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Fluorescein Isothiocyanate Linked Immunoabsorbent Assay Based on Surface-Enhanced Resonance Raman Scattering

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By using fluorescein isothiocyanate (FITC) as a Raman probe, we have developed a simple and sensitive method for an immunoassay based on surface-enhanced resonance Raman scattering (SERRS). For the first time, a SERRS-based immunoassay on the bottom of a microtiter plate is reported. We have applied the main pretreatment method of enzyme-linked immunoabsorbent assay (ELISA) to the present study. In this method, SERRS spectra of FITC are measured after several continuous steps of antigen coating, blocking, antibody adding, and colloidal silver staining. Human immunoglobulin G (IgG) and FITC−antihuman IgG are used for the immunoreaction. The proposed method has several advantages for immunoassay. First, we can determine the concentration of antigens via the intensity of a SERRS signal of FITC molecules that are attached to antibodies without an enzyme reaction, and thus the process is simple and reagent saving. Second, one can obtain SERRS spectra of FITC directly from silver aggregates on the bottom of a microtiter plate without displacement. Third, by using SERRS of FITC, the present method is sensitive enough to detect antigens at the concentration of 0.2 ng/mL, which is comparable to ELISA. Results are presented to demonstrate that the proposed SERRS-based immunoassay may have great potential as a high-sensitivity and high-throughout immunoassay.

Recently, surface-enhanced Raman scattering (SERS) and surface-enhanced resonance Raman scattering (SERRS) have widely been used as a powerful tool for ultrasensitive chemical analysis down to single-molecule detection,[1−10] and it expands its realm of applications from chemical−biochemical analysis to nanostructure characterization and biomedical applications.[9,10] SERRS signals do not overdevelop as in systems based on optical absorbance, and unlike fluorescence readout systems, SERS-active groups do not self-quench.[11,12] Therefore, the SERRS method holds considerable promise for immunoassay. Several SERS or SERRS-based immunoassays have already been reported. They utilized various SERS probes, for example, products of enzyme reactions,[11,13] Raman dye-labeled gold nanoparticles,[14−20] or rhodamine B-labeled Ag/SiO_2 core/shell nanoparticles.[21] Label-free immunoassays based on SERS were also reported.[22,23] All these studies have demonstrated the great potential of SERS and SERRS in immunoassay.

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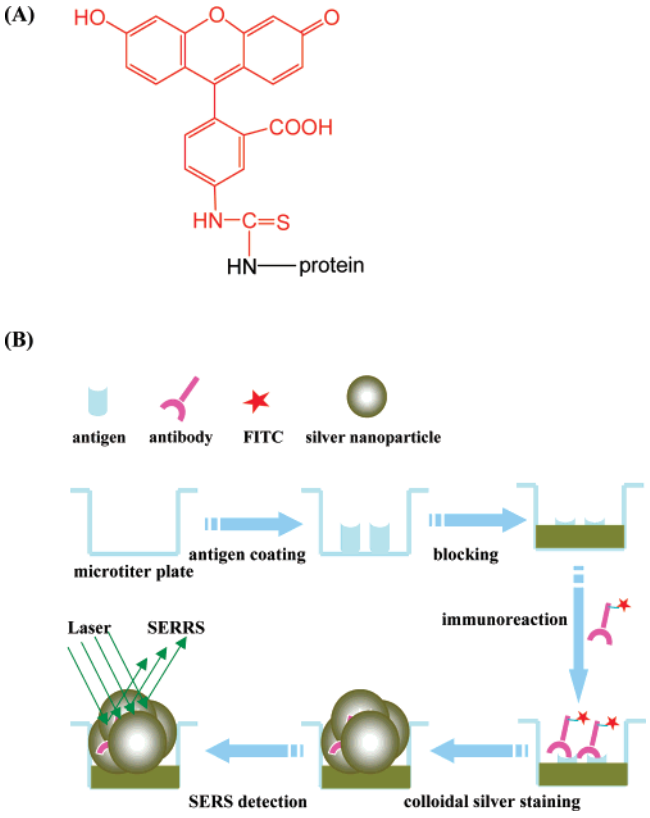
Scheme 1. (A) Structure of FITC and (B) Newly Proposed Procedure for SERRS-Based Immunoassay on a Microtiter Plate



