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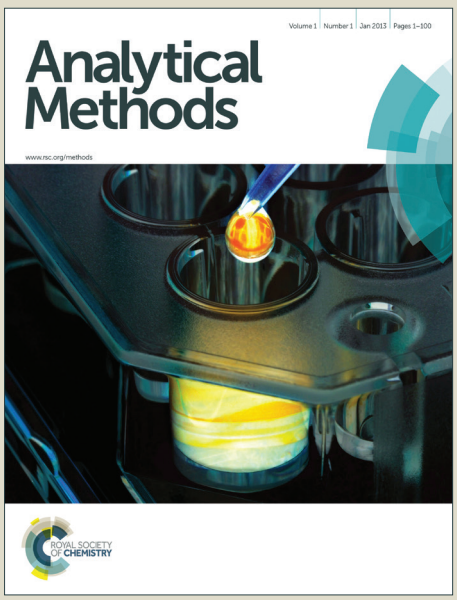

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ARTICLE TYPE

Ultrasensitive Surface-Enhanced Raman Scattering Detection of Alkaline Phosphatase

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In this paper an ultrasensitive surface-enhanced Raman scattering (SERS) detection of alkaline phosphatase (ALP) has been developed, in which Nile blue A (NBA) was chosen to replace nitro blue tetrazolium chloride (NBT) in a previous reactive system of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), NBT, and ALP. In the reactive process, NBA was converted to a low SERS-active molecule, and its SERS intensity at 592 cm^{-1} decreased when NBA was reduced by 5-bromo-4-chloro-3-indolyl which converted from BCIP by ALP. The SERS signal of NBA was inversely proportional to the amount of ALP in the reactive system. Based on convenient SERS materials of gold nanoshells absorbed on acupuncture needles, the detectable concentration range of ALP was 1 -10^4 mU/L in the reactive system of BCIP, NBA, and ALP.

1. Introduction

Alkaline phosphatase (ALP) is a hydrolase enzyme to remove phosphate groups from biomolecules in body, and it was involved in many biochemical reactions of energy metabolism, signal transduction, and etc. The change in its activity or content would indicate physiological and pathological evolution, and thus, it is important to detect ALP in body fluids for diagnosis in many diseases. [1] Besides, ALP is also used as a marker reagent for biological studies. [2] Compared with horseradish peroxidase (HRP), ALP has the advantage of high stability, high sensitivity when it was used as a marker reagent in enzyme immunoassays. It was also used as a powerful quantitative indicator in gene expression researches. [3,4] Here, it is more important for above researches to develop an ultrasensitive ALP activity detection method. Currently, the activity of ALP was detected by measuring the optical density of colored products or the intensity of emitted fluorescence or chemiluminescence of products. The limit of detection is about 500 mU/L and 20 mU/L, respectively. [5,6] Besides, detection methods of ALP based on nanomaterials also have been reported. [7-10]

Surface enhanced Raman scattering (SERS) techniques are powerful tools for ultrasensitive detection. [12] In applications, the strong SERS signals were always collected from molecules with both high affinity to SERS materials and strong intrinsic Raman activity. Many strategies have been developed for other molecules without affinity to SERS materials or strong intrinsic Raman activity. [12,13] In SERS detection of enzyme activity, when substrates and products of enzymes cannot provide strong SERS signal, masked-dyes (synthesized substrates containing a dye group) were used for obtaining strong signal. [12,14]

5-bromo-4-chloro-3-indolyl phosphate (BCIP) is a commercial chromogenic substrate used in detection of ALP activity, and it was converted to 5-bromo-4-chloro-3-indole (BCI) and inorganic phosphate. In immunoblotting, in situ hybridization, and immunohistochemistry, the produced BCI reduces nitroblue tetrazolium (NBT) to form insoluble colored precipitates to indicate the existence of ALP. Although the SERS detection method of ALP has been developed by collecting the SERS signal of BCI (dimers), [15] here, Nile blue A (NBA), a molecule with stronger intrinsic Raman activity, was used in developing a SERS detection method of ALP.

NBA is often used as a Raman active molecule to characterize enhancement effect of SERS materials due to its high affinity to SERS materials and strong intrinsic Raman activity. In previous report, NBA was used as a mediator for studying NADH oxidation. [16] In our finding, the collected SERS signal of NBA was decreased after NBA was reduced by the produced BCI. We speculated that the decrease of SERS intensity at 592 cm^{-1} was attributed to its decreased affinity to gold substrates due to its charged quaternary ammonium group replaced by tertiary ammonium group after reduced. As illustrated in Fig. 1, when oxidized form of NBA was converted to reduced form, the amount of NBA absorbed on the SERS materials would decrease, and the collected SERS signal also would decrease. When the amount of BCIP was abundant, the amount of produced BCI would be proportional with ALP, and the collected SERS intensity of NBA would be inversely proportional with ALP. Here, based on BCIP and NBA as substrates of ALP, a novel detection method of ALP activity would be developed.

