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A sensitive surface-enhanced Raman scattering enzyme-catalyzed immunoassay of respiratory syncytial virus

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ABSTRACT

Respiratory viruses have become a major global health challenge which would benefit from advances in screening methods for early diagnosis. Respiratory syncytial virus (RSV) is one of the most important pathogen causing severe lower respiratory tract infections. Here we present a novel surface-enhanced Raman scattering (SERS) enzyme-catalyzed immunoassay of RSV by employing peroxidase substrate 3, 3′–5, 5′-tetramethylbenzidine (TMB) as Raman molecule. Horseradish peroxidase (HRP) attached to the detection antibody in a novel sandwich immunoassay catalyzes the oxidation of TMB by H_2O_2 to give a radical cation (TMB^+), which could be easily adsorbed on the negatively charged surface of silver nanoparticles (AgNPs) through electrostatic interaction, inducing the aggregation of AgNPs and thus giving a strong SERS signal. A linear relationship was obtained between the Raman intensity and the amount of RSV in the range from 0.5 to 20 pg/mL, and the minimum detectable concentration of this SERS-based enzyme immunoassay was 0.05 pg/mL, which was 20 times lower than that found in the colorimetric method.

1. Introduction

Respiratory syncytial virus (RSV), a global infectious agent, is the major cause of acute lower respiratory tract infections in infants as well as an important cause of morbidity and mortality in adults, which leads to the growing health public burden and enormous economic losses [1,2]. According to the World Health Organization (WHO), RSV has been estimated to infect nearly 64 million people each year, with 160,000 deaths [3]. Furthermore, initial RSV infection fails to induce an efficient immunological memory so that repeated infections are frequent throughout life [4–6]. Despite decades of research, there are still no commercially available vaccines against RSV. Current treatments are effective only if provided early in the course of infection [7,8]. Therefore, an accurate and sensitive detection method for early diagnosis is crucial due to the severity and sequels of the disease caused by RSV.

Enzyme-linked immunoabsorbent assay (ELISA) is the most common technique used as a diagnostic tool in medical and biochemical fundamental researches, as well as a quality control check in various industries [9]. In a typical sandwich ELISA, it relies on the antibody-antigen specific interaction where capture antibody adsorbed on a solid surface (such as a microtiter plate) will binds with its antigen, which will then react with a detection antibody that is linked with enzyme. The enzyme activity can consequently be recorded by addition of the chromogenic substrate, generating a color product which is correlated to the amount of antigen. Common enzymes used in ELISA include horseradish peroxidase (HRP), alkaline phosphatase (ALP), β-galactosidase. Among them, HRP is the most desirable label because it is the smallest and most stable [10]. The substrate used extensively in HRP-based detection systems is 3, 3′, 5, 5′-tetramethylbenzidine (TMB), which is less toxic than *o*-phenylenediamine (OPD) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Although many advances have been achieved in conventional ELISA [11–15], there is still a challenge for new protocols to improve the sensitivity and simplicity.

Raman scattering spectroscopy, which can provide rich structure information as well as quantitative and qualitative information about the molecular samples by the sharp and distinguishable vibrational bands in a nondestructive manner, has been an invaluable technique to investigate biological materials [16,17]. However, conventional Raman spectroscopy is hampered for the use as a readout method in biological analysis by its limited

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sensitivity [18]. In recent years, surface-enhanced Raman scattering (SERS) and surface-enhanced resonance Raman scattering (SERRS) have attracted great interest and become powerful techniques in numerous biochemical systems because of their extraordinary sensitivity [19–23]. SERS enables the strong enhancement of Raman signal and allows obtaining intrinsic molecular "fingerprint" information even to the single molecule level, providing a unique opportunity for immunoassay [24]. Recently, many reports of SERS based immunoassays have already been reported. They utilized various SERS nanotags, for example, Raman dye-labeled gold nanomaterials [25–27] or silver nanoparticles [28–30] or gold@silver nanoparticles [31,32] and 4-aminobenzenethiol (4-ABT)-tagged multilayer SiO_2@(AgNPs/PEI) particles [33]. In these SERS-based immunoassays, nanomaterials should be first functionalized with Raman reporter, which was time-consuming, complex and harder to handle [34]. Thus by utilizing the sensitivity afforded by SERS coupled with efficient enzyme-catalyzed oxidation system commonly used in ELISA, a convenient, simple, and reliable SERS-based immunoassay would be constructed. The first proof of concept of this immunoaasy using HRP and the enzyme reaction product azoaniline as SERS probe was explored by Dou et al. [35]. However, the measurement range was relatively narrow (0.158–2.5 ng/mL). In this paper, we describe the use of SERS as a readout technique in a HRP-based sandwich immunoassay using TMB as the substrate for the highly sensitive detection of RSV and their application to the measurement of RSV in cell lysate.

