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Detection of Alkaline Phosphatase Using Surface-Enhanced Raman Spectroscopy

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A new approach was developed to detect the activity of alkaline phosphatase (ALP) enzyme at ultralow concentrations using a surface-enhanced Raman scattering (SERS) technique. The approach is based on the use of gold nanoparticles as a SERS material whereas 5-bromo-4-chloro-3-indolyl phosphate (BCIP) is used as a substrate of ALP. The enzymatic hydrolysis of BCIP led to the formation of indigo dye derivatives, which were found to be highly SERS active. For the first time, we were able to detect ALP at a concentration of $\sim 4 \times 10^{-15}$ M or at single-molecule levels when ALP was incubated with BCIP for 1 h in the Tris-HCl buffer. The same technique also was successfully employed to detect surface-immobilized avidin, and a detection limit of 10 ng/mL was achieved. This new technique allows the detection of both free and labeled ALP as a Raman probe in enzyme immunoassays, immunoblotting, and DNA hybridization assays at ultralow concentrations.

Alkaline phosphatase (ALP) is one of the most commonly used biomarkers in enzyme immunoassays, gene assays, histochemical staining, and related affinity sensing methods for monitoring proteins, nucleic acids, drugs, enzymes, and other analytes.[1−5] ALP is also an enzyme found in human serum and assayed in routine clinical analysis, because ALP is an indicator of hepatobiliary and bone disorder.[6] Therefore, needs exist to detect ALP sensitively and selectively in many diagnostic and clinical assays. Current monitoring techniques for ALP commonly use an enzymatic substrate of ALP, and upon reactions with ALP, products of the substrate or its derivatives give strong chemiluminescent,[7] colorimetric,[8] fluorometric,[9] or electrochemical signals.[10,11] The same technique is also used to quantify levels of ALP present in sample solutions or on various substrate surfaces.

Raman spectroscopy has been an invaluable technique in studies of various chemical and biological systems and has become widely accepted as an analytical characterization methodology.[12] Raman spectroscopy can give narrow characteristic bands and the detailed fingerprint of the target molecule in a mixed sample without tedious separation steps. Furthermore, Raman spectra can be collected directly in aqueous solution because of a weak Raman scattering from water molecules. On the other hand, normal Raman spectroscopy is also known to be insensitive because of its inherently small Raman scattering cross sections exhibited by most molecules.[13] In recent years, however, surface-enhanced Raman scattering (SERS) has overcome the weakness of insensitivity for normal Raman spectroscopy and become one of the most sensitive techniques capable of detecting single molecules or single nanoparticles.[14] For example, SERS has been used extensively in studies of signal transduction mechanisms in biological and chemical sensing applications.[15−18] One of these applications takes advantage of the binding properties of antibody and antigen molecules (or DNA strands) and uses metal nanoparticles coated with Raman-active chromophores as tags to detect antigen molecules or genes.[19−23] SERS also has been used to detect the activity of hydrolases at ultralow concentrations.[24]