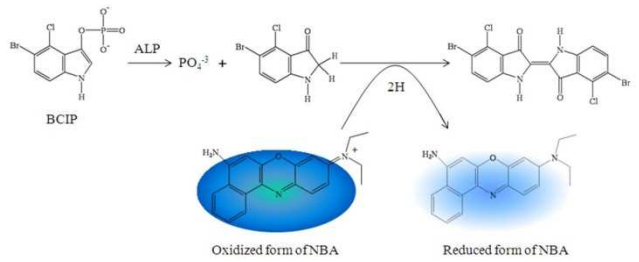

Fig. 1. Illustration of a chemical equation of BCIP and NBA catalyzed by ALP (in the catalytic process, the amount of oxidized form of NBA decreased, that is, the amount of NBA absorbed on SERS substrates decreased and thus, the collected SERS intensity also decreased).

2. Materials and methods

2.1. Materials

Stainless-steel acupuncture needles of 0.20 mm in diameter were purchased from Suzhou Tianxie Acupuncture Instruments Co. Ltd. (China). The suspensions of silica colloidal particles of 55 nm in radius were from Nissan Chemical Corporation (Japan). 3-mercaptopropyltriethoxysilane (MPTES) was from Xiya Reagents (China). $HAuCl_4$ was from Guoyao Group (China). Alkaline phosphatise (ALP) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Sangon Biotech (Shanghai) Co., Ltd. (China). Nile blue A (NBA) was from Sigma-Aldrich. All other reagents used were analytical grade。

2.2. Fabrication of SERS-active Needles

First, the gold nanoshells (GNSs, [r1, r2]=[55, 80] nm) were fabricated as blocks to construct SERS-active needles. [17] The SERS-active needles were fabricated as previous reported. Briefly, stainless-steel acupuncture needles were functionalized by immersed in 10% (v/v) of MPTES ethanol solution for 48 h at 4 ℃; Second, after rinsed with ethanol for three times, the functionalized needles were immersed in GNSs suspension for 24 h at 4℃. Under the interaction of covalent bond between thiol group of the surface of needles and Au layer of GNSs, GNSs were adsorbed on the functionalized needles to form a SERS-active needle.

2.3. Characterizations and Measurements

Zeiss ULTRA-plus scanning electron microscopy (SEM) was used to characterize GNSs on acupuncture needles. A UV-vis spectrometer (UV3150, Shimadzu) was used to obtain absorbance spectra. Renishaw Invia microRaman spectroscopy was used to measure SERS spectra at room temperature (~20 °C) using a 785 nm excitation laser. The laser was focused onto the sample surface by using a 50× long working distance objective. The extinction power and the acquisition time were 600 μW and 10 s in all measurements, respectively.

The SERS-active needles were immersed in NBA aqueous solutions for 5 min and then, they were taken out for collecting SERS signal. ALP was diluted with pH 9.5 tris-HCl buffer solutions containing 5 mmol/L $MgCl_2$ and 100 mmol/L NaCl. BCIP aqueous solution and NBA aqueous solution were subsequently added into ALP solutions, and the initial concentration of BCIP in reactive systems is 4.8×10^{-4} mol/L. After the reactive systems were incubated at 37 °C for 30 min, the SERS-active needles were immersed in the systems for 5 min and then, they were taken out for collecting SERS signal.

3. Results and discussions

3.1. Fabrication of SERS-active needles

As our previous reports, GNSs were absorbed on functionalized acupuncture needle to structure SERS-active needles (here, the aim of integrating GNSs on acupuncture needle is to operate nanomaterials conveniently during applications). Fig. 2(A) showed part of a typical SERS-active needle, the surface of which was covered by GNSs completely. Fig. 2(B) showed that GNSs were close-compact arranged on the needle body, which guarantee not only to collect large SERS signal but also to endow repeatability at different domains on the SERS materials. Fig. 2(C) showed a typical SERS spectrum of NBA obtained with 600 μW laser in 10 Sec acquisition time from a SERS-active needle immersed in 4.0×10^{-6} mol/L for 5 min, which suggested that the SERS-active needles is an excellent SERS substrate. As shown in Fig. 2(D), the SERS signals are well reproducible from spot to spot (148000 ± 15600) and from needle to needle (153000 ± 18600), which are from eight random spots of one needle and from eight different needles, respectively.

