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Multianalyte immunoassay based on surface-enhanced Raman spectroscopy

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In this paper, two immunoassay methods based on SERS are developed for multiplex analysis, both of which stemmed from the concept of forming a sandwich structure 'capture antibody substrate/antigen/Raman-reporter-labeled immuno-nanoparticles'. They are two-molecule labeled one-nanoparticle and one-molecule labeled two-nanoparticle methods. In both the methods, two different antibodies covalently bound to a solid substrate can specifically capture two different antigens from a sample. The captured antigens in turn bind selectively to their corresponding antibodies immobilized on Raman-reporter-labeled nanoparticles. Multianalyte immunoassay is successfully demonstrated by the detection of characteristic Raman bands of the probe molecules only when the antigen and antibody are matched. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: surface-enhanced Raman spectroscopy; multianalyte; immunoassay; $Ag_{core}Au_{shell}$ bimetallic nanoparticles

INTRODUCTION

Immunoassay is a fast, cost-effective, and reliable method for identifying and quantifying the presence of target compounds in samples.[1] The strong specific binding of an antibody to its antigen has been widely exploited in biochemical studies, clinical diagnostics, food safety, sensor design, and environmental monitoring etc. Simultaneous multianalyte immunoassay (SMIA), in which two or more analytes are measured simultaneously in a single assay, has been continuously developed with the growing demands for diagnostic, drug discovery, and biodefense applications.[2,3] Compared with parallel single-analyte immunoassay methods, a multianalyte immunoassay offers some remarkable advantages, such as high sample throughput, improved assay efficiency, low sample consumption, and reduced overall cost per assay.[4] In a typical SMIA progress, different antibodies are first immobilized on a solid substrate by methods, such as physical adsorption, covalent linkages, indirect anchors, or laser printing and stamping.[5] Then, a mixture of antigens is reacted with the multicomponent antibodies, captured on the substrate and immobilized on the substrate. Afterwards, the immobilized antigen is reacted and detected with the labeled antibodies. Detection has been routinely achieved using radiometric, enzymometric, gravimetric, amperometric, or fluorometric labels.[6] Among these, most efforts have focused on multicolor fluorescent detection (in connection with different organic dyes).[7] However, multicolor fluorescence labeled immunoassays often suffer from problems, such as narrow excitation bands (a need of multi excitation sources) and broad emission spectra (because of the spectral overlapping, the throughput in multiplex assay is determined by the spectral width of each emission spectrum). These two factors make the detection of multiple light emitting probes difficult.[8] Furthermore, the low resistance of the fluorescent labels to chemical degradation and photodegradation restricts its development. One should be aware of the rapid development using quantum dots as fluophors with broad excitation and narrow emission.[9,10]

We are exploring the concept of multianalyte immunoassay based on multiple Raman labels that can be detected by surface-enhanced Raman spectroscopy (SERS). In comparison with other spectroscopic methods, several characteristics of SERS warrant it to be developed further for practical application.[11–13] Firstly, SERS will not be interfered by the presence of water commonly existing in all

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biological samples. Secondly, Raman bands are generally 10–100 times narrower than most fluorescence bands, minimizing the possible overlapping of different labels in a given spectral region, which is especially advantageous for a multiplexed detection. The optimal excitation wavelength for SERS is not strongly dependent on the adsorbed molecule (for nonresonant molecules) but on the structure of nanoparticles, allowing the use of a single excitation source for multiple species. In addition, Raman scattering is not sensitive to humidity or affected by oxygen and other quenchers, facilitating its application in a variety of environments. SERS has been a useful tool for immunoassay, but most works were focused on the single-analyte immunoassay.[14–19] Ni *et al*.[13] demonstrated a dual-analyte assay, based on SERS for rat IgG and rabbit IgG detection by using thiophenol (TP) and 2-naphthalenethiol (NT) as probe molecules, but the nonspecific interactions, which appeared in their assay, probably result from the nonspecific adsorption and/or the cross-reactivity of polyclonal antibodies.