Enzyme-linked immunoabsorbent assay (ELISA) is a powerful biochemical technique, which is used mainly in immunology to detect antibodies or antigens in samples quantitatively and qualitatively. It has played a great role in the fundamental researches and clinical medicine.[24−26] In general, ELISA uses a polystyrene microtiter plate as a solid support, which can absorb proteins nonspecifically and is made up of many wells. Since it was established as a method of immunoassay, many improved ELISAs have been proposed (e.g., "sandwich ELISA", "competitive ELISA", and "fluorescence ELISA"[27,28]). However, for ELISA, an enzyme acts as an amplifier, and thus the procedure for ELISA consists of immunoreaction, enzyme reaction, and spectrophotometer or spectrofluorometer analysis, which is very cumbersome and reagent consuming. Furthermore, any factor that may affect the enzyme reaction will directly affect results of ELISA. Inevitably, the same problem lies in the SERS-based enzyme immunoassay. Moreover, the complex procedure of synthesis of labeled nanoparticles and the nonquantitative detection of SERS-based label-free immunoassay may restrict their applications to high-throughout immunoassay.

In the present paper, we report a novel and simple SERRS-based immunoassay on a microtiter plate, using fluorescein isothiocyanate (FITC, Scheme 1A) as a Raman probe. On one hand, we use the same process as ELISA including antigen coating, blocking, and immunoreaction on a microtiter plate. Furthermore, the microtiter plate is also used as a solid phase for SERRS detection (Scheme 1B). On the other hand, we employ FITC molecules, which are directly linked to antibodies, instead of products of an enzyme reaction as a probe for the SERRS-based immunoassay. In the present study, silver nanoparticles play a role as an amplifier for the resonance Raman (RR) signals of FITC by colloidal silver staining after immunoreaction. The proposed method has several advantages for immunoassay. First, we can determine the concentration of antigens via the intensity of a SERRS signal of FITC molecules without the process of enzyme reactions, and thus the process of the present immunoassay is simple and reagent saving. Second, one can obtain SERRS spectra of FITC directly from silver aggregates on the bottom of a microtiter plate without displacement. Third, by using SERRS of FITC, the present method is sensitive enough to detect antigens at the concentration of 0.2 ng/mL, which is comparable with ELISA. Therefore, the proposed method has great potential in high-sensitivity and high-throughout immunoassays.

EXPERIMENTAL SECTION

Materials. Human immunoglobulin G (IgG), FITC−antihuman IgG, and bovine serum albumin (BSA) were obtained from Sigma Co., Ltd. and used without further purification. Human serum is from the College of Animal Science and Veterinary Medicine, Jilin University, Changchun, P. R. China. Eight-well microtiter plates were purchased from Beijing Dingguo Biological Reagent Co., Ltd. Tris (hydroxymethyl) aminomethane was from Roche Co., Ltd., and silver nitrate was purchased from Aldrich Co., Ltd. All other chemicals are from Beijing Chemical Reagent Co., Ltd. Triply distilled water was used throughout the present study.

Preparation of Silver Colloid. Colloidal silver was prepared by the aqueous reduction of silver nitrate (10^{-3} M, 200 mL) with trisodium citrate (1%, 4 mL) using a method referred to by Lee and Meisel,[29] The plasmon absorption maximum of the silver colloid we prepared was located at 415 nm.

Immunoreaction Protocol. (1) Antigen Coating. A human IgG powder was first dissolved with a coating buffer (0.16% Na_2CO_3, 0.292% $NaHCO_3$, 0.016% NaN_3, pH 9.0), and then a microtiter plate was coated with human IgG solutions of different concentration (0.2, 2.2, 4.2, 6.2, 8.2 ng/mL), 100 μL per well. Last, the microtiter plate was incubated for 2 h at 37 °C. After that, the microtiter plate was washed three times by a washing buffer (0.8% NaCl, 0.02% KH_2PO_4, 0.02% KCl, 0.12% $Na_2HPO_4 \cdot 12H_2O$, 0.05% Tween-20, 0.1% NaN_3) to remove unbound human IgG and impurities.

(2) Blocking. We prepared a phosphate buffer (pH 7.0) containing 1% BSA and 0.1% NaN_3 and used it as a buffer for blocking the nonspecific adsorption of FITC−antihuman IgG to the plate. To each well was added 100 μL of blocking, and then the plate was incubated for 12 h at 37 °C. After that, the plate was washed three times by a washing buffer.