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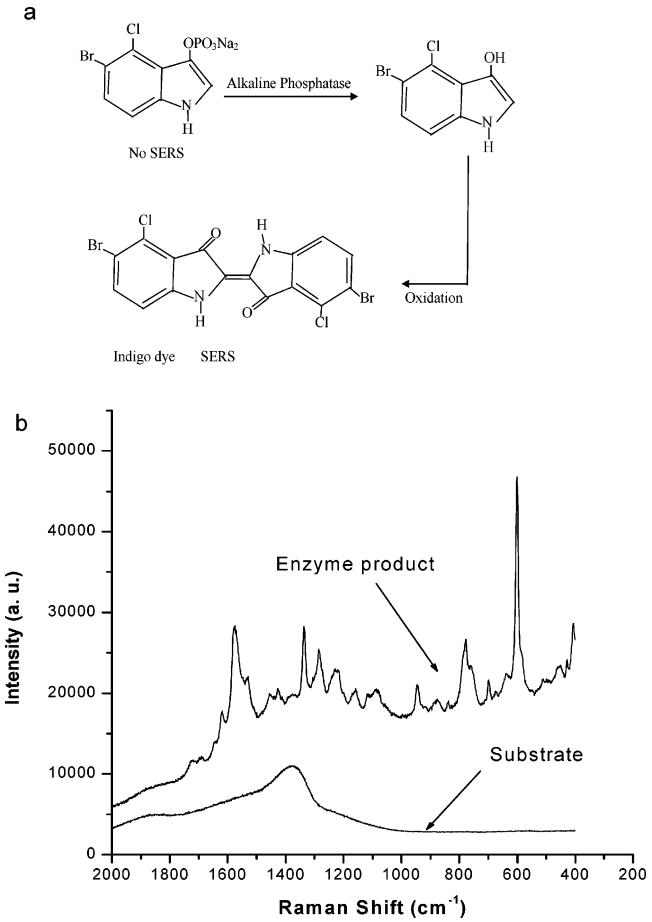

Figure 1. (a) Structure of 5-bromo-4-chloro-3-indolyl phosphate and its enzymatic reaction products, BCIP dimer; (b) corresponding SERS spectra of BCIP (substrate) at 0.3 mg/mL and its reaction products. ALP concentration 10^{-10} M, reaction time 1 h. Laser 785 nm, ~1.5 mW. Acquisition time 10 s.

Similar techniques have been used in enzyme immunoassays or in the detection of an antigen−antibody complex by SERS without using chromophore-tagged secondary antibodies.[25,26] However, despite these technological advances, relatively few studies have examined the use of SERS in immunoassays and DNA hybridization assays, perhaps because of the requirements of high detection sensitivity and reproducibility. Few studies have achieved a sensitivity of better than 10^{-14} M for Raman tags using the SERS technique. As such, detections by fluorescence, chemiluminescence, and electrochemical and UV−visible absorption signals are among the most widely used diagnostic techniques in enzyme-linked immunosorbent assays based on enzyme amplification reactions.

In this work, we report the development of a SERS-based assay technique that allows rapid and ultrasensitive analysis of ALP at concentrations as low as $\sim 4 \times 10^{-15}$ M using gold nanoparticles as a SERS substrate. For the first time, we demonstrate that 5-bromo-4-chloro-3-indolyl phosphate (BCIP), a substrate of ALP, becomes highly SERS active upon exposure to active alkaline phosphatase. Exposure of BCIP to ALP induces the hydrolysis of the phosphate moiety of BCIP and generates an enol intermediate compound, which is subsequently oxidized in air to insoluble blue BCIP dimers (Figure 1a). The BCIP dimer is a member of the class of indigo dyes, and its coupling with nitro blue tetrazolium, a color enhancer, has been widely exploited to locate enzymatic activity on cell walls, tissues, membranes, and chromatographic gels because it gives the intense color in the vicinity of the enzyme.[27−30] In comparison with these conventional assay techniques, SERS enables the detection of BCIP dimer or ALP activity directly at ultralow concentrations. Furthermore, a system based on the biological binding reaction of avidin/ALP−biotin is demonstrated for detecting immobilized biomolecules on the glass surface in a way similar to those of membrane-based immunoblotting protocols.

EXPERIMENTAL SECTION

Chemicals and Biochemicals. Alkaline phosphatase (5.8 mg/mL) from bovine intestinal mucosa was purchased from Sigma (No. P5521) in a solution containing 3.2 M $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$. Gold(III) chloride trihydrate, BCIP, trisodium citrate (98%), sodium borohydride (98%), (3-aminopropyl)triethoxysilane (99%), and other reagent chemicals and solvents were purchased from Aldrich (St. Louis, MO). Sodium phosphate dibasic (GR) and sodium phosphate monohydrate (GR) were bought from EM Science (Cherry Hill, NJ). Bovine serum albumin, glutaric dialdehyde (25%), tris(hydroxymethyl)aminomethane, and hydrogen peroxide (30%) were purchased from J. T. Baker (Phillipsburg, N. J.). Alkaline phosphatase-conjugated biotin (1 mg) and unconjugated avidin (10 mg) were purchased from Rockland Immunochemicals (Gilbertsville, PA).