In this work, we made some efforts to choose a suitable substrate and monoclonal antibodies to minimize the nonspecific interactions and to optimize the strategies for the multianalyte immunoassay. Two strategies proposed are shown in Scheme 1. The common procedure for the two strategies are: mixed antibodies are immobilized on a solid substrate, which will then interact with the corresponding antigens in a sample containing antigens mixture to form a composite substrate; then, the immobilized antigens can capture Raman-reporter-labeled nanoparticles, modified with corresponding antibodies in a solution containing a mixture of reporter-labeled immuno-nanoparticles, forming a sandwich-type complex. The final complex was then detected by Raman spectroscopy. The difference in the two strategies is: in Strategy A, the Raman-reporter-labeled immuno-nanoparticles were typically performed by two kinds of Raman-reporter molecules and Au nanoparticles, whereas only one kind of molecule and two kinds of different nanoparticles ($Ag_{97}Au_3$ bimetallic nanoparticles and Au nanoparticles) were adopted in strategy B. The potential of using the two strategies for effective detection methods for multianalyte immunoassay is discussed.

EXPERIMENTAL

Apparatus

Raman spectra were obtained using a confocal microprobe Raman system (LabRam I, Dilor).[20] It is a single spectrograph instrument equipped with a holographic notch filter and a CCD detector. The sizes of slit and pinhole were 200 μm and 800 μm, respectively. A long working distance 50 × objective was used to collect the Raman scattering signal. The excitation wavelength was 632.8 nm from a He–Ne laser. Scanning electron microscopic (SEM) images were taken with a field-emission microscope (FE1530, Leo) operated at an accelerating voltage of 20 kV.

Reagents

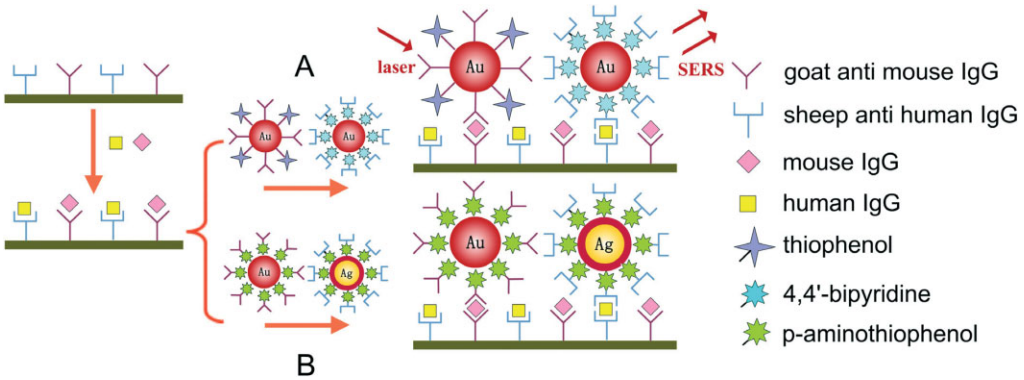
Goat antimouse IgG, sheep antihuman IgG, bovine serum albumin (BSA) and Tween 80 were purchased from Sino-American Biotechnology Co. Mouse IgG and human IgG were acquired from Sigma. *P*-aminothiophenol (PATP) was obtained from Lancaster. Hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$), trisodium citrate, tris(hydroxymethyl) aminomethane (Tris), and TP were purchased from Shanghai Reagents Company (Shanghai, China). Chloroauric acid ($HAuCl_4{\cdot}4H_2O$), 4,4′-bipyridine (BiPy), and silver nitrate ($AgNO_3$) were obtained from Sinopharm Chemical Reagent Company, Ltd. Ultrapure water (18 MΩ cm^{-1}) was used in all experiments.

Buffers

The following buffer solutions were used: borate buffer (BB, 2 mM, pH = 9), PBS buffer (KH_2PO_4/K_2HPO_4, 150 mM NaCl, pH = 7.6), TBS buffer (10 mM Tris, 150 mM NaCl, pH = 7–8), and TBS/0.1% Tween buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween, pH = 7–8).

Synthesis of nanoparticles

Au nanoparticles with a target diameter of 50 nm were synthesized according to literature procedures.[21] All glasswares



Scheme 1. Schematic illustration of two strategies for multianalyte immunoassay based on a sandwich structure concept.

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were cleaned rigorously and rinsed with water prior to use. In a 250 ml glass flask, 100 ml of 10^{-4} g/ml $HAuCl_4$ was brought to boiling under magnetic stirring. Upon boiling, 0.9 ml of 1% sodium citrate was rapidly injected and the resulting mixture was kept boiling for 15 min.