(3) Antibody Adding. FITC−antihuman IgG solution was 100-fold diluted by a phosphate buffer (pH 7.0) before being used,

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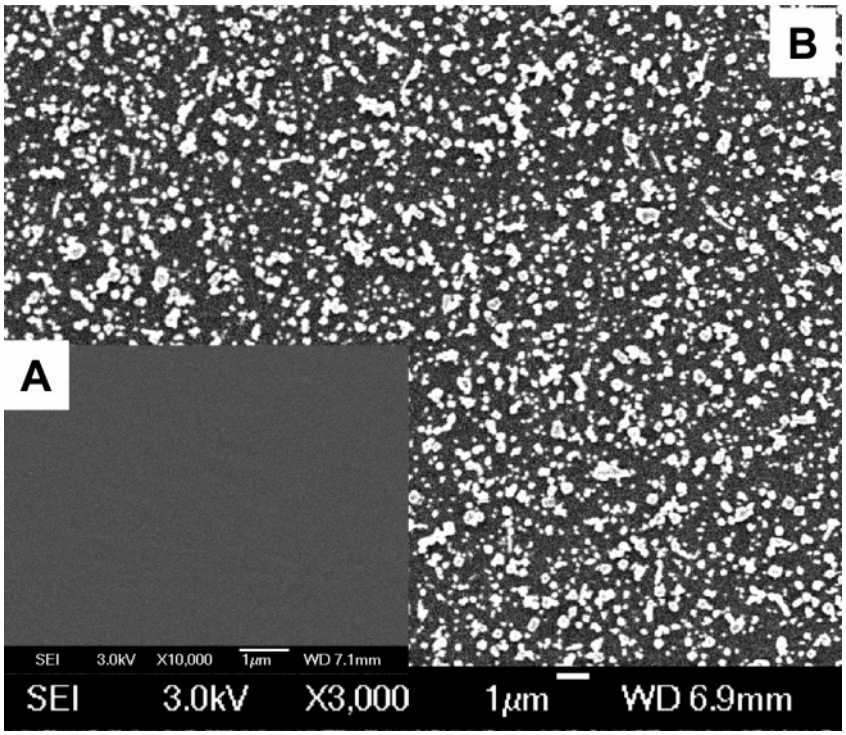

Figure 1. FE-SEM images of the bottom of a microtiter plate (A) before and (B) after colloidal silver staining.

and 100 μL of the diluted solution was then added to each well of the plate. After that, the plate was deposited hermetically for 2 h at room temperature. Finally, nonspecifically absorbed antibodies were washed by a washing buffer.

Colloidal Silver Staining. After the immunoreaction, 100 μL of colloidal silver was added to each well of the microtiter plate. Four hours after the deposition of the plate at room temperature, the colloidal silver was removed, and then the microtiter plate was washed by using a washing buffer followed by triply distilled water three times. Field emission scanning electron microscope (FE-SEM) images of the bottom of the microtiter plate before and after colloidal silver staining were measured with a JEOL JSM-6700F FE-SEM with an accelerating voltage of 3 kV.

SERRS Measurement. SERRS spectra of FITC on the bottom of a microtiter plate were measured with a Renishaw 1000 model confocal microscopy Raman spectrometer equipped with a CCD detector and a holographic notch filter. Radiation of 514.5 nm from an air-cooled argon ion laser was used for the SERS excitation with the power of 5 mW at the sample position. The microscope attachment based on a Leica DMLM system and a 50× objective was used to focus the laser beam onto a spot of approximately 1 μm in diameter. The typical accumulation time for each SERRS measurement in this study was 20 s.

RESULTS AND DISCUSSION

Scheme 1B depicts the main procedure of the proposed immunoassay. A multiwell microtiter plate is first coated with samples of unknown antigen concentration, and then a blocking buffer is used to block the area where there is no nonspecific antigen absorbing on the plate surface. After that, an FITC-labeled IgG solution is added for specific immunoreaction, and the bound and free antibodies are separated by a washing step to remove the nonspecifically absorbed molecules of antibodies. Finally, by colloidal silver staining, one can directly obtain a SERRS spectrum of the FITC probe on the bottom of the microtiter plate. By this way, unknown antigens can be analyzed qualitatively and quantitatively.

Colloidal Silver Staining. We prepared a turbid gray silver colloid following the method of Lee and Merisel,[29] and the plasmon absorption maximum of the silver colloid is located at 415 nm. Figure 1 shows FE-SEM images of the bottom of a microtiter plate before and after colloidal silver staining. Figure 1A shows a flat surface of the microtiter plate without any obvious particles, while one can observe the aggregates of silver nanoparticles with the diameter ranging from 200 to 400 nm in Figure 1B, which probably originate from the interactions between absorbed proteins and silver nanoparticles. It is well-known that salts and surfactants can induce the formation of aggregates of silver particles, which can make it possible to tune colloidal silver substrates for the maximum SERS signal intensity[30−33] Therefore, it is very likely that the salts and surfactants used in the washing buffer also induce the aggregation of the silver nanoparticles. Figure 1B also confirms that the formation of silver aggregates results in electromagnetic (EM) enhancement of Raman and resonance Raman scattering.