Solutions and Buffers. Tris-HCl (0.05 M, pH 9.8) buffer was prepared by dissolving tris(hydroxymethyl)aminomethane in deionized water. Phosphate buffer (PBS; 0.01 M, pH 7.2, 0.15 M NaCl) was prepared by dissolving an equal amount of sodium phosphate monohydrate and sodium phosphate dibasic in deionized water. The pH values of buffer solutions were adjusted using dilute NaOH or HCl.

Preparation of Au Colloids. Colloidal Au nanoparticles were prepared according to previously published methods[31,32] with minor modifications. Briefly, all glassware used in the following procedures was cleaned in a bath of freshly prepared 3:1 HCl (36.5%)/HNO_3 (69%) and rinsed thoroughly in water prior to use. Au "seed colloids" suspension was prepared by mixing 1 mL of 1% aqueous $HAuCl_4 \cdot 3H_2O$ with 100 mL of H_2O under vigorous stirring, and at 1 min apart, 1 mL of 1% trisodium citrate and 1 mL of 0.075% $NaBH_4$ in 1% trisodium citrate were added. The reaction was allowed to continue for an additional 5 min to complete, and the solution (with Au seed colloids) was stored at 4 °C until use. Au nanoparticles of ~54-nm diameter were then prepared by refluxing 4 mL of 1% $HAuCl_4 \cdot 3H_2O$ in 900 mL of H_2O, followed by the addition of 1 mL of the above "seed colloid"

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solution and 4 mL of a 1% trisodium citrate solution. The mixture was refluxed for 10 min at boiling temperature and allowed to cool with continuous stirring.[31] The synthesized Au colloids were concentrated by centrifugation at 17 000 rcf for 10 min, and the volume was reduced to ~2 mL prior to use. The size and size distribution of Au nanocolloids were determined by means of dynamic light scattering using a ZetaPlus particle-size analyzer (Brookhaven Instruments Corp., Holtsville, NY). Data were collected at room temperature for every batch of Au colloids.

ALP Enzyme Assays. Stock solution of ALP (4.1×10^{-5} M) was diluted with Tris-HCl buffer solution (pH 9.8) to give concentrations ranging from 4.1×10^{-10} to 4.1×10^{-15} M. Solutions of 0.05 M $MgCl_2$ (100 μL) and BCIP (100 μL at 3 mg/mL) were subsequently added into 800 μL of ALP solutions, respectively. The mixture was incubated at room temperature for 1 h, followed by the addition of 50 μL of 1 M H_3PO_4 solution to each vial to stop enzymatic reaction and to adjust the pH of the solution. Finally, 100 μL of concentrated Au colloids (~2.5×10^{-3} M) was added, and 10 μL of each resulting solution was transferred onto glass slides for SERS measurements.

ALP as a Biomarker for Avidin Assays. Small glass tubes (8 mm diameter × 7.5 mm long) were first thoroughly washed following procedures reported by Zheng et al.[33] The washed tubes were silanized by immersing them into 100 mL of methanol solution containing 1% (3-aminopropyl)triethoxysilane and 0.1 mL of 0.01 M HCl for overnight at room temperature. They were then immersed into 25% glutaric dialdehyde solution for 1 h to obtain aldehyde surface functionalized tubes, which were necessary to assay avidin sorbed on glass surfaces. Avidin was diluted with PBS buffer to concentrations ranging from 100 μg/mL to 1 ng/mL, and deionized water was used as a control. One milliliter of each avidin solution was then added into aldehyde functionalized tubes and allowed to react for 2 h at room temperature. Subsequently, 1 mL of 3% bovine albumin was added into each tube to react for an additional 2 h to block free aldehyde functional groups on glass surfaces, and the tubes were washed thoroughly with water to remove excess albumin. To assay adsorbed avidin on glass surfaces at varying concentrations, 100 μL of ALP-conjugated biotin solution (1:1000 dilutions) was added into each tube and allowed to react with adsorbed avidin on glass for 1 h. Excess amounts of ALP-conjugated biotin were washed off thoroughly with water. Finally, a Tris-HCl buffer solution (pH 9.8) containing 0.3 mg/mL BCIP and 0.005 M $MgCl_2$ was added to each tube and allowed to react with ALP for 30 min. The reaction products were assayed similarly as described earlier.