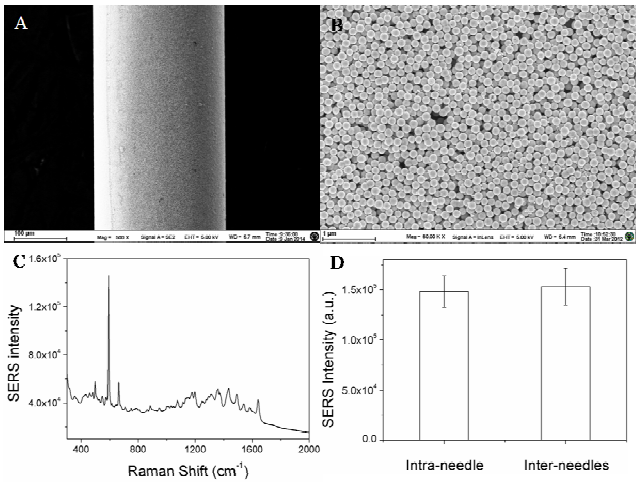

Fig. 2. (A) A SEM image of part of an acupuncture needle covered by GNSs. (B) A SEM image of GNSs on the SERS-active needle. (C) A typical SERS spectrum of NBA from the SERS-active needle. (D) SERS intensity distribution of NBA at the line of 592 cm^{-1} of intra-needle and inter-needles, respectively.

3.2. Absorbance spectra evolution of mixtures of NBA, BCIP or ALP

As shown in Fig. 3, BCIP has no absorbance peaks in visual range, while NBA has its typical absorbance peak at about 636 nm. Compared with Fig. 3, there is only the absorbance peak of NBA appeared in the spectra of a mixture of BCIP and NBA in Fig. 4(A), and its intensity also did not change in 30 min, which suggested that the concentration of NBA did not change after mixed with BCIP. From Fig. 4(B), although the mixture solution contained ALP, the absorbance intensity of NBA did not change in 30 min, suggested that the concentration of NBA also did not

change after mixed with ALP. While, from Fig. 4(C), new peaks

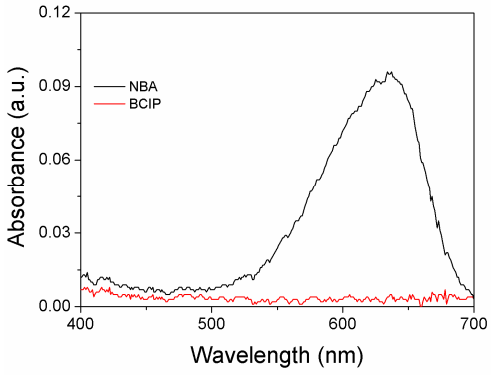

Fig. 3. Absorbance spectra of NBA (4.0×10^{-6} mol/L) and BCIP (4.8×10^{-4} mol/L), respectively.

appeared in the absorbance spectra of the mixture solution of BCIP and ALP, and the peak intensity is stronger, suggested that ALP converted colorless BCIP to colored BCI. From the absorbance spectra evolution of mixture of NBA, BCIP and ALP shown in Fig. 4(D), at 5 min, the peak intensity almost did not change, but the shape of absorbance peak changed. At 10 min, the peak shape did not match with the typical peak shape of NBA. At 30 min, the peak shape became similar to that in the spectrum of Fig. 4(C) at 10 min. We speculated that at 30 min, there is only the peak of BCI appeared in the spectra of the mixture, and that NBA was almost reduced completely by the produced BCI to form colorless products.

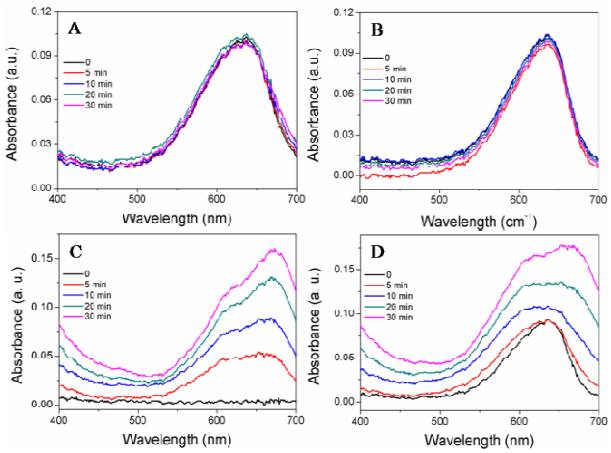

Fig. 4. (A) Absorbance spectra evolution of mixture of NBA (4.0×10^{-6} mol/L) and BCIP (4.8×10^{-4} mol/L). (B) Absorbance spectra evolution of mixture of NBA (4.0×10^{-6} mol/L) and ALP (10 U/L). (C) Absorbance spectra of mixture of BCIP (4.8×10^{-4} mol/L) and ALP (10 U/L). (D) Absorbance spectra evolution of mixture of NBA (4.0×10^{-6} mol/L), BCIP (4.8×10^{-4} mol/L) and ALP (10 U/L).

