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A SERS nano-tag-based magnetic-separation strategy for highly sensitive immunoassay in unprocessed whole blood

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ABSTRACT

Assay technologies capable of detecting biomarker concentrations in unprocessed whole blood samples are fundamental for applications in medical diagnostics. SERS nano-tags integrated magnetic-separation biosensor (MSB) was realized for the first time for immunoassay in whole blood. The reliability and sensitivity of this method rely, in a large extent, on the quality and properties of the SERS nano-tags. The constructed silicacoated Ag SERS nano-tags as labels were used in a rapid and specific MSB immune sensor to detect Matrix Metalloproteinases 9 (MMP-9) in unprocessed blood samples. With fast screening ability and outstanding sensitivity, we anticipate that this method would greatly promote practical application of stroke-based early-stage cancer diagnosis.

1. Introduction

Over the last decade the matrix metalloproteinases (MMPs) have been widely investigated for their role in disruption of the blood-brain barrier (BBB), particularly the extracellular matrix (ECM), following stroke and other cerebral pathologies such as traumatic brain injury and neoplasm [1–5]. Early clinical diagnosis is very significant and essential in improving the survival rate and evaluating the recovery of stroke patients. However, there are many challenges in the early diagnosis of stroke because it's often silent with non-specific symptoms [6,7]. At least 23 MMPs have been identified to date, with MMP-2 and MMP-9 the most widely studied in stroke. In particular, MMP-9 has been implicated, not only in the pathogenesis of BBB breakdown and subsequent vasogenic edema formation following stroke [8,9]. Conventional approaches for MMP-9 detection, including enzyme-linked immuno sorbent assay and western blotting, are generally complex, time-consuming and poorly reproducible. Therefore, the development of new method for simple, rapid and sensitive detection of MMP-9 is highly desirable [10]. In comparison with these immunological methods, surface-enhanced Raman scattering (SERS) sensors for immunoassays has attracted increasing attention due to its sensitive and convenient label-free advantages [11–13]. Meanwhile, SERS immunoassays based on AgNPs for highly sensitive detection of MMP-9 has not been reported.

SERS has become a powerful and sensitive analytical technique for chemical and biomedical analysis compared with traditional immunoassays, SERS has some especial advantages [14–16]. Firstly SERS has better anti-interference resistance to other molecules in the complex sample system due to its fingerprint characteristic. Second SERS can highly enhance the Raman signals of good stability, convenience and reproducibility. As a result, SERS has been widely used in the detection of proteins for immunoassays [17–20].

More recently, more and more studies have reported the reliable immunoassays based on SERS using SERS tags and magnetic microspheres [21]. The whole reaction was occurred in solution system, overcomed the slow reaction problems of diffusion-limited kinetics on the solid substrate. The AgNPs show strong enhancement effects from individual particles due to their ability to localize the surface electromagnetic fields through the hot spots in their structures [22,23]. Hence, they can be used as SERS tags for sensitive immunoanalysis of biomarkers [25,26]. The previous AgNPs were always fabricated by one-step synthesis. The one-step method was relatively simple, however, the surface of the prepared AgNPs were rough with relatively poor controllability and reproducibility [27,28]. The methods can not obtain monodispersed AgNPs, which have an adverse influence on quantitative immunoassay [29,30].

In this paper, taking advantage of the intrinsic properties of the SERS nano-tags and magnetical capturing, we have developed a SERS nano-tag-based magnetic separation strategy to be successfully used for immunoassay in the unprocessed whole blood that overcomes the current assay limitations. This method largely relies on the properties of

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SERS nano-tags. Considering the easy oxidation of silver nanomaterial, We have prepared the polyvinylpyrrolidone (PVP) modified silver nanomaterial is more stable, which is more suitable for biological monitoring. We have prepared SERS nano-tags, comprised of one SERS-active Ag nanoparticle (NP) and a sub-monolayer of 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) Raman molecules adsorbed to the AgNP surface. The as-constructed Ag@DTNB nano-tags displayed stronger SERS signals with the enhancement factor, and processed excellent stability in the unprocessed whole blood solutions. We further integrated these Ag@DTNB SERS tags with magnetic separation to construct an immuneassay for direct measuring stroke biomarker, MMP-9 in unprocessed whole blood solutions. The results demonstrated the constructed in whole blood held sensitive and reproducible response to the MMP-9 concentration in the range up to 100 ng/mL with a detection limit of 1 pg/mL. As a result, the SERS method can be utilized to evaluate MMP-9 using SERS signals as the indicator.