SERRS Detection. FITC (Scheme 1) is currently one of the most commonly used fluorescent dyes in many biotechnologies. It is a small organic molecule and is typically conjugated to a protein via primary amines (i.e., lysines). Because of its visible

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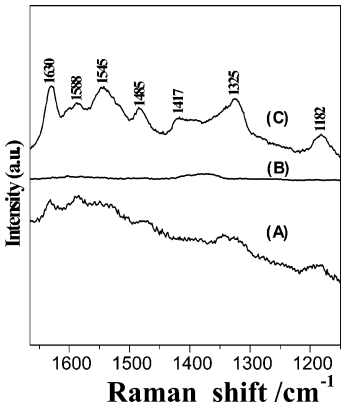

Figure 2. (A) SERRS spectrum of FITC−antihuman IgG on a silver colloid, (B) SERS spectrum of a protein mixture without immunoreaction on a microtiter plate, and (C) SERRS spectrum of FITC-labeled immunocomplex on a microtiter plate.

absorption maximum at about 490 nm, we can obtain SERRS spectra of FITC by using 514.5 nm excitation.

Before we applied FITC to the SERRS-based immunoassay, we measured two SERS spectra independently, to explore if there are any contributions from the microtiter plate and some reagents added to the present system to the SERRS spectrum of the FITC-labeled immunocomplex. Figure 2A shows a SERRS spectrum of FITC−human IgG, which is obtained from a mixture of a silver colloid and a 100-fold diluted solution of FITC−human IgG (volume ratio 1:1). Furthermore, we also measured the SERS spectrum of the protein mixture (8.2 ng/mL human IgG and 1% BSA) on a microtiter plate by the method depicted in Scheme 1, in which the step of immunoreaction is omitted (see Figure 2B). Figure 2C shows a SERRS spectrum of FITC-labeled immunocomplex (8.2 ng/mL human IgG) obtained from the whole present system shown in Scheme 1. From these spectra, one can see that there is nearly no obvious signal in the SERS spectrum of the protein mixture without immunoreaction, and that spectrum A in Figure 2 is very similar to spectrum C in Figure 2 despite some differences in SERRS intensities. Therefore, it is little doubt that the SERRS signals arise from FITC, which is linked to antihuman IgG, and that other reagents do not show any detectable SERS signals.

Figure 3 exhibits the dependence of the SERRS spectrum of the FITC-labeled immunocomplex on the concentration of the antigens. The antigen (human IgG) solutions with the different concentrations of 0.2, 2.2, 4.2, 6.2, and 8.2 ng/mL were examined by the method depicted in Scheme 1, and their SERS spectra are shown in Figure 3a−e. Note that the higher the concentration of the human IgG, the more intensive the SERRS signals. Therefore, one can determine the concentration of certain antigen indirectly by the intensity of a SERRS band of FITC. The detection limit of the present method is found to be as low as 0.2 ng/mL, which is comparable to that of ELISA.[34] Moreover, we repeated the same immunoassay for human IgG in human serum with different concentrations by the present method. The intensity of a peak at 1630 cm^{-1} versus the concentration of human IgG is plotted in Figure 4. We have found that for human serum, the average peak intensity at 1630 cm^{-1} is weaker than that for human IgG. It is

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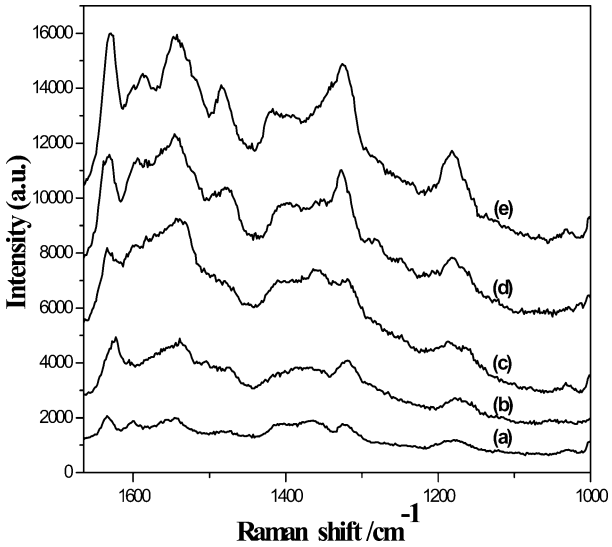

Figure 3. SERRS spectra of FITC-labeled immunocomplexes (the concentration of human IgG: (a) 0.2, (b) 2.2, (c) 4.2, (d) 6.2, and (e) 8.2 ng/mL).

