Quantitative Detection of Immunoreaction using Magnetite Nanoparticles and Raman Scattering Spectroscopy

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We developed a new quantitative detection method for an immunoreaction combining magnetite (Fe$_3$O$_4$) nanoparticle labeling with confocal Raman spectrometry. Magnetite nanoparticles are transparent, Raman active, and can be controlled easily by magnets in bimolecular reactions. Human Chorionic Gonadotropin (hCG) was selected as a target molecule for this investigation. Sandwich immunoreaction was performed on a dot-patterned substrate using two anti-hCG antibodies, which were the antibody immobilized on the substrate and the biotinylated antibody. The immunoreaction was microscopically visualized by reacting streptavidin-modified magnetite nanoparticles with the biotinylated antibody on the substrate. The adsorbed magnetite nanoparticles on the dot pattern were detected by Raman intensity imaging. A good linear relation between the integrated Raman intensity spectra at 220 cm$^{-1}$ and hCG concentration was gained. This method may be applied toward the detection of other target molecules in the fields of biotechnology and biomedicine.

Keywords: Immunoreaction; Magnetite nanoparticle; Raman scattering spectroscopy; Biosensing and devices; Iron oxide; Self-assembly

I. INTRODUCTION

In the past, fluorescence and luminescence molecules have been used as labels for bimolecular interaction. However, the common drawback of these markers is that they can be photobleached by exposure to light and the passing of time, which means they are not suitable for long-term preservation. Recently, because their magnetic signals are stable, magnetic particles have been proposed for use as labels to detect bioassays [1, 2]. In addition, the magnetism of the particles simplifies the usually complicated process of sample preparation, including the concentration of trace amount of targets and immobilization of molecules on the particles [3, 4]. The detection of the specific bimolecular interaction between biotin immobilized on a monolayer-modified substrate and streptavidin-modified magnetite nanoparticles has been previously reported [5, 6]. When magnetic signals from the particles attached on the substrate were detected by a magnetic force microscope (MFM), the signal that was mainly derived from the surface neighborhood was determined. The magnetic signals could be detected, but it was difficult to detect them quantitatively because magnetite nanoparticles formed rough piles on the surface of the substrate. Further investigation is required of the quantitative measurements made with this assay system.

Recently magnetic sensors such as giant magnetoresistive (GMR) sensors [7] and Hall sensors [8] have been used for immunosensing because of their high sensitivity. However, they are easily affected by geomagnetism and are strongly dependent on their own magnetic characteristic, which means that they must be strictly controlled.

To solve these problems of magnetic detection systems, we focused on the Raman spectroscopic technique, which is a powerful optical detection technique for providing the vibration and structural properties of materials. Analysis of Raman spectra enables quantitative determination of permeable iron oxide magnetic materials regardless of their surface structure. Furthermore, specific detection of the target material can be carried out by selecting the appropriate wavelength of the Raman scattering spectrum.

In the work reported herein, we used confocal Raman spectroscopy to conduct an immunoassay of magnetite nanoparticles. Human chorionic gonadotropin (hCG), which has widely been employed as a marker for the diagnosis of pregnancy and/or gonadal tumors, was used as
a target molecule.

II. EXPERIMENTAL

A. Material

Human chorionic gonadotropin (hCG), antibody, FITC-streptavidin, glutaraldehyde, and bovine serum albumin (BSA) were purchased from Wako Chemical. 3-aminopropyltriethoxysilane (APS) and n-octadecyltrimethoxysilane (ODS) were purchased from Gelest Inc. Sulfo-NHS-LC-LC-biotin was obtained from Pierce. Magnetite nanoparticles, 40 nm in size, were obtained from TODA Kogyo Corp. Other reagents were of analytical-reagent or laboratory grade. Ultra pure water was used in all procedures.

B. Preparation and patterning of ODS monolayer-modified substrate

A p-type Si (100) wafer covered with thermally grown silicon oxide was used in this study. The wafer was dispersed in an SPM solution (H₂SO₄:H₂O₂=4:1) and then placed into a vial with a bottle full of organosilane liquid, n-octadecyltrimethoxysilane (ODS) and heated for 3 h at 110°C [9]. After that, the substrate was cleaned with trichloroethylene in an ultrasonic bath for 20 min to remove the residual organic molecules.

The ODS-modified substrate was covered with conventional UV-resist, and patterning was carried out with 350 nm UV light irradiation. The substrate was exposed with O₂ plasma to form patterns on it under the following conditions: input power of 300 W and O₂ flow rate of 80 sccm for 1 min.