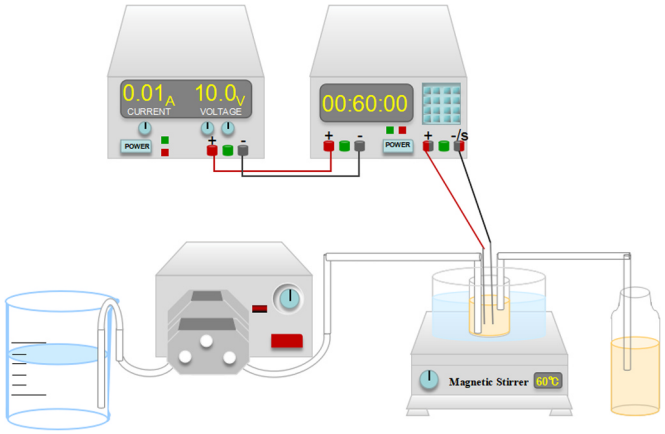


Fig. 1. AgNPs were synthesized by a continuous flow electrochemical process.

2. Experimental

2.1. Reagents and instrumentation

silver nitrate ($AgNO_3$), polyvinylpyrrolidone (PVP), 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ethylene glycol, 2-Mercaptoethanol (ME), tetraethyl orthosilicate (TEOS), EDC, N-Hydroxysuccinimide (NHS) were analytical grade. MMP-9 antigen, anti-MMP-9 McAb (detection antibody), anti-MMP-9 PcAb (capture antibody) were purchased from Shanghai Linc-Bio Science Co. LTD, phosphate buffer saline (PBS, pH 7.4, 0.01 M) and bovine serum albumin (BSA) were purchased from Sigma, Aqueous solutions used in the experiments were prepared by Milli-Q water from Milli-Q system (resistivity > 18 MΩ), Human blood samples were kindly provided by the Affiliated Hospital of Taishan Medical University.

2.2. Preparation of silver nanospheres (AgNPs)

In the current study, AgNPs were synthesized by a continuous flow electrochemical process as reported previously [24,25], in which only polyvinylpyrrolidone (PVP) and two silver rods were used as the stable agent and electrodes, respectively. Firstly, the silver electrodes were polished and washed, and then fitted on the airtight container's cover. Subsequently, the 5 mg/mL PVP solution was injected into the container continuously by a syringe at the flow rate of 100 mL/h. Finally, the collected colloidal silver solution was purified with 0.22 mm filter membrane. The reaction temperature was 90 °C and the electrolytic voltage was 15 V. During the reaction, the polarity direction of the electrodes was alternately changed every minute. Fig. 1

2.3. Synthesis of Fe_3O_4 microspheres

The magnetic particles were prepared by means of a solvothermal reaction as reported previously [26]. Approximate 0.74 g of $FeCl_3{\cdot}6H_2O$ and 1.44 g of sodium acetate were added in sequence in 20 mL of ethylene glycol under vigorous stirring. After 2 h, the solution was transferred to a stainless-steel autoclave, heated at 200 °C for 12 h. The resulting black magnetite particles were washed five times with ethanol and water respectively, and then dried in vacuum at 60 °C for 12 h.