3.3. SERS spectra of mixtures of NBA, BCIP or ALP

As shown in Fig. 5, NBA and BCIP have typical SERS spectra from SERS-active needles at the concentration of 4.0×10^{-6} mol/L and 4.8×10^{-4} mol/L, respectively. Although its concentration is two orders of magnitude lower than that of BCIP, because of its strong intrinsic Raman activity, the collected SERS intensity of NBA is stronger than that of BCIP.

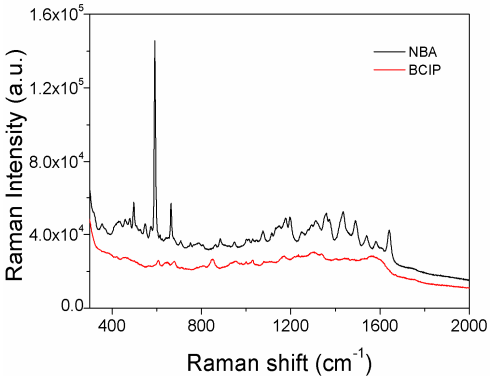

Fig. 5. SERS spectra from SERS-active needles immersed in the solutions of NBA (4.0×10^{-6} mol/L) and BCIP (4.8×10^{-4} mol/L), respectively.

After absorbance spectra evolution of mixtures of NBA, BCIP or ALP has been collected, SERS-active needles were immersed in the above solutions for 5 min for obtaining SERS spectra. Fig. 6(A) showed that a SERS spectrum collected from the mixed solution of NBA and BCIP, and only the SERS signal of NBA appeared in the spectrum. Combined with the absorbance spectra in Fig. 4(A), we speculated that BCIP did not change the structure of NBA in the mixture. Fig. 6(B) also showed that only SERS spectrum of NBA appeared in the spectrum collected from the mixture of NBA and ALP, and considering the results of Fig. 3(B), we speculated that ALP also did not change the structure of NBA in the mixture. Although absorbance spectrum of the mixture of BCIP and ALP suggested that ALP catalyzed BCIP to form colored product BCI, Fig. 6(C) suggested that both BCIP and its colored product have no strong typical SERS peaks from the SERS-active needles. Fig. 6(D) showed that only a SERS signal of NBA appeared in the spectrum from the mixed solution of NBA, BCIP, and ALP. Compared with Fig. 6(B), the SERS intensity in Fig. 6(D) was weaker. From the results of Fig. 4 and 6, we concluded that the SERS intensity at 592 cm^{-1} decreased only when NBA was reduced by BCI, which converted from BCIP by ALP. Thus, if the amount of BCIP is enough, the collected SERS intensity of NBA was inversely proportional to the amount of ALP. That is, based on the detection of changed amount of NBA, a novel SERS method can be developed to detect ALP.

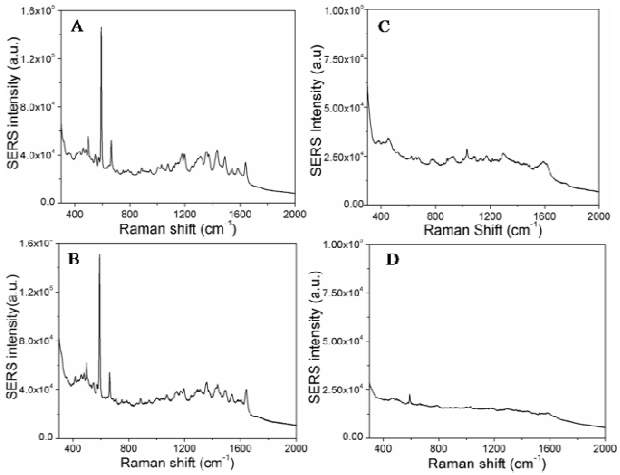

Fig. 6. (A) A SERS spectrum of mixture of NBA and BCIP. (B) A SERS spectrum of the mixture of NBA and ALP. (C) A SERS spectrum of the mixture of BCIP and ALP. (D) A SERS spectrum of the mixture of NBA, BCIP, and ALP.