2. Material and methods

2.1. Materials

An ELISA test kit comprised a microtiter plate coated with anti-RSV, RSV standard, HRP-labeled antibody (anti-RSV-HRP), TMB, hydrogen oxide (H_2O_2), and washing buffer was purchased from Fengxiang Biological Technology Co., Ltd. (Shanghai, China). Silver nitrate ($AgNO_3$) was purchased from Ruijinte Chemical Group Co., Ltd. (Tianjin, China). Sodium hydroxide (NaOH) and NaCl were obtained from Chuandong Chemical Group Co., Ltd. (Chongqing, China). Ascorbic acid (AA) and sodium citrate were obtained from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Polyvinylpyrrolidone (PVP) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All the chemicals were used as received without further purification. Ultrapure water obtained from a Millipore water purification system (≥ 18 MΩ, Milli-Q, Millipore, Billerica, USA) was used as a solvent.

2.2. Apparatus

Absorbance measurements were recorded at 650 nm using an Epoch microplate spectrophotometer (Biotek, USA). UV–vis absorption spectra were acquired by a Hitachi U-3010 spectrophotometer (Tokyo, Japan). Raman spectra were collected using a LabRam HR800 Laser confocal Raman microprobe (Horiba Jobin Yvon, France) equipped with a 532 nm helium–neon laser for excitation. Scanning electron microscopy (SEM) images were obtained using a Hitachi S-4800 scanning electron microscope (Tokyo, Japan) operating at 30.0 KV. The dynamic light scattering (DLS) measurements were conducted using a Zetasizer Nano ZS System (Malvern, UK) at room temperature.

2.3. Synthesis of silver nanoparticles (AgNPs)

AgNPs were synthesized by using AA and sodium citrate to reduce AgCl colloid in aqueous solution. Briefly, AgCl colloid was first prepared by mixing 17.0 mg of PVP and 17.0 mg of $AgNO_3$ in 4.0 mL of ultrapure water under vigorous stirring. Then, 40 μL of NaCl (5 mol/L) was added rapidly to the mixture and allowed to stand for 15 min in the dark. To prepare regular AgNPs, 20 mL of AA (50 mmol/L) and 2.7 mL of NaOH (0.5 mol/L) were mixed under magnetic stirring. 2.5 mL of freshly prepared AgCl colloid solution was added all at once and a color occurred after 30 min, followed by addition of 2 mL of a 1% solution of sodium citrate in water. The final mixture was left stirring for 1 h at room temperature. Residual reactants were removed by centrifuging at 5000 rpm for 5 min. The supernatant was carefully removed and the AgNPs were resuspended in ultrapure water.

2.4. Preparation of virus

HEp-2 human epithelial cells (1×10^5 cells/mL) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (6 mg/mL) and streptomycin (10 mg/mL) at 37 °C in 5% CO_2. When monolayer was formed, the medium was removed from the cells and washed with PBS buffer one time. Virus was then added and allowed to adsorb at 37 °C for 2 h before adding fresh medium. When a high degree of cytopathic effects (CPE) appeared, the cells were subjected to two freeze-thaw cycles and the cell debris was removed by centrifugation at 3000 g for 10 min. The harvested RSV was stored at −80 °C for further use.