C. Preparation of biotinylated antibody

Anti-hCG antibody (Rabbit-Poly) was dissolved in a carbonate buffer (pH 9.2), and 1 mg of sulfo-NHS-LC-LC-biotin was dissolved in 100 µL of dimethylsulfoxide (DMSO). An antibody solution (95 %) and a biotin (5 µL) solution were mixed and reacted for 2 h. After the reaction, unreacted biotin was removed by using a spin column according to the manufacturer's instructions. The biotinylated antibody was stored in PBS.

D. Preparation of streptavidin-modified magnetite particle

The magnetite nanoparticles were dispersed in an APM solution (NH₄OH:H₂O₂:H₂O=1:1:5) and heated at 100°C for 20 min. The nanoparticles were then collected using a neodymium-boron magnet, washed with methanol at 60°C for 1 h to remove the residual solvent. The nanoparticles then were resuspended in 5 % (v/v) APS dissolved in 20 mL of toluene. The bottle containing the APS-modified nanoparticles was placed in an ultrasonic bath and kept at 65°C for 8 h. Then, Sulfo-NHS-LC-LC-biotin was added and allowed to react for 1 h, to further modify the nanoparticles. After washing, the nanoparticles were suspended in 1 mL of PBS buffer containing 50 µg/mL streptavidin and incubated for 1 h. Finally, the nanoparticles were modified and washed using an ultrasonic bath.

E. Immunoreaction on ODS patterned substrate

First, a 0.03 mg/mL solution of BSA (10 µL) was absorbed onto the ODS substrate for 10 min by hydrophobic interaction. Next, 1 % glutaraldehyde (10 µL) was reacted with the substrate for 10 min. Then, the substrate was reacted with 10 µL/mL anti-hCG IgG antibody (Mouse-monomonal) for 30 min. For the immunoreaction, hCG diluted in difference concentrations ranging from 10 ng/mL to 1 mg/mL was added onto the substrate surface and incubated for 30 min. After the substrate was rinsed with PBS, it was reacted with 10 µg/mL of biotinylated polyclonal anti-hCG IgG antibody for 30 min. Finally, the substrate was incubated into streptavidin-modified magnetite nanoparticles (10 µg/mL) for 30 min (Fig. 1).

F. Raman spectroscopy of magnetite nanoparticles on the substrate

Figure 2 shows a schematic diagram of the experimental imaging and measurement system used in the present study to carry out Raman experiments. The system has two basic subsystems: a spectrometer, including a CCD detector, and a confocal microscope. The originating apparatus of the system is a Nd:YAG laser diode, which has strong outputs at a wavelength of 532 nm. The laser light is incident on a 50 % beam-splitter which diverts half of the beam (with a diameter of around 1 µm or less) through a pinhole into a 100× confocal microscope objective through which it is focused with an irradiation power of 9 mW onto the sample. The microscope objective col-
lects the excitation beam and the back-scattered Raman light. Then, both the light beams pass through the beam-splitter and an edge filter where the excitation light is cut off. The Raman back-scattered light is then passed into a grating spectrometer where the collected light is dispersed. The dispersed light is then measured with a CCD detector.

Raman spectra are then acquired with the aid of a computer as a function of wave-number separated from the detector. The dispersed light is then measured with a CCD detector.

FIG. 2: Schematic illustration of confocal Raman spectrometer.

III. RESULTS AND DISCUSSION

A dot pattern of ODS on the SiO$_2$ substrate was used to immobilize biomolecules. BSA was adsorbed onto the ODS surface by hydrophobic interaction. Adsorption of proteins strongly depends on the nature of the substrate surface. Previous researchers have reported that BSA adsorbs to a hydrophobic surface at solution concentrations below 50 $\mu$g/mL in any pH range [10]. We therefore used a 30 $\mu$g/mL BSA solution. Amino residues of BSA attached on the substrate surface were reacted with a cross-linker, glutaraldehyde, and then immobilized with anti-hCG antibody. The modified substrate was reacted with hCG so that the activity of the antibodies immobilized on the substrate could be examined. Then hCG was reacted with the antibodies on the substrate. In addition, anti-hCG primary antibody and Cy5-labeled anti-IgG secondary antibody were subsequently reacted on the substrate. As a result, the dots were clearly seen as bright spots on a dark background under the fluorescence microscope. In contrast, no dot pattern was observed in the reaction in the absence of hCG (Fig. 3). Alternatively, we have examined direct immobilization of hCG antibody on the ODS surface by hydrophobic interaction. However, reduction of the antibody activity that was observed may be the result of the collapse of the molecular structure of the antibody (Data not shown). We therefore used BSA as an intermediate layer for immobilization on the substrate to stabilize the antibody structure and activity.