Surface-Enhanced Raman Scattering Measurements. Raman spectra were obtained through a Renishaw micro-Raman system equipped with a 300-mW near-infrared diode laser at a wavelength of 785 nm for excitation (Renishaw Inc., New Mills, U.K.). The laser beam was set in position through a Leica Imaging Microscope objective (50×) at a lateral spatial resolution of ~2 μm on the sample. A charge-coupled device array detector was used to achieve signal detection from a 1200 grooves/mm grating light path controlled by Renishaw WiRE software and analyzed by Galactic GRAMS software. An intensity of <1% of the laser power was used at the exit of the 50× objective. For enzyme assays, 10 μL of above solutions with varying concentrations of ALP was transferred onto the glass slides, and samples were dried in the air for 30 min before SERS analysis.

Table 1. Assignments of Selected Raman Vibrational Bands for Enzyme Reaction Product (BCIP Dimer)

vibrational frequency, cm^{-1}	band assignment[a]
1620	ν (C=C), δ (C−H)
1576	ν (C=C), ν (C=O)
1336	δ (N−H), δ (C−H)
1285	δ (C−C)
1225	δ (C−H)
1158	δ (C−C), ν (C−C) ring
1087	γ (C−H)
945	γ (C−H), δ (C−C) ring
778	δ (C−H), δ (C−N−C)
699	δ (C−C)
600	δ (C=C−CO−C)

[a] Key: ν, stretching; δ, in-plane rocking or scissoring; γ, out-of-plane wagging or twisting.

RESULTS AND DISCUSSION

SERS Spectra of Substrate and Enzymatic Reaction Products. SERS spectra of both BCIP and its enzymatic reaction product, dimer of BCIP, were collected first in order to examine the feasibility and potential interferences in detecting ALP using BCIP as a substrate (Figure 1b). Results indicate that BCIP substrate itself was not SERS active or gave no discernible SERS signal except a broad peak at ~1400 cm^{-1}, which is assigned to the background scattering from glass slides. This feature ensured that the BCIP substrate itself was unable to complex with Au nanoparticle surfaces and thus made it "invisible" to SERS. Such a low background signal is essential for the development of an assay technique for sensitive detection of ALP.

On the other hand, BCIP dimers, the enzymatic reaction products, generated an intense SERS spectrum with the vibrational fingerprint of the molecule clearly observable. The strong vibrational bands of enzymatic reaction product adsorbed onto Au colloids were observed at 600, 699, 778, 945, 1087, 1158, 1225, 1285, 1336, 1529, 1576, and 1619 cm^{-1} (Figure 1b). This SERS spectrum of BCIP dimers on Au colloids is similar to the Raman spectra of indigo dyes reported previously, except for the enhanced Raman shift at 600 cm^{-1}.[34,35] It confirms that indigo dye derivatives were formed during the enzymatic reaction. The assignments of selected Raman bands for BCIP dimers are listed in Table 1.[34,36−38] These assignments of Raman shifts were referenced from Raman spectra of indigo dye derivatives in the literature because no SERS spectra of BCIP dimers are available. The strongest Raman shift at 600 cm^{-1} was assigned to the C=C−CO−C bending vibration.[34] The fact that this band was significantly enhanced in the presence of Au colloids may be