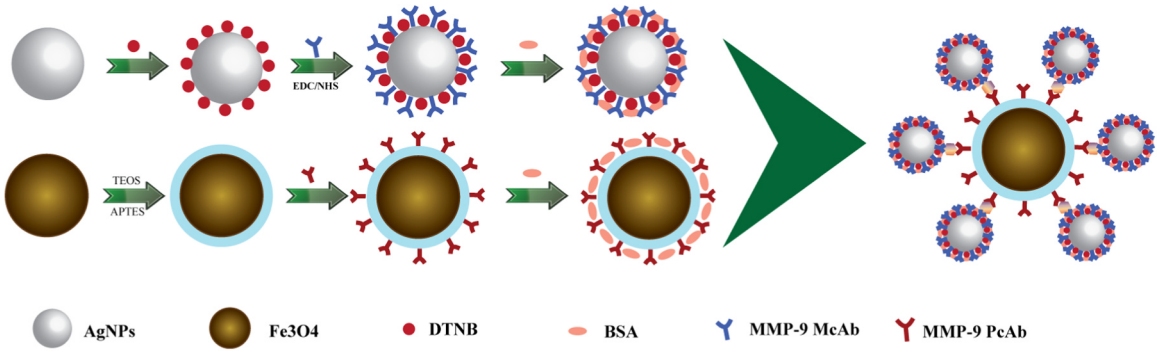
2.4. Preparation of MMP-9 McAb-conjugated AgNPs

AgNPs-based SERS tags were prepared as shown in Scheme 1. First, DTNB was chosen as Raman reporter as well as a coupling reagent to bond MMP-9 McAb. About 10 μL of DTNB (10^{-3} M) was added to 10 mL of AgNPs (OD = 1.0). After reacting for 30 min under stirring, the Raman reporter was absorbed by the AgNPs with the -SH of the DTNB chemically bonded to the Ag on the AgNPs surface. And then 2.0 μL of BSA (1 mM) was used to coat the nonspecific sites remained on the surface of the AgNPs for preventing nonspecific interactions with other proteins. what's more, it could improve the stability of AgNPs. The solution was centrifuged to remove residual BSA, and then washed three times by PBS buffer and redispersed in PBS buffer through ultrasonicator. Finally, 5 μL of EDC (1 mM) and 5 μL of NHS (1 mM) were added and allowed to react for 30 min to activate the -COOH of the DTNB. Finally, 50 μL MMP-9 McAb (40 μg/mL) was added to the NHS-activated AgNPs solution and reacted for 2 h. Antibody immobilization onto the surfaces of the AgNPs was achieved by displacing the NHS group.

2.5. Preparation of MMP-9 PcAb-conjugated Fe_3O_4 microspheres

As shown in Scheme 1, the schematic illustration for the preparation of MMP-9 PcAb-conjugated magnetic nanoparticles. Here, 0.01 g of Fe_3O_4 (above mentioned) were dispersed in 5 mL ethanol by ultrasonicator, 10 μL of APTES was added to the solution under stirring for 2 h, and then 10 μL of succinic anhydride (0.1 M, freshly prepared) was added to the mixture under stirring for 7 h. Then, washed three times with copious ethanol and PBS buffer successively, the carboxy group modified silica-coated magnetic nanoparticles were isolated from the mixture with a magnet and redispersed in PBS buffer. Lastly, 100 μL of EDC (1 mM, freshly prepared) and 100 μL of NHS (1 mM, freshly prepared) were added and allowed to react for 1 h to activate the -COOH group, follow by 100 μL of MMP-9 PcAb (20 μg/mL) was added to NHS-activated magnetic beads and reacted over night, and the final nanoprobes were washed twice with PBS buffer. Subsequently, these substrates were incubated in MMP-9 coating antibody molecules for 4 h and then washed with phosphate buffer saline to remove free antibody molecules, respectively. Finally, in order to avoid nonspecific binding during immunoassays, the substrates were immersed into bovine serum albumin solutions (1% in phosphate buffer saline) to block the residual vacant portion and then washed with phosphate buffer saline.