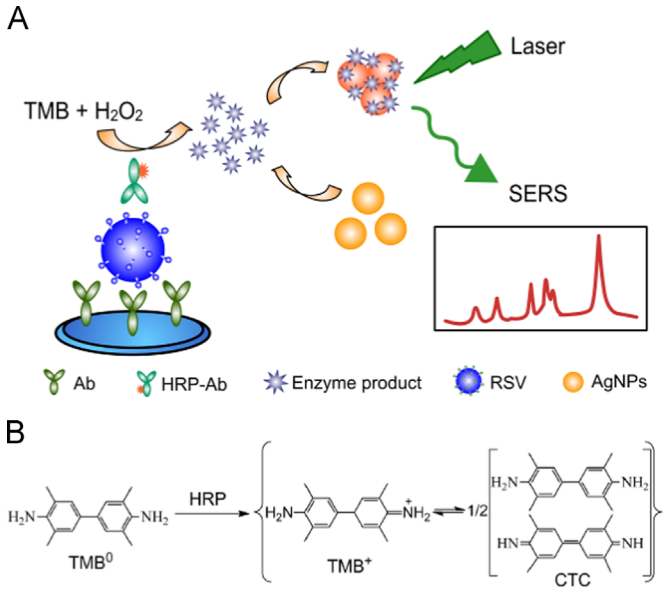
2.5. Enzyme-based SERS immunoassay

The ELISA test kit was used according to the manufacturer's protocols. Briefly, RSV standards, control samples and cell lysate samples (50 μL) were added to the wells containing immobilized capture antibody against RSV and allowed to react at 37 °C for 30 min. Then, the wells were washed five times with washing buffer to separate bound and free RSV. Next, anti-RSV-HRP conjugate (50 μL) was added to each well and incubated for 30 min. Following five washings, substrate solution (the mixture of TMB and H_2O_2, 100 μL) was added, and the reaction was left to proceed for 15 min at 37 °C. Absorbance measurements of the enzyme reaction product were carried out at this stage, while the Raman spectrum were recorded by a LabRam HR800 Raman spectrometer (28 mW, 532 nm, 5 s) after incubation with AgNPs (45 μL) 10 min.

3. Results and discussion

3.1. Enzyme-based SERS immunoassay design

Our developed strategy combines the advantages of the high specific enzyme immunoassay and highly sensitive SERS-based assay. As illustrated in Scheme 1A, RSV is first captured with specific antibody on a solid substrate and subsequently with an HRP-labeled antibody, forming sandwich complex. A colored product is generated by the HRP-catalyzed oxidation of TMB by H_2O_2, providing a convenient mean for quantitative analysis. The HRP oxidizes the parent diamine (TMB^0) to a radical cation (TMB^+) that exists in rapid equilibrium with a blue charge transfer of the diamine (CTC) (Scheme 1B). The TMB substrate exhibits low SERS signals. However, the positively charged enzymatic reaction products could induce the aggregation of citrate-capped negatively charged AgNPs through the electrostatic interactions, producing highly enhanced Raman scattering due to the formation of hot spots. The concentration of RSV could be quantified by SERS signals produced from the oxidized product, avoiding the complicated modification process of Raman reporter.



Scheme 1. Principle of the SERS-based immunoassay. (A) Sandwich-like immunoassay for RSV sensitive detection using enzyme-catalyzed reaction. (B) Oxidation of TMB by H_2O_2 in the presence of HRP.

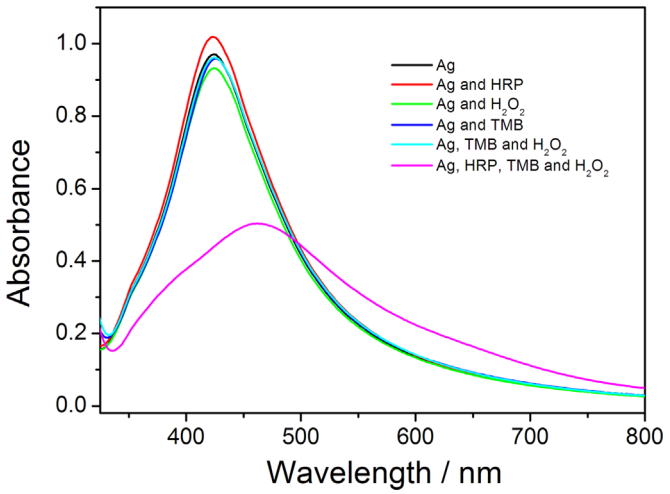


Fig. 2. UV–vis absorption spectra of the AgNPs in solution upon addition of TMB, H_2O_2, HRP, and the enzyme reaction product. 45 μL of AgNPs, 100 μL of substrate solution, and 5 μL of HRP (10 ng/mL) were diluted to 500 μL and the spectra taken after 10 min of incubation.