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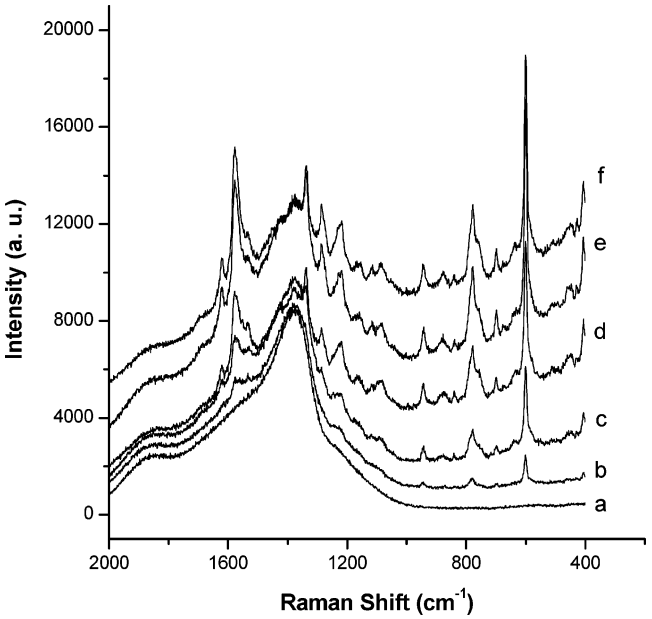

Figure 2. Time-dependent SERS spectra of alkaline phosphatase-catalyzed hydrolysis of BCIP. The initial BCIP concentration was 0.4 mg/mL. ALP enzyme was at 4.1×10^{-10} M. (a) Background without ALP; (b) 1, (c) 5, (d) 10, (e) 30, and (f) 45 min. Laser 785 nm, ~1.5 mW. Acquisition time 10 s.

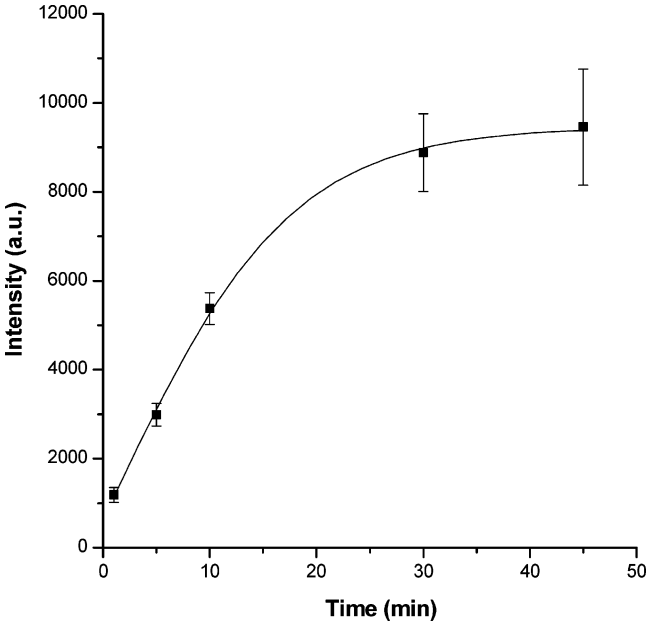

Figure 3. Peak intensity of the Raman band at 600 cm^{-1} as a function of incubation time. The initial BCIP concentration was 0.4 mg/mL. ALP enzyme was at 4.1×10^{-10} M. Each data point represents an average of 5−7 measurements (error bar is the standard deviation).

attributed to its adsorption onto Au colloids. These observations demonstrate that BCIP dimer released from the enzymatic reaction is SERS active, and the Raman shift at 600 cm^{-1} can be used as a characteristic peak to quantify ALP and the dynamics of these enzymatic reactions.