In our sandwich-structured SERS tag, the AgNPs is vital to ensure the stability of the SERS probe in the real-world blood plasma that typically exhibits high ionic strength. Under the optimal experiment condition, the developed SERS tags was employed to detect various concentrations of MMP-9 biomarker spiked into a mixture of 20% PBS solution and 80% whole blood. It is noted that measurement of MMP-9 in a diluted blood can be finished within 30 min. Prior to MMP-9 measurement, the baseline concentration of MMP-9 in the blood plasma was measured by a commercial MMP-9 ELISA kit. Three ELISA measurements were performed. None of the tests showed any signal response, which indicated that the MMP-9 content in blood plasma was lower than the LOD of the ELISA Kit.



Scheme 1. Illustration of SERS based Immunoassay for MMP-9 detection: a fabrication process of SERS tags and fabrication process of MMP-9 PcAb-conjugated magnetic beads and the resulting sandwich for SERS detection.

2.6. Characterization

Transmission electron microscopy (TEM) was taken with a JEM-2100EX (JEOL) transmission electron microscope operated at 200 kV. The microstructures of our nanoparticles were observed on a Zeiss Ultra Plus field emission scanning electron microscope (SEM) at 15 kV (Ultra Plus, Zeiss, Germany). The synthesized AgNPs were primarily characterized by UV–vis spectroscopy (Shimadzu UV-3600, Japan) spectrophotometer in a range of 300–800 nm. All Raman spectra were using Renishaw Invia microscope Raman spectrometer to carry Raman experiments. Each SERS spectrum was averaged from 5 measurements. Results were given as means ± the standard deviation (SD). All Raman experiments were carried out at room temperature.

3. Results and discussion

3.1. Characterization of AgNPs

In the current study, an electrochemical method was used to prepare uniform AgNPs of 2 nm, 15 nm and 40 nm by tuning the voltage and flow rate of electrolytic solution. Besides, PVP, which is one of the most commonly applied stabilizers and protective agents for AgNPs synthesis, was used as the protecting agent during the synthesizing to prevent aggregation and promote the nucleation of AgNPs. Fig. 2 shows the TEM image and the absorbance spectra revealed that the AgNPs in the present study were in approximately spherical shape and the plasmon absorbance intensity of them was changed. AgNPs with three sizes displayed various absorption peaks, which were 428 nm for 2 nm AgNPs, 421 nm for 15 nm, AgNPs, and 430 nm for 40 nm AgNPs, which were in accordance with the typical surface plasmon resonance (SPR) absorption band of AgNPs proved to be a very useful technique for the analysis of nanoparticles.

As shown in Fig. 3(A-C), the size distribution pattern the mean sizes of AgNPs in the current study were 2.44 ± 0.78 nm, 15.17 ± 4.08 nm and 40.03 ± 4.57 nm, which were statistically obtained by analysis of the recorded TEM images. Fig. 3(D-F) shows SERS spectra for DTNB-labeled AgNPs of 2 nm, 15 nm and 40 nm. The result displays that the Raman intensity of DTNB labeled AgNPs of 2 nm, 15 nm and 40 nm at 1332 cm^{-1} is 10 times, which indicating the excellent SERS enhancement effect of AgNPs. Through the comprehensive comparison TEM and plasmon absorbance intensity and SERS intensity we select 15 nm for the following experiments.

3.2. Characterization of AgNPs tags and PcAb-conjugated magnetic microspheres substrate

AgNPs can be easily functionalized with DTNB through the Ag-S bond, the formed Ag-S bond is stable in physiological conditions.