The procedure for preparing $Ag_{97}Au_3$ bimetallic nanoparticles has been reported previously,[18] i.e. Ag nanoparticles (average diameter 50 nm), synthesized by reducing silver nitrate with trisodium citrate[22] were coated with Au by simultaneously adding aqueous $HAuCl_4$ and $NH_2OH{\cdot}HCl$ in a neutral aqueous solution.[23] Briefly, 0.5 ml of 6.25×10^{-3} M $NH_2OH{\cdot}HCl$ and 0.5 ml 4.65×10^{-4} M $HAuCl_4$ were added dropwise (*ca* 2 ml/min) to a 12.5 ml of Ag colloid diluted with 10 ml of ultrapure water by two separate pipettes upon vigorous stirring. The stirring was continued for 45 min.

Preparation of Raman-reporter-labeled immuno-nanoparticles

Four kinds of Raman-reporter-labeled immuno-nanoparticles have been designed: TP/goat antimouse IgG-labeled immuno-Au nanoparticles, BiPy/sheep antihuman IgG-labeled immuno-Au nanoparticles, PATP/goat antimouse IgG-labeled immuno-Au nanoparticles, and PATP/sheep antihuman IgG-labeled immuno-$Ag_{97}Au_3$ bimetallic nanoparticles. The first two immuno-Au nanoparticles were designed for the immunoassay protocol A (Scheme 1), and the rest two PATP-labeled immuno-nanoparticles were designed for the protocol B (Scheme 1), based on the fact that the PATP molecule will present different spectral features when adsorbed on the $Ag_{97}Au_3$ bimetallic nanoparticles and Au nanoparticles. Raman-reporter-labeled immunoassay nanoparticles were prepared in a two-step process. In the first step, the uncoated colloids were labeled with Raman-reporters though the spontaneous adsorption of molecules on nanoparticles. TP,[24] BiPy[25,26] and PATP[27] were chosen as reporters because of good SERS signal from these molecules and a minimal overlapping of their spectral features. Typically, 1 µl of 1 mM probe molecule (TP or PATP) in ethanol was added to 1.0 ml of Au nanoparticles or $Ag_{97}Au_3$ bimetallic nanoparticles and the resultant mixture was allowed to react for 1h. In the case of BiPy, 0.5 µl of 0.5 mM BiPy was added to 1.0 ml of Au nanoparticles and the resultant mixture was allowed to react for 10 min. The reporter-labeled nanoparticles were then separated from the solution by centrifugation at 6000 g for 20 min and resuspended with 1.0 ml borate buffer.

The second step involved the immobilization of different IgG proteins on the reporter-labeled colloids. 20 µl of 1.0 mg/ml goat antimouse IgG was added to 1.0 ml of TP-labeled Au nanoparticles or PATP-labeled Au nanoparticles, 6 µl of 3.34 mg/ml sheep antihuman IgG was added to 1.0 ml BiPy-labeled Au nanoparticles or PATP-labeled $Ag_{97}Au_3$ bimetallic nanoparticles. After incubation at room temperature for 1 h, the Raman-reporter labeled immuno-nanoparticles were purified by centrifugation and resuspended with 1.0 ml of the borate buffer. To make sure that no bare sites were left, 10 µl of BSA (2% m/m) was added to the above Raman-reporter-labeled immunoassay nanoparticles. The mixture was incubated for 60 min at room temperature, and then centrifugated and resuspended in 1.0 ml borate buffer respective.

Preparation of capture antibody substrates for multianalyte immunoassay analysis

The substrates were microscopic glass slides coated with multiple layers of materials, as described below and were donated by Full Moon BioSystems. The slide surface was first coated with a buffer layer of Ni–Cr using a vacuum deposition process and then coated with a thin layer of silver. After activation, the surface was covered with a polymer layer, which contains specifically designed functional groups that can bind to the –COOH groups of antibodies. This particular binding arrangement allows antibodies to be erected on the surface without compromising their biological activities. The specific surface modification will reduce the nonspecific adsorption as found in Ref. 13.