Quantification of ALP. *Kinetics of Enzymatic Hydrolysis of BCIP.* The above technique was subsequently used to detect the kinetics of enzymatic hydrolysis of BCIP. Experiments were performed at a fixed concentration of ALP (4.1×10^{-10} M) and BCIP (0.4 mg/mL) in 1 mL of Tris-HCl buffer solution containing 0.005 M $MgCl_2$ at room temperature. At different time intervals, SERS spectra of samples were recorded after mixing with 50 μL of 1 M H_3PO_4 and 100 μL of Au colloids. Results (Figure 2) showed a consistently increased intensity of SERS spectra with time, which is indicative of efficient enzymatic turnover of the substrate leading to a continuous buildup in the concentration of indigo dye. After 1-min reaction, the reaction product was already detectable by measuring the Raman band at 600 cm^{-1}. The peak intensity and area of this Raman band increased consistently, and detailed vibrational fingerprints of indigo dye molecules became clearer with an increased reaction time. A plot of the peak intensity at 600 cm^{-1} as a function of time (Figure 3) indicated a first-order or exponential decay of BCIP substrate ($R^2 = 0.98$). This observation is in agreement with that of Ashrafi et al., who reported the initial zero-order reaction kinetics of ALP with *p*-nitrophenyl phosphate disodium as an enzymatic substrate within the first 25 min.[39] No significant changes in Raman intensities were observed after ~30 min of the reaction (Figure 3), suggesting that the maximum coverage of indigo dyes on Au nanoparticles could have been achieved under the given experimental conditions. On the other hand, no Raman bands were observed in the absence of either ALP or BCIP even at an incubation time of 45 min (controls), indicating that the nonenzymatic hydrolysis of BCIP is negligible, and the enzymatic reaction is the sole route for observed SERS signal of indigo dyes.

ALP Assay. Quantification of ALP in samples was carried out by recording SERS spectra of ALP at different concentrations ranging from 4.1×10^{-10} to 4.1×10^{-15} M after incubation with BCIP in pH 9.8 Tris-HCl buffer containing 0.005 M $MgCl_2$ for 1 h (Figure 4). Again, intensities of the characteristic Raman band at 600 cm^{-1} were used to quantify the concentrations of ALP in samples, and a linear relationship was observed (Figure 5). Note that each data point in Figure 5 represents an average of five to seven measurements across the SERS specimen. Variations in signal intensities of different sample spots were typically <20%, shown as error bars in the plot. This is in contrast to previous findings that SERS detection usually relies on a few "hot spots",[40,41] and we found that this methodology is quite reproducible.

On average, the SERS intensities increased as the enzyme concentration increased, suggesting that more indigo dye was produced in the presence of higher concentrations of ALP enzyme. This linear relationship holds over 5 orders of magnitude concentrations of ALP (from 4.1×10^{-11} to 4.1×10^{-15} M). However, the relationship became nonlinear at higher concentrations of enzyme, which may be attributed to limited surface coverage of indigo dyes on Au colloids. Because of low background interferences, the characteristic Raman band at 600 cm^{-1} could be clearly discerned even at an ALP concentration of 4.1×10^{-15} M (after incubation with BCIP for 1 h). This concentration is more than three times the standard deviation of background noise and can thus be regarded as the detection limit of free ALP

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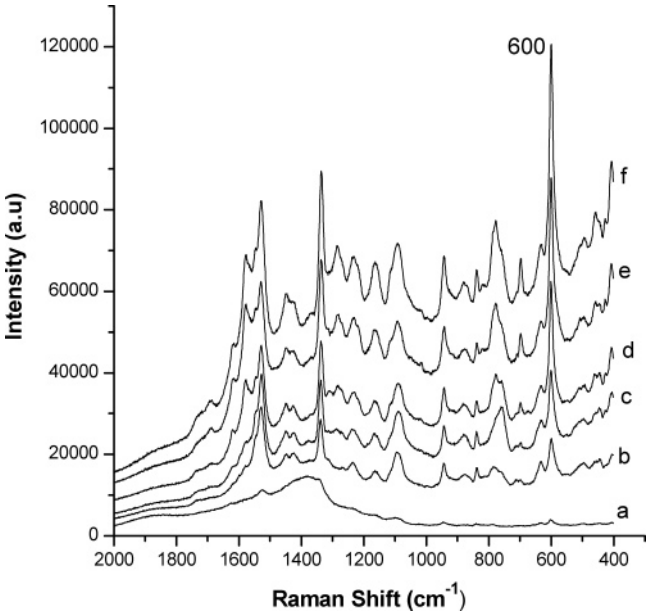