Streptavidin-modified magnetite nanoparticles were prepared through the use of a biotin-streptavidin reaction. Biotin is a small molecule (244 Da) that specifically binds to streptavidin. Biotin was attached to the nanoparticles through the cross-linking reaction of sulfo-NHS-LC-LC-biotin with surface amino groups. The biotin-modified particles were then allowed to react with streptavidin. Streptavidin is a globular protein (approximately 60 kDa) with four biotin binding sites on its opposing sides. The free binding sites, which were available for reaction with other biotin molecules, were used for the recognition of biotinylated antibodies reacted on the substrate.

Sandwich immunoassay was performed using the anti-hCG antibody-modified substrate and the streptavidin-modified magnetite nanoparticles. hCG was reacted with the antibody immobilized on the substrate at concentrations of 10 ng/mL to 1 mg/mL. Biotinylated anti-hCG antibody was reacted with hCG bound to the substrate surface. Streptavidin-modified magnetite nanoparticles were then reacted with the biotinylated antibody. Anti-hCG antibody-modified magnetite nanoparticles can be directly reacted with hCG captured by the antibody immobilized on the substrate. However, the reaction specificity (kinetics) between biotin and streptavidin is much higher than the reaction specificity between antigen and antibody. We therefore used a biotin-streptavidin reaction for the attachment of magnetite nanoparticles to finish the immunoassay. Figure 4(A) shows optical images of the magnetite nanoparticles attached on the substrate after the sandwich reaction. The nanoparticles on the pattern are clearly identifiable after the reaction in the presence of hCG. On the other hand, as Fig. 4(B) shows, there are no identifiable nanoparticles after the reaction in the absence of hCG. These results indicate that the particles that remained on the substrate were formed by an immunoreaction.
The magnetite nanoparticles that reacted with antibodies at the dot region of the substrate were imaged by confocal Raman microscopy. Raman spectra were collected from a single dot region of the substrate. The peaks observed at 950 cm$^{-1}$, 400 cm$^{-1}$, 300 cm$^{-1}$, and 220 cm$^{-1}$ are all assigned to magnetite (Fig. 5(A)) [11]. The most intense band, which was observed at 510 cm$^{-1}$, is derived from the silicon substrate. A band at 220 cm$^{-1}$ was then selected to produce a Raman image of the magnetite nanoparticles by scanning the substrate. As a result, a Raman image was observed that corresponded to the optical image of magnetite nanoparticles attached on the substrate (Fig. 5(B)). The bright red and yellow pattern indicates magnetite particles, whereas the dark region represents the substrate. Nonspecific adsorption of nanoparticles outside the dot pattern also was observed. This adsorption may be derived from the nonspecific adsorption of hCG and/or the streptavidin particles modified with bare silicon oxide, which have a slightly hydrophobic nature. The immunoreaction between hCG and monoclonal anti-hCG IgG antibody was quantitatively examined using the confocal Raman system. Solutions containing various concentrations of hCG were prepared by serial dilution in PBS of a 1 mg/mL hCG standard to cover the range from 10 ng/mL to 1 mg/mL. As was observed under the optical microscope, increasing the hCG concentration increased the number of magnetite nanoparticles attached on the substrate. Corresponding to this increase, it also was determined that the Raman intensity at 220 cm$^{-1}$ increased in a linear fashion when the hCG concentration was increased (Fig. 6(A)). Figure 6(B) is a dose-response curve showing Raman spectra at the intensity at 220 cm$^{-1}$ as a function of hCG concentration. The relation between hCG concentration and Raman intensity of magnetite nanoparticles shows linear response. Given all these findings, it is clear that the new quantitative detection method we developed for an immunoreaction could be useful to detect various analytical targets in the biotechnology and biomedical fields.

IV. CONCLUSION

We demonstrated a procedure to detect hCG by using magnetite nanoparticles and Raman spectroscopy. Sandwich immunoreaction was successfully demonstrated between the hCG captured by the antibody immobilized on the substrate and the biotinylated antibody. A linear relation between the intensity from Raman spectra at 220 cm$^{-1}$ and hCG concentration was determined. This method may be applied toward the detection of other target molecules.
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