The mixture of goat antimouse IgG (100 µg/ml in 0.1M borate buffer, pH 9) and sheep antihuman (100 µg/ml in 0.1M borate buffer, pH 9) was dropped onto the 1 cm^2 substrate. After placing in a chamber with a relative humidity of 65–75% for over 12 h, the substrates were allowed to dry at room temperature for 30 min. The substrates were then incubated in 5% BSA for 1 h to block active sites between antibodies, rinsed with water and dried under nitrogen.

Immunoassay protocol

The immunoassays were conducted following the typical procedure for a sandwich-type assay. Mouse IgG (100 µg/ml), human IgG (100 µg/ml), and a 1:1 mixture of mouse IgG (50 µg/ml) and human IgG (50 µg/ml) were used as test antigens. In each case, the capture antibody-coated substrate was immersed in the above solution. After gently shaking the tube for about 2 to 4 h on a shaker at room temperature, the substrates were taken out and washed three times with TBS/0.1% Tween at room temperature and then washed two times with TBS. After rinsing with copious amounts of water, the substrates were placed in a tube containing Raman-reporter-labeled Immuno-nanoparticles. The tube was gently shaken at room temperature for 2 h, and then all the samples were rinsed with TBS/0.1% Tween, TBS, deionized water successively and then dried under nitrogen gas.

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RESULTS AND DISCUSSION

Multianalyte immunoassay using one type of nanoparticles and two reporter molecules

In our assay, TP and BiPy were adopted as probe molecules for the following reasons. First, the characteristic bands of two molecules are well separated. Second, the two molecules have relative large Raman cross sections and can provide high detection sensitivity. In a typical process, TP and BiPy were first adsorbed separately on the gold nanoparticles to form Raman-reporter molecules. Then, goat antimouse IgG and sheep antihuman IgG were added to the TP-labeled Au nanoparticles and BiPy-labeled Au nanoparticles, respectively. Finally, the two Raman-reporter-labeled immuno-Au nanoparticles were mixed in equal amounts for the following immunoassay analysis experiments. The SER spectra of such Raman-reporter-labeled immuno-Au nanoparticles are shown in Fig. 1. Figure 1(a) shows the SERS spectrum of BiPy/sheep antihuman IgG-labeled immuno-Au nanoparticles. The bands at 1610, 1510, 1294, 1229, 1071 and 1019 cm^{-1} are similar to those reported in the literature.[25] Similarly, the main SERS bands of TP/goat antimouse IgG-labeled immuno-Au nanoparticles (Fig. 1(b)) at 1570, 1070, 1018 and 994 cm^{-1} are in accord with the main bands of TP molecule.[24] Fig. 1(c) shows the SERS spectrum of the 1 : 1 mixture of BiPy/sheep antihuman IgG-labeled immuno-Au nanoparticles and TP/goat antimouse IgG-labeled immuno-Au nanoparticles. As can be seen from the SER spectra, the bands of both reporters did not interfere with each other when the two immuno-Au nanoparticles were mixed. The result demonstrates that the location of the vibrational bands of the TP and BiPy is well suitable for a multianalyte immunoassay, which requires minimal spectral overlapping between different labels. On the other hand, it should be noted that the SERS signal of BiPy molecule is stronger than that of TP. Therefore, in order to obtain comparable signal from two cases, the labeling time for BiPy case should be shorter and the concentration lower.

The immunoreaction was conducted by using the procedure for the sandwich-type and the selective antibody–antigen interaction can be confirmed by the appearance the SERS spectral feature of the Raman-reporter. The substrates coated with a mixed layer of goat antimouse IgG and sheep antihuman IgG were used to capture antigens from solutions. BSA was used to block the nonspecific binding site. After the blocking step, the substrates were exposed to solutions of mouse IgG (100 μg/ml), human IgG (100 μg/ml), or a mixture of mouse IgG (50 μg/ml) and human IgG (50 μg/ml). After incubation for several hours, the substrates were developed in the preformed Raman-reporter-labeled immuno-Au nanoparticles. If the corresponding antigen was captured, a sandwiched structure 'capture antibody substrate/antigen/Raman-reporter-labeled immuno-Au nanoparticles' will be constructed, and the nanoparticles linked with antibodies and Raman-reporter molecules will be assembled on the capture antibody substrates and can not be washed away by routine washing process.