Figure 4. SERS spectra of enzyme reaction products at different ALP concentrations: (a) background without ALP; and (b) 4.1×10^{-15}, (c) 4.1×10^{-14}, (d) 4.1×10^{-13}, (e) 4.1×10^{-12}, and (f) 4.1×10^{-11} M. BCIP concentration 0.3 mg/mL. Reaction time 1 h. Laser 785 nm, ~ 1.5 mW. Acquisition time 10 s.

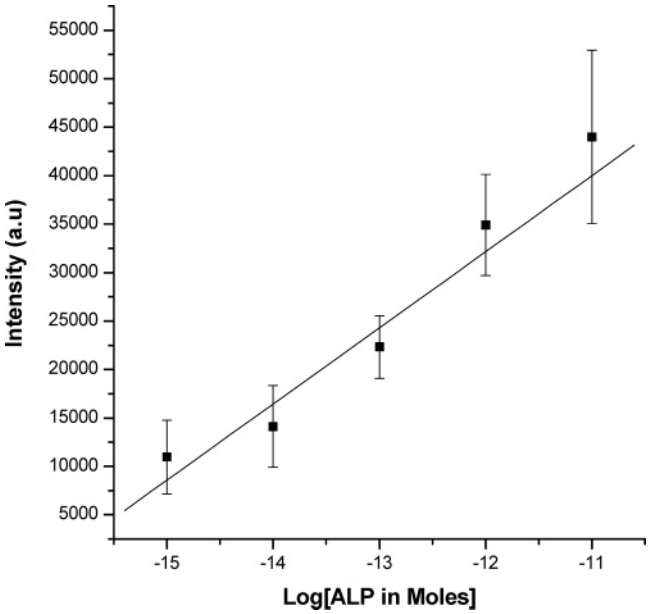

Figure 5. ALP assay by measuring peak intensities of the SERS band at 600 cm^{-1} (shown in Figure 4). Each data point represents an average of 5−7 measurements (error bar is the standard deviation).

by SERS. It is comparable with or better than some commonly used techniques, such as electrochemical assay (with a calculated detection limit of 10^{-14} M)[42] and enzyme biosensors (with a detection limit of 6.7×10^{-14} M.[43]

ALP as a Biomarker for Avidin Detection. The importance of ALP as a biomarker in immunoassays and DNA hybridization

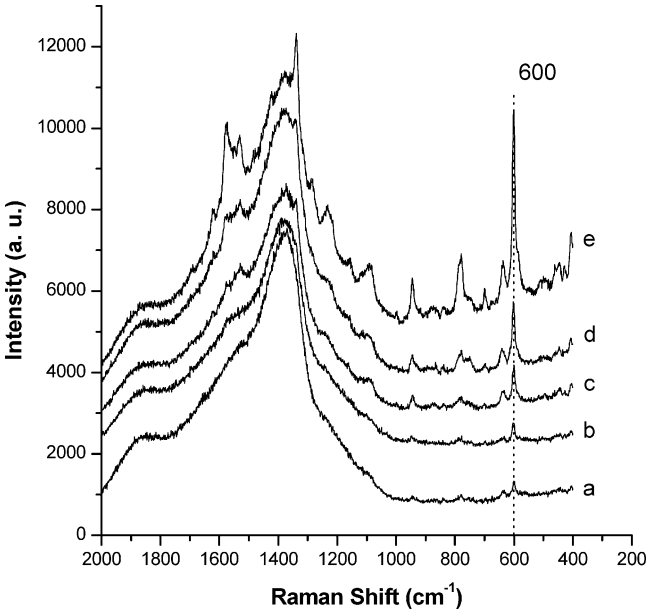

Figure 6. SERS spectra of ALP reaction products corresponding to varying avidin concentrations, which were immobilized on glass surfaces. (a) Background; (b) 1, (c) 10, and (d) 100 ng/mL; (e) 1 μg/mL. Laser 785 nm, ~1.5 mW. Acquisition time 10 s.