3.2. The study of enzyme-catalyzed reaction by SERS

As a starting point, we attempted to investigate the SERS spectra of both TMB and its oxidized product to examine the feasibility of our design. Results in Fig. 1A show that no peak was observed in the spectrum of the substrate solution (TMB and H_2O_2). And the spectrum of enzyme reaction product without adding AgNPs does not elicit distinctive Raman bands. In sharp contrast to these spectra, apparent Raman signals can be observed only in the presence of AgNPs. The Raman peaks at 1191 cm^{-1}, 1346 cm^{-1}, and 1606 cm^{-1} can be assigned as the CH_3 bending modes, inter-ring C–C stretching modes, and a combination of ring stretching and C–H bending modes, respectively [36]. The number and position of the bands are agreement with previous studies, proving that oxidation of the TMB has taken place. Based on these results, one important conclusion can be given that only the oxidized product of TMB shows a strong SERS spectrum, thus the concentration of the HRP can be investigated directly. Subsequently, different amounts of HRP were added to the substrate solution and incubated to observe the SERS signal change. As shown in Fig. 1B, the control group displays a weak feature of Raman spectrum. As a result of adding HRP into the substrate solution, the SERS intensities increase dramatically as the concentration of HRP increased from 0.02 to 0.5 ng/mL, building the foundation of RSV quantification.

3.3. Aggregation of AgNPs induced by enzyme reaction product

To validate the mechanism of SERS, UV–vis absorption spectra, SEM, DLS and zeta potential measurements were used. As can be seen in Fig. 2, the as-prepared AgNPs shows a characteristic absorption peak at 420 nm, which is ascribed to the surface plasmon resonance (SPR) of the AgNPs. The addition of H_2O_2, TMB, and HRP alone to the AgNPs solution leads to almost no change in the spectra, whereas the enzyme raction product dampens and broadens the spectrum of the AgNPs, which is indicative of aggregation of AgNPs [37]. SEM observations show that the citrate-capped AgNPs are highly dispersed in substrate solution due to the electrostatic repulsion between AgNPs and the average size is about 50 nm, while strong aggregation of AgNPs is observed in the enzyme reaction mixture (Fig. 3). DLS measurements further supported the aggregation of AgNPs. The average hydrodynamic diameter of bare AgNPs is 88.5 ± 5.4 nm, and then it increases to 645 ± 18.6 nm after incubation with enzyme reaction products, in good agreement with the SEM results. Besides, zeta potential measurements, correlating with the surface charge and the local environment of AgNPs, was also used to compare the surface charge on AgNPs before and after interaction with the enzyme

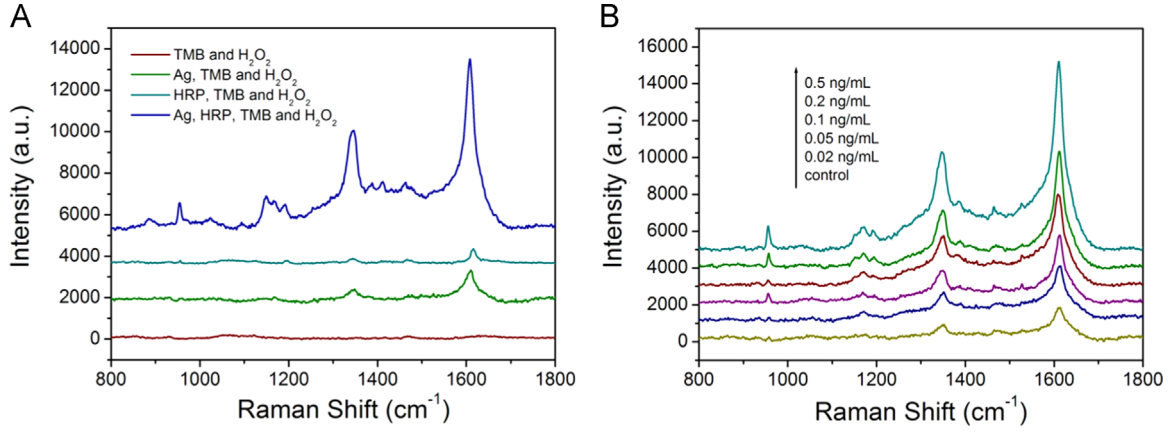


Fig. 1. (A) Typical SERS responses obtained in TMB oxidation process. (B) SERS signals in response to HRP at different concentrations: 0, 0.02, 0.05, 0.1, 0.2, and 0.5 ng/mL. The Raman spectra were recorded after 10 min of incubation.