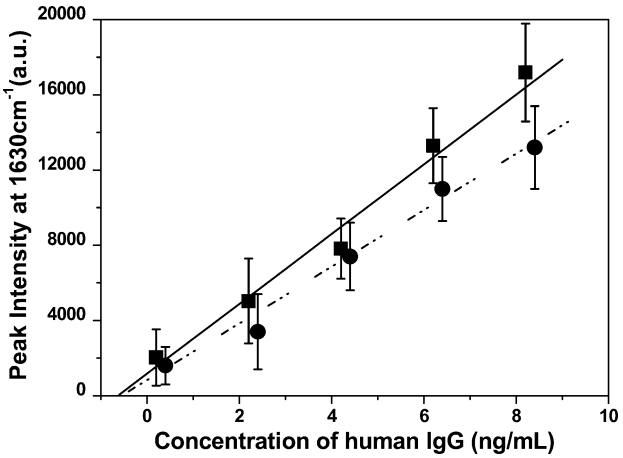

Figure 4. SERRS intensity of a peak at 1630 cm^{-1} from FITC-labeled immunocomplex vs the concentration of human IgG, detected in human IgG (■) and human serum (●). Each data point represents an average of 5−7 measurements (each error bar indicates the standard deviation).

very likely that other components of human serum affect the absorption of human IgG on the microtiter plate.

In comparison to fluorescence measurement of FITC, the present immunoassay holds advantages in sensitivity and stability. We cannot observe the fluorescence of FITC in the same immnuoassay without colloidal silver staining for the human IgG concentration ranging from 0.2 to 8.2 ng/mL. Figure 5 depicts the time-dependent SERRS intensity of the FITC-labeled immunocomplex. It is noted that the average SERRS intensity of FITC can remain stable over several days.

CONCLUSION

The proposed method for immunoassay, which combines the techniques of ELISA and SERS, has taken the advantages of the good solid supports of ELISA and the high sensibility of SERRS together. FITC, which is easily linked to proteins and commonly used as a fluorescent probe in many biochemical studies, was used as a SERRS probe in the present study. Moreover, colloidal silver

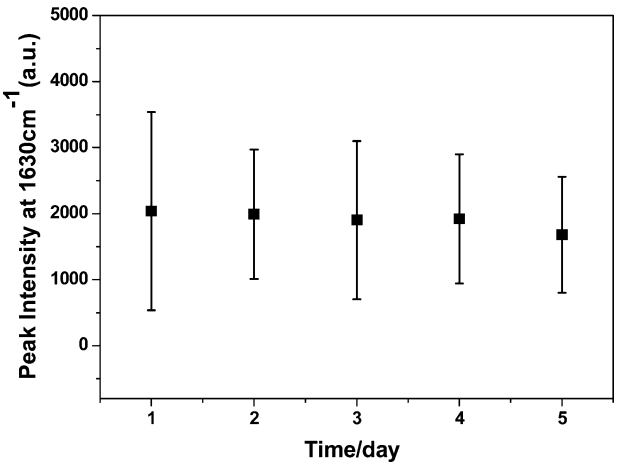

Figure 5. Time-dependent SERRS intensity of the FITC-labeled immunocomplex. The concentration of human IgG was 0.2 ng/mL. Each data point represents an average of 5−7 measurements (each error bar indicates the standard deviation).

staining was employed to offer a SERRS-active substrate for FITC, and the results have confirmed that the aggregates of silver nanoparticles significantly enhance resonance Raman signals of FITC. A good linear relationship was obtained between the intensity of a SERRS peak at 1630 cm^{-1} from FITC and the concentration of human IgG according to a calibration curve. The detection limit of the present method is found to be as low as 0.2 ng/mL. Therefore, on the bottom of a microtiter plate certain antigens can be detected quantitatively by the intensity of SERRS signals of FITC. All the results described in this paper show that FITC is not only a good fluorescent probe for fluorescent-based immunoassays but also a good SERRS probe for SERRS-based immunoassays, and this new SERRS-based method has great potential in high-throughout quantitative and qualitative immunoassays. Furthermore, even higher sensitivity might be expected if one could find a more proper fluorescent molecule and apply it to the system that we have presented.

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