugated HGNs and magnetic beads, has several advantages over previously reported SERS detection using the immobilization of an immunocomplex on a solid substrate with respect to good reproducibility and fast assay time. Therefore, this novel immunoassay technique is expected to be a powerful clinical tool for fast and reliable cancer diagnosis.

ACKNOWLEDGMENT

This research was supported by projects of the Korea Science and Engineering Foundation (Grants R01-2007-000-20238-0, R11-2008-044-01002-0, and 2007-04431), the National Cancer Center of Korea (Grant 0620400-1), and the Seoul Research and Business Development Program (Grant No. 10574).

Received for review December 22, 2008. Accepted February 27, 2009.

AC802722C