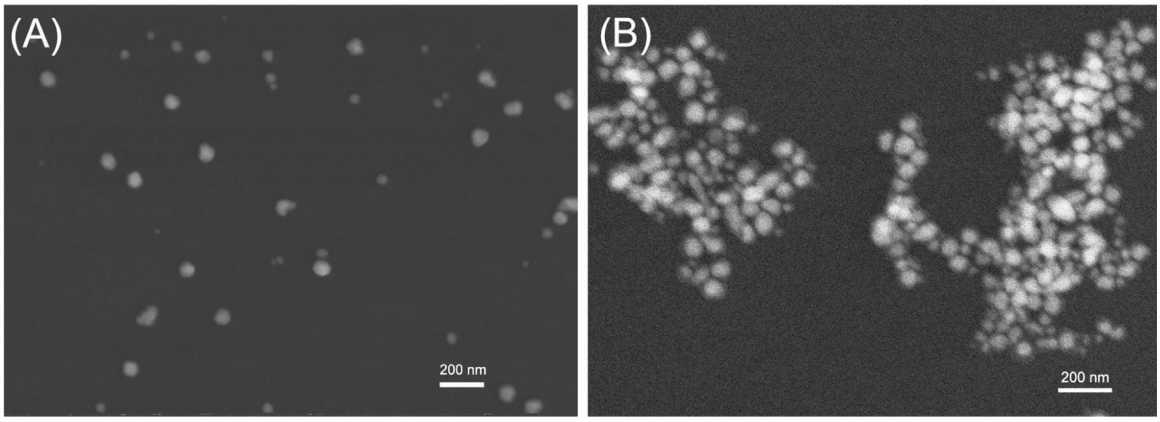


Fig. 3. Typical SEM images of AgNPs in the substrate solution (A) and the enzyme reaction product solution (B) after 10 min of incubation.

reaction product. The zeta potential of well-dispersed AgNPs is about -26.9 ± 0.42 mV, while that of aggregates of AgNPs increases to be approximately -11.3 ± 3.3 mV. Overall, in the absence of HRP, the TMB^0 can not be enzymatically converted into TMB^+, thus AgNPs remain well-dispersed and there is no obvious signal in the SERS spectrum. However, in the presence of HRP, TMB^+ is generated and can be adsorbed on the surface of negatively charged AgNPs through electrostatic interaction, inducing the aggregation of AgNPs and producing strong SERS signals.

3.4. Enzyme-based SERS immunoassay for RSV

This strategy was subsequently adapted into the sandwich ELISA for RSV detection. Fig. 4A depicts the typical SERS spectral signals in this immunoassay against RSV concentration. One can see the remarkable increase in the SERS intensities with the increasing concentration of RSV, which demonstrates the ability of the proposed strategy in quantification of RSV. Strong and reproducible SERS signals at 1606 cm^{-1} versus the RSV concentrations is plotted in Fig. 4B. A satisfying linear relationship is obtained over the range of 0.5–20 pg/mL with the correlation coefficient of 0.995. The minimum detectable concentration is as low as 0.05 pg/mL, confirming the high sensitivity of SERS-based assay. As a comparison, absorbance measurements of the enzyme reaction products were taken in parallel at 650 nm (Fig. 5). The signal becomes distinguishable from the control at 1.0 pg/mL and exhibits a linear to RSV within the concentration range from 2 pg/mL to 20 pg/mL. Overall, the SERS-based enzyme immunoassay is more sensitive compared to the conventional colorimetric method.