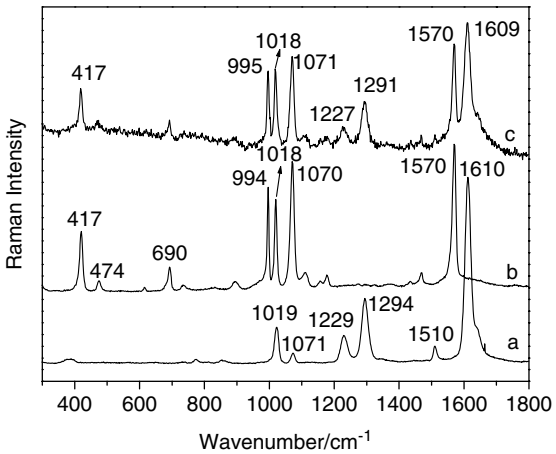

Figure 1. SER spectra of Raman-reporter-labeled immuno-Au nanoparticles: (a) BiPy/Sheep antihuman IgG-labeled immuno-Au nanoparticles; (b) TP/goat antimouse IgG-labeled immuno-Au nanoparticles; (c) the mixture of (a) and (b). The acquisition time is 50 s.

Figure 2(a) shows the spectrum from the substrate exposed to mouse IgG and the mixture of the TP/goat antimouse IgG-labeled immuno-Au nanoparticles and BiPy/sheep antihuman IgG-labeled immuno-Au nanoparticles. This sample should capture only the colloids labeled with goat antimouse IgG, so the spectral feature of only one molecule should be present. The presence of the strong bands at 994 and 1570 cm^{-1} for TP is consistent with this expectation. Similarly, only bands diagnostic of BiPy (Fig. 2(b)) should be found for the sample exposed to human IgG. The presence of the strong bands at 1609, 1227 and 1291 cm^{-1} agrees with this prediction. In comparison, when equal amounts of mouse IgG and human IgG are exposed to the capture antibodies substrates, both the TP bands at 994 and 1570 cm^{-1} and BiPy bands at 1610, 1227 and 1291 cm^{-1} appear in the spectrum (Fig. 2(c)). This finding indicates that both TP/goat antimouse IgG-labeled immuno-Au nanoparticles and BiPy/sheep antihuman IgG-labeled immuno-Au nanoparticles have bound to the substrates, demonstrating that the two analytes can be simultaneously detected using easily distinguishable Raman scatters as labels.

Multianalyte immunoassay using one molecule and two types of nanoparticles

Although the above mentioned strategy has some advantages, such as minimum overlapping of Raman-reporters and simultaneous detection of different analytes, there is one severe limitation, that is, it is difficult to find a suitable molecule with comparable SERS intensity with good spectral separation. For example, different molecules have

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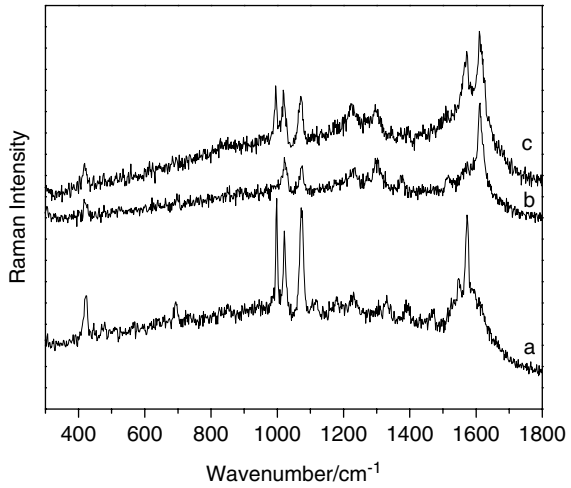

Figure 2. Sandwich immunoassay demonstration of dual-analyte assay based on SERS using strategy A. Gold nanoparticles conjugated with TP/goat antimouse IgG and gold nanoaprticles conjugated with BiPy/sheep antihuman IgG were mixed in a 1 : 1 ratio and used as reporter-labeled immunogold colloids. The substrates, modified with the mixture of goat antimouse IgG and sheep antihuman IgG, were used as capture antibody substrates. SERS spectra of substrates exposed to different samples: (a) mouse IgG (100 μg/ml), (b) human IgG (100 μg/ml), (c) a mixture of mouse IgG (50 μg/ml) and human IgG (50 μg/ml).