assays prompted us to examine a biological binding reaction between avidin (or streptavidin) and biotin by using ALP as a biotin conjugate. Avidin, found in the whites of chicken eggs, is a glycoprotein with a molecular mass of 68 kDa. The interaction between avidin and biotin results in the strongest affinity (dissociation constant, $K_d \sim 10^{-15}$ M) known between a ligand and a protein.[37] The reaction also is highly specific and has resulted in the avidin−biotin system being extensively used in biological applications such as affinity-based separation and diagnostic assays.[44] For the first time, we introduce a SERS-based methodology to detect immobilized avidin (on glass surfaces) using ALP conjugated biotin as a probe. ALP bound on the glass surface through avidin−biotin reaction can be detected by catalytic hydrolysis of BCIP, and the reaction products of ALP are detected by SERS. Figure 6 shows the results of the SERS-based determination of immobilized avidin on glass tubes. Clearly, intensities of Raman spectra increased consistently with the concentration of immobilized avidin, and a linear relationship between peak intensities at 600 cm^{-1} and avidin concentrations was observed in the range from 10 ng/mL to 1 μg/mL (Figure 6c). At 1 ng/mL avidin (Figure 6b), the peak intensity at 600 cm^{-1} generated no significant differences from the background. A small band at 600 cm^{-1} was observed in the background, suggesting the nonspecific adsorption of ALP on the modified glass surface. Such a nonspecific adsorption is quite common and often difficult to eliminate in most immunoassays or biological binding assays.[45] In this approach, although 3% bovine serum albumin was used to block nonspecific adsorption of biotin conjugated ALP, the nonspecific adsorption still could be detected due to a high sensitivity of SERS for indigo dye derivatives. We therefore claim a detection limit at

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~10 ng/mL avidin in this study, but an even lower detection limit could be achieved with further development of the methodology. Our results demonstrate that the SERS technique could potentially be used as a sensitive tool to detect surface-immobilized avidin and other biological agents by using conjugated ALP as a probe. The major advantages of using SERS to detect these biological agents are its high sensitivity, specificity, versatility (either in solids or liquid for many bioagents), and short analytical time.

CONCLUSIONS

A new SERS-based approach was developed to detect the activity of ALP at ultralow concentrations or single-molecule levels. ALP was detected through the hydrolysis of BCIP to form indigo dye of BCIP dimers, which were subsequently adsorbed on surface-modified gold nanoparticles as a SERS substrate. The detection limit of ALP was determined to be $\sim 10^{-15}$ M upon 1-h incubation in basic Tris-HCl solution containing $MgCl_2$. The technique is equivalent to or better than the most commonly used fluorescence assay techniques with a greatly reduced analytical time. It is also worth drawing attention to the sampling setup of the Raman system, in which the exciting light is focused onto the sample by a 50× microscope objective and the SERS spectra collected at 180°. The lens thus interrogates a small proportion of the actual sample with an estimated volume at picoliters to femtoliters.[24] This translates that we are likely to be sampling only reactions arising from tens or a few hundreds of enzyme molecules, at most. In other words, the technique allows the detection at single-molecule levels and thus has attractive possibilities with respect to moving toward in vivo monitoring of enzyme reactions. This same technique also was successfully employed to detect surface-immobilized avidin through ALP−avidin−biotin binding reactions. Therefore, apart from its potential usage as a SERS probe in studies of enzyme activity in living cells and gene diagnosis, the technique could be used as a new immunoassay tool (i.e., SERS immunoassay) with improved sensitivity in real-time analysis.

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