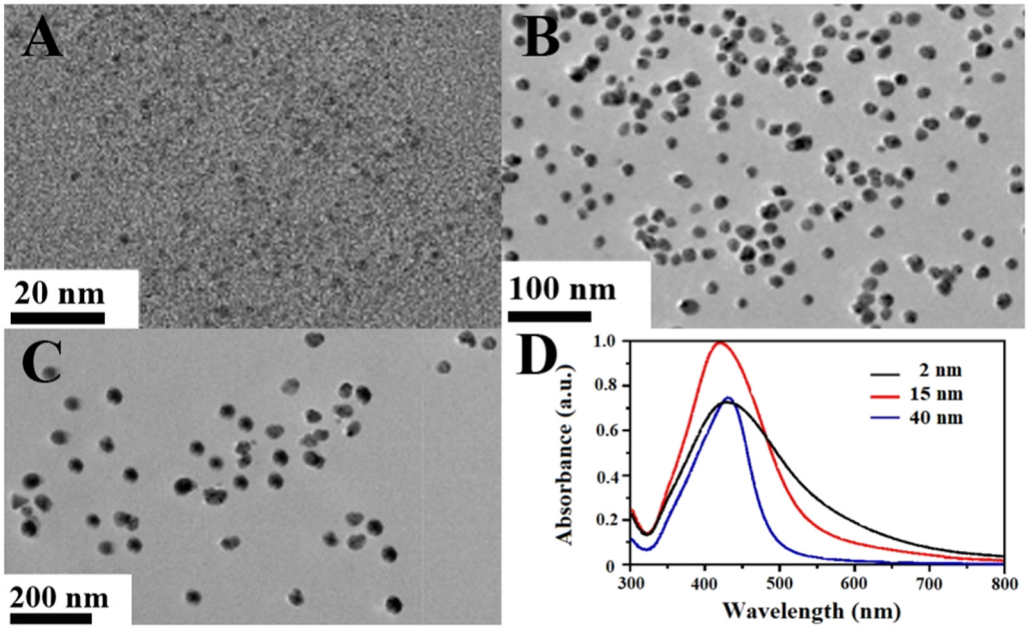


Fig. 2. TEM images of (A) 2 nm AgNPs and (B) 15 nm AgNPs and (C) 40 nm AgNPs prepared by electrochemical method. D UV–Vis absorption spectrum of AgNPs.