different Raman scattering sections, and may present different intensity, especially in SERS. To overcome this problem, one has to adjust the labeling time or solution concentration to achieve a similar SERS intensity. In our experiment, the label time for TP molecule (1 mM) on Au nanoparticles is 1 h, but 10 min for BiPy (0.5 mM) to achieve comparable signal intensity. The difference in the preparing time will make the process more complicated and it will be hard to keep the two molecules at the same intensity in every assay. Core shell nanoparticles have unique catalytic, electronic and optical properties distinct from those of the corresponding monometallic particles.[28,29] Recently, we have demonstrated that $Ag_{core}Au_{shell}$ bimetallic nanoparticles can be used as a new SERS-active and biocompatible substrate for SERS immunoassay.[18] In our SERS study of PATP adsorbed on the $Ag_{97}Au_3$ bimetallic nanoparticles and Au nanoparticles, we found that PATP gives a different spectral feature on the two nanoparticles, but presents almost the same intensity when they are adsorbed on the two nanoparticles with the same labeling time. We considered using $Ag_{97}Au_3$ bimetallic nanoparticles and Au nanoparticles labeled with PATP to overcome the limitation of different detection sensitivity in the two-label approach.

The SERS spectra of the PATP adsorbed on the gold nanoparticles and $Ag_{97}Au_3$ bimetallic nanoparticles are shown in Fig. 3. The spectrum obtained on the $Ag_{97}Au_3$ bimetallic nanoparticles (Fig. 3(a)) is similar to that observed by Osawa *et al*.[27] on an electrochemically roughened silver electrode with 514 nm excitation. The detected bands can be classified into two sets: one set at 1004, 1077, 1176 and 1578 cm^{-1}, which belongs to a_1 modes of the PATP molecule, and the other set at 1147, 1393 and 1438 cm^{-1}, which are assigned to b_2 vibrational modes of the PATP molecules. When PATP was adsorbed on the Au nanoparticles (Fig. 3(b)), only three major bands at 1578, 1076 and 390 cm^{-1} that are assigned to a_1 vibrational modes of PATP molecules were observed. The apparent enhancement of b_2 modes has been interpreted by Osawa *et al*.[27] using the photon-induced charge transfer from metal to an affinity level of the adsorbed molecule and the four b_2 modes gain their intensity via a Herzberg-Teller contribution. The different feature of PATP adsorbed on $Ag_{97}Au_3$ and Au nanoparticles might also be explained similarly. The fact that one molecule adsorbed on two different nanoparticles can exhibit different bands but presents almost the same intensity affords a new concept for dual-analyte assay.

The PATP-labeled Au nanoparticles and $Ag_{97}Au_3$ bimetallic nanoparticles have been used for the dual-analyte immunoassay. Figure 4(a) shows the spectrum from the substrates exposed to mouse IgG and the mixture of PATP/goat antimouse IgG-labeled immuno-Au nanoparticles and PATP/sheep antihuman IgG-labeled immuno-$Ag_{97}Au_3$ nanoparticles. This sample should capture only the colloids labeled with goat antimouse IgG, so a spectrum containing only the features in Fig. 3(b) should be obtained. The presence of the strong bands at 390, 1077 and 1578 cm^{-1} for PATP is consistent with this expectation. Similarly, the bands diagnostic of PATP adsorbed on the $Ag_{97}Au_3$ nanoparticles (Fig. 4(b)) should be found for the sample exposed to human IgG. The presence of the strong bands at 1147, 1393, 1438 and 1578 cm^{-1} agrees with this prediction. This finding indicates

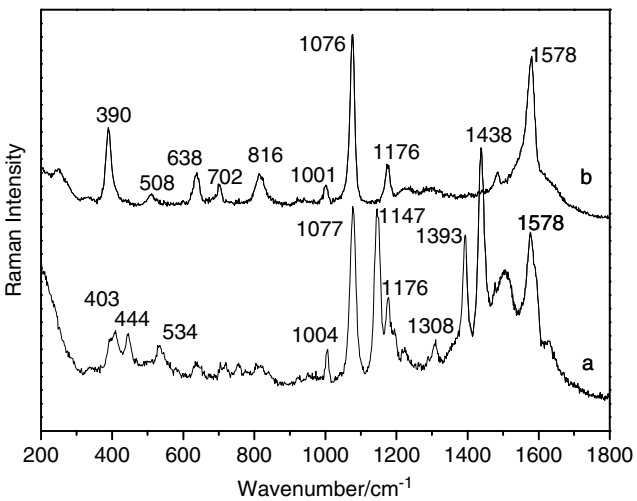

Figure 3. SERS spectra of PATP adsorbed on the $Ag_{97}Au_3$ bimetallic nanoparticles (a), and Au nanoparticles (b). The acquisition time is 50 s.