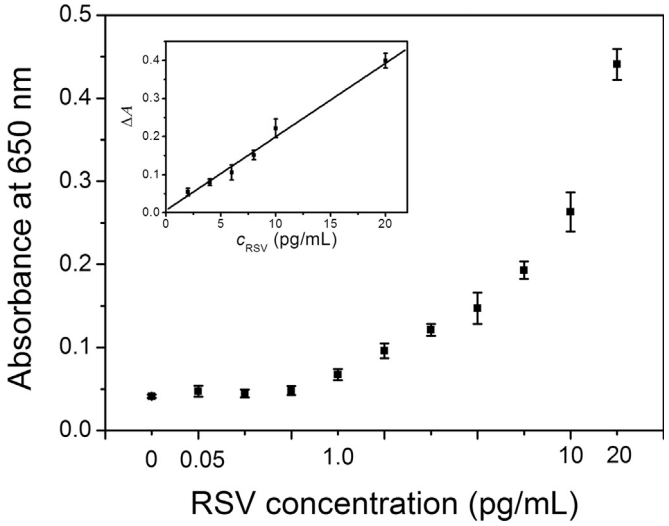


Fig. 5. The absorption change at 650 nm versus RSV concentration. Inset shows a linear relationship between the absorbance and the concentration of RSV within the range of 2 to 20 pg/mL (coefficient of determination, R=0.994). Error bars indicate standard deviations from three measurements.

Besides, the dynamic range obtained using the developed assay is wider than that of absorbance method.

3.5. Detection of RSV in cell lysates

To assess the practicality of the developed method, RSV in cell

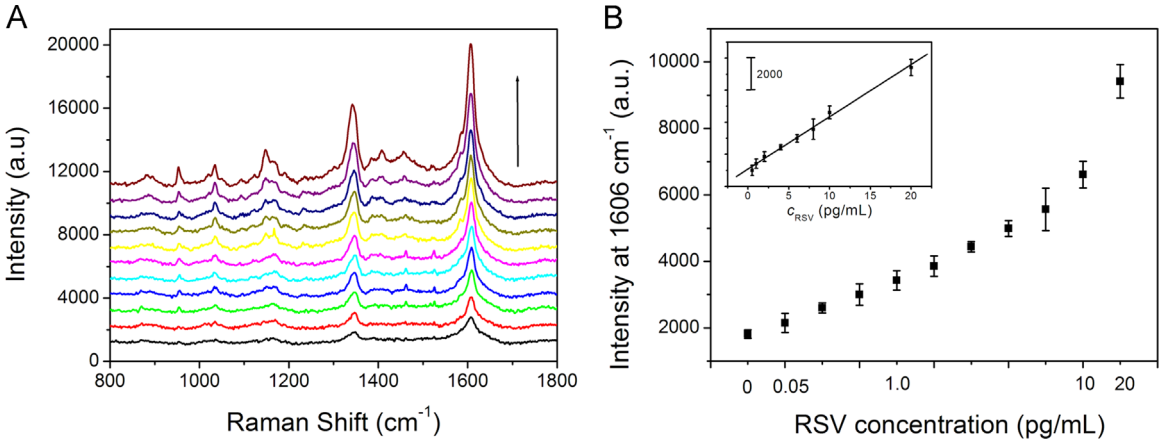


Fig. 4. SERS-based assay for RSV. (A) SERS spectra for increasing concentrations of RSV and (B) corresponding intensity of the SERS signal at 1606 nm. Inset shows a linear relationship in the concentration range from 0.5 to 20 pg/mL (coefficient of determination, R=0.995). Error bars indicate standard deviations from three measurements.

Table 1
Comparison assay results of cell lysates samples for SERS immunoassay and colorimetric method ($n=3$).

Samples	SERS immunoassay (pg/mL)[a]	Colorimetric method (pg/mL)[b]
1	12.86 ± 1.16	12.13 ± 0.71
2	18.35 ± 1.57	17.51 ± 0.68
3	15.17 ± 2.23	14.22 ± 0.94

[a] All the measurements are performed using SERS intensity at 1606 cm^{-1} and using the linear fitting equation shown in Fig. 4B.
[b] All of the abosorbance measurements are recorded at 650 nm and using the linear fitting equation shown in Fig. 5.

lysate was analyzed by the traditional colorimetric immunoassay method and SERS assay. Table 1 shows the comparison of the colorimetric detection results with those of SERS data obtained from cell lysates samples. It is clear that enzyme-based SERS assay is also suitable for the determination of RSV in complicated biological samples.

4. Conclusions

In summary, we described a new enzyme immunoassay employing SERS spectra of the enzyme reaction product for RSV detection. Because of its high sensitivity, the SERS-based immunoassay has been applied toward the detection of RSV in cell lysate, showing its great potential for target pathogens in complicated biological samples. Although only RSV was detected through the present assay, the technique would be extended toward the detection of a variety of other pathogens as well as RSV when using TMB as substrate in ELISA.

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