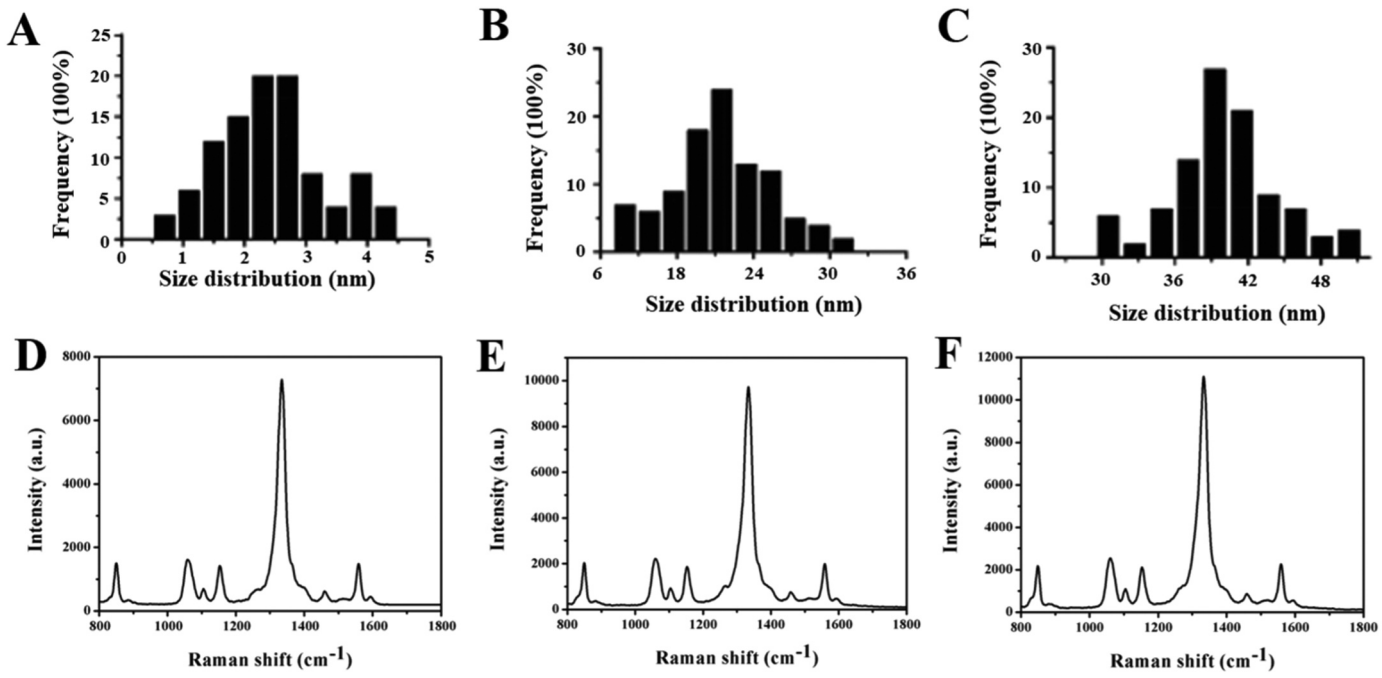


Fig. 3. Size distribution histograms images of (A) 2 nm AgNPs and (B) 15 nm AgNPs and (C) 40 nm AgNPs. (D-F) SERS spectra for DTNB-labeled AgNPs.

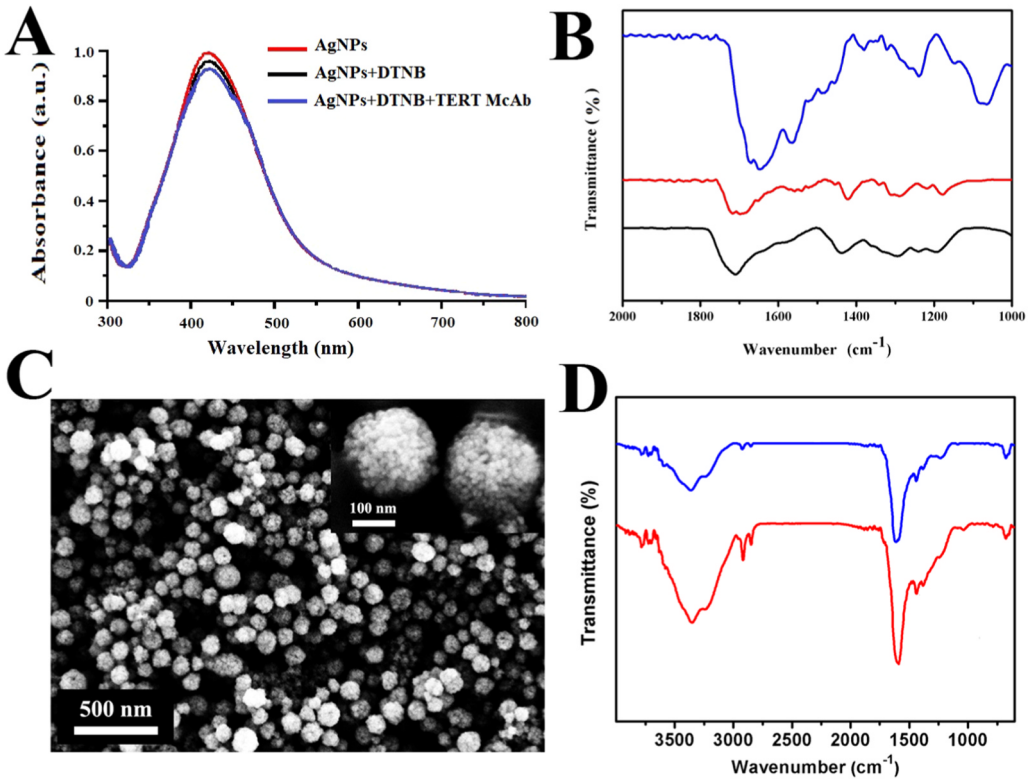


Fig. 4. (A) UV–visible spectra of AgNPs tags. (B) FT-IR spectra of McAb-conjugated AgNPs tags. (C) SEM images of Fe_3O_4. (D) FT-IR spectra of MMP-9 PcAb-conjugated magnetic microspheres.