3.4. SERS Detection of ALP

It is a common strategy in detecting enzyme activity to using the color or intensity change of chromogenic substrates in colorimetric detection, such as in the detection of HRP activity, 3,3N-diaminobenzidine tertrahydrochloride (DAB), o-phenylenediamine dihydrochloride (OPD), or 3,3',5,5'-tetramethylbenzidine (TMB) were usually used as chromogenic substrates, which can be converted to colored producers in catalyzed reaction of HRP. In the detection of ALP activity, BCIP and NBT were used as chromogenic substrates. Here, to develop a more sensitive method of ALP activity based on SERS detection, NBT was replaced by NBA to detect ALP activity.

As shown in Fig. 7, based on the optimized parameters above, the developed SERS detection method can detect ALP at a range of 1-10^4 mU/L. At 10^4 mU/L, the amount of ALP perhaps nearly saturated relative to the concentration of NBA, which resulted in a nonlinear relationship between the amount of ALP and exhausted NBA at high concentration of ALP. Since the concentration of NBA was adjusted by the concentration of ALP in the developed method, if prolonging or shortening the reactive time of the system, the detected range of ALP would be lower or higher than the above detected range. Using SERS substrates with higher SERS-activity, the detectable lowest concentration of ALP would decrease further. Besides, if the analytical method was used in biological samples, the other molecules would absorb on the SERS substrates and charged biomolecules would also absorb NBA, which also decreased the absorbed amount of NBA on the substrates. Thus, false positive results would be obtained in biological samples. For address this question, additional control experiments should be provided, such as adding inhibitors of ALP into biological samples as a negative control.

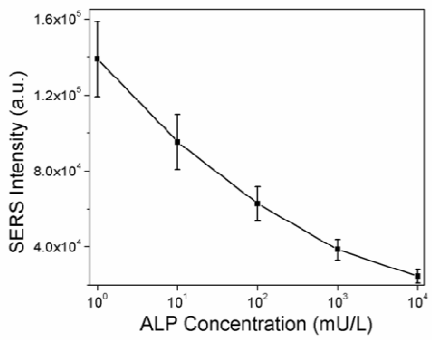

Fig. 7. A plot of SERS intensity at 592 cm-1 line of NBA versus ALP activity.

Compared with common colorimetric detection even if combined with nanomaterials, [18] the sensitivity of the developed method is higher because the SERS detection of NBA is more sensitive than the common colorimetric detection. Compared with SERS detection of enzymes previous reported, [14] BCIP and NBA used were commercial chemicals, and it is not necessary to synthesize novel chemicals laboriously. Compared with the SERS detection using substrates of BCIP and NBT, [15] the SERS intensity of NBA at 592 cm^{-1} decreased while no visual Raman peak of BCI (dimers) appeared in the SERS spectra shown in Fig. 6(D), and as shown in Fig. 8, even at 120 min the SERS signal of NBA at 592 cm^{-1} almost disappeared while a weak SERS signal of BCI (dimers) at about 600 cm^{-1} appeared, which suggested that the sensitivity of our developed method is higher based on the SERS-active needles.

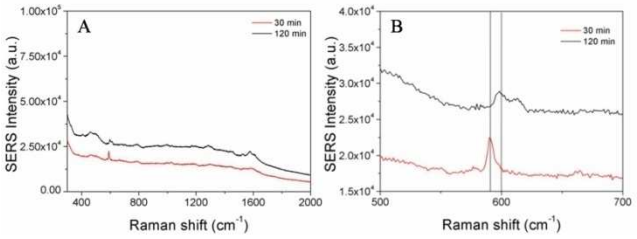

Fig. 8. (A) SERS spectra of the mixture of NBA, BCIP, and ALP at 30 and 120 min, respectively. (B) Enlarged parts of (A) to show detailed SERS peaks of the mixture (the two vertical lines show SERS peaks of NBA and BCI dimers, respectively).

4. Conclusions

In summary, based on the finding of the reduction of NBA by BCI and the change in SERS intensity of NBA after reduced, NBA was chosen to replace NBT as substrates of ALP. GNSs were absorbed on acupuncture needles as convenient SERS materials for detecting the SERS signal of NBA during the catalyzed process. Although BCIP and BCI have their typical SERS spectra, the SERS line of NBA at 592 cm^{-1} dominated the collected SERS spectra from the catalyzed system due to its stronger intrinsic Raman activity. The SERS intensity at 592 cm^{-1} decreased only when NBA was reduced by BCI, which converted from BCIP by ALP. Based on the SERS detection of NBA in catalyzed system, the detectable concentration range of ALP was

1-10^4 mU/L.

Notes and references

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