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that using one molecule and two kinds of nanoparticles can also be successfully applied to immunoassay.

This novel single labeling approach offers a simple way for multianalyte immunoassay detection. However, a drawback of this single-probe detection method is the limitation for the choice of probe molecules. Usually, molecules that can present two different features with large separation on different nanoparticles are very seldom. Furthermore, when the two samples appear in the solution at the same time, overlapping of the bands make it difficult to discriminate the detection events. Considering the assay of strategy B as example, if the capture antibodies substrates were exposed to the mixture of mouse IgG and human IgG, the Au nanoparticles labeled with PATP/goat antimouse IgG and $Ag_{97}Au_3$ bimetallic nanoparticles labeled with PATP/sheep antihuman IgG should be captured, and the SERS spectra of PATP adsorbed on the Au nanoparticles and adsorbed on the $Ag_{93}Au_7$ bimetallic nanoparticles should be detected at the same time. However, the overlapping of the characteristic bands of PATP adsorbed on the Au nanoparticles and bands of PATP adsorbed on the $Ag_{97}Au_3$ bimetallic nanoparticles made it difficult to distinguish whether the analytes contained human IgG or the mixture of human IgG and mouse IgG. Although there exist some disadvantages, we still believe this single labeling method will exploit a new concept for multianalyte immunoassay, and the limitation of overlapping of the bands will be overcome by the quantitative analysis intensity of the bands.

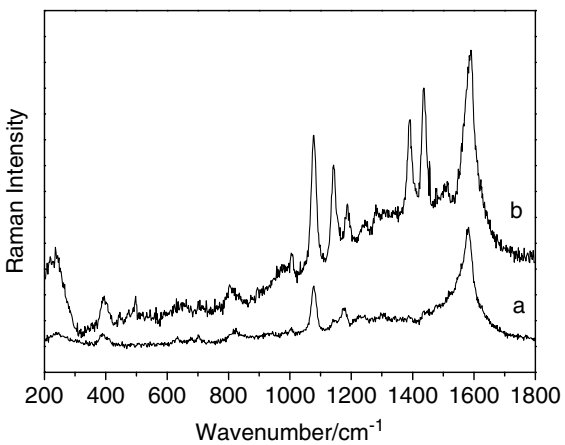

Figure 4. Sandwich immunoassay demonstration of dual-analyte assay based on SERS using strategy B. Gold nanoparticles conjugated with PATP/goat antimouse IgG and $Ag_{core}Au_{shell}$ nanoaprticles conjugated with PATP/sheep antihuman IgG were mixed in a 1 : 1 ratio and used as reporter-labeled immuno-colloids. The substrates modified with the mixture of goat antimouse IgG and sheep antihuman IgG were used as capture antibody substrates. SERS spectra of substrates exposed to different samples: (a) mouse IgG (100 μg/ml), (b) human IgG (100 μg/ml).

CONCLUSION

Two methods have been proposed for multianalyte immunoassay based on SERS, which are based on the two kinds of probe molecules immobilized on one type of nanoparticles and one type of probe molecules immobilized on two types of nanoparticles, respectively. Both methods have been found to work successfully for simultaneous dual-analyte assay, with well separated Raman bands. The nonspecific adsorption has been markedly suppressed by the functionalized substrate with suitable binding group with the antibody. The advantage of one-nanoparticle two-molecule method is the wide choice of probe molecules, however, it may suffer from different intensity response. The advantage of two-nanoparticles and one-molecule method is the comparable SERS intensity from two systems. One molecule can seldom present more than two types spectral feature with acceptable separation even on different substrates limits to some extent the selection of molecules. Although some problems still exist, this study points promising future of using SERS for multianalyte immunoassay.

Acknowledgements

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