Firstly, the DTNB was modified on AgNPs, and then modified MMP-9 McAb. The UV–vis spectra of the AgNPs tags is shown in Fig. 4A. The extinction spectrum of AgNPs tags also proves that the surface modification procedures do not disrupt the stability of the AgNPs, because the SPR band of the AgNPs-tags only exhibited a slight red-shift while no obvious tailing in the longer wavelength is observed in Fig. 4A. The red-shift of the spectrum is the increase in refractive index around AgNPs, which is caused by the modification of DTNB and MMP-9 McAb. Fig. 4B gives the FT-IR spectra of DTNB-AgNPs (red) and McAb-conjugated AgNPs tags (black). The bands at 3729 cm^{-1} and 675 cm^{-1} appeared in the two spectra are assigned to in plane bending vibration of OH (alcohol) and out plane bending vibration of OH, respectively. The peak at 1600 cm^{-1} also appeared in two spectra corresponding to the benzene skeleton vibration of C=C from DTNB, but the peaks at

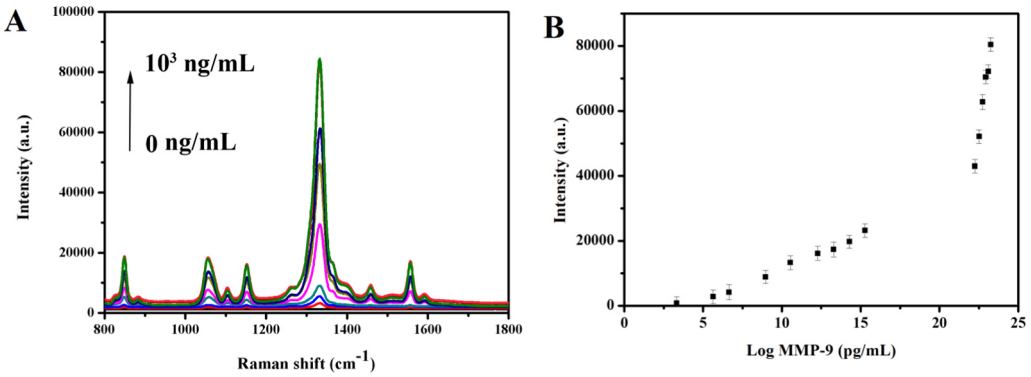


Fig. 5. SERS spectra of sandwich immunocomplexes from AgNPs-based SERS tags and magnetic microspheres with serial concentrations of MMP-9 (Concentration of MMP-9 from bottom to up is blank、1×10^{-3}、1×10^{-1}、1×10^{0}、1×10^{1}、1×10^{2}、 and 1×10^{3} ng/mL).

1126 cm^{-1} and 1374 cm^{-1} assigned to primary amine C-N stretching mode and acid amides C-N only appeared in the spectrum of McAb-conjugated AgNPs. The existence of amide bond shows that anti-MMP-9 are successfully conjugated to DTNB. As shown in Fig. 4C, the SEM images of prepare uniform Fe_3O_4 microspheres. Fig. 4D shows the FT-IR spectra of magnetic microspheres (red) and MMP-9 PcAb-conjugated magnetic microspheres (black). The bands at 1018 cm^{-1}, 1131 cm^{-1}, 1241 cm^{-1}, and 1407 cm^{-1} were assigned to primary amine C-N stretching mode and acid amides C-N, respectively. The existence of amide bond shows that anti-MMP-9 was successfully conjugated to magnetic particles. Overall, the FT-IR spectra provided evidence that McAb-conjugated AgNPs tags and MMP-9 PcAb-conjugated magnetic microspheres have been successfully prepared.

3.3. AgNPs-based sandwich immunoassays

Fig. 5 shows the results of AgNPs-based sandwich immunoassays. The relative Raman intensity of DTNB at 1332 cm^{-1} was used as a quantitative evaluation of the MMP-9 antigen levels. As shown in Fig. 5A, when the concentrations of MMP-9 increased from 1 pg/mL to 100 ng/mL, the Raman intensity of DTNB at 1332 cm^{-1} increased along with the concentration of MMP-9, implied that more sandwich immunocomplexes were formed in high concentration of MMP-9 solutions. The Fig. 5B shows of calibration curve shows that the SERS intensity at 1332 cm^{-1} is proportional to the low and high MMP-9 concentration and shows a very good linear reaction within the detected range. In this case, the limit of detection (LOD) was estimated to be 1 pg/mL. This SERS proved that the synthesizing SERS tags used in SERS-detected sandwich immunoassays.

The details of the SERS immunoassay for MMP-9 detection are shown in Scheme 1. Based on the specific combination of MMP-9 antigen and MMP-9 antibody, SERS tags can conjugate with magnetic beads. During the process of immunoassays, the MMP-9 antigen molecules with different concentrations (0 pg/mL, 1 pg/mL, 5 pg/mL, 10 pg/mL, 50 pg/mL, 100 pg/mL, 500 pg/mL, 1.5 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL) were detected with magnetic beads immunoassays. Under light excitation, the Raman signal of DTNB used as the Raman reporter and linking group is greatly amplified and influenced by the concentration of MMP-9 antigen molecules. The SERS spectra in Fig. 6A indicate that there is a positive correlation between the SERS peak intensity at 1332 cm^{-1} for DTNB and MMP-9 concentration raging from 0 pg/mL to 40 ng/mL. Furhermore, Fig. 6B shows a good linear relationship, which is described by the equation $y = 5596\times + 6439.7$ with a correlation coefficient (R2) of 0.99. From the results, we can learn that the SERS immunoassay for MMP-9 antigen based on AgNPs is sensitive and effective.

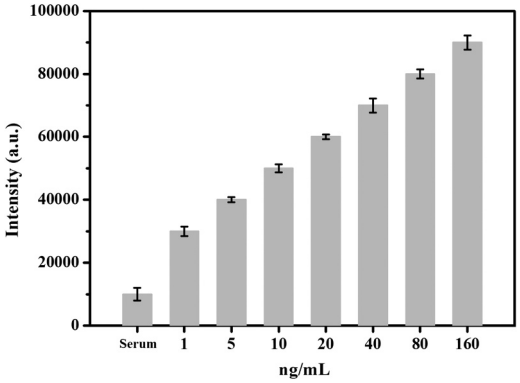


Fig. 7. SERS intensities of 1332 cm^{-1} for various concentrations of MMP-9 in human whole blood.

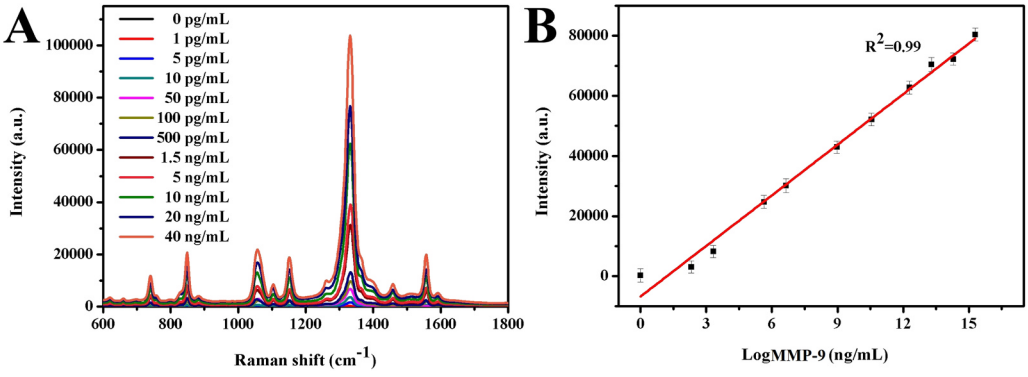


Fig. 6. (A) Average SERS spectra for the MMP-9 antigen molecules with different concentrations (0 pg/mL, 1 pg/mL, 5 pg/mL, 10 pg/mL, 50 pg/mL, 100 pg/mL, 500 pg/mL, 1.5 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 40 ng/mL) were detected with magnetic beads immunoassays. (B) The calibration plots of the SERS peak intensity at 1332 cm^{-1} versus the logarithm value of MMP-9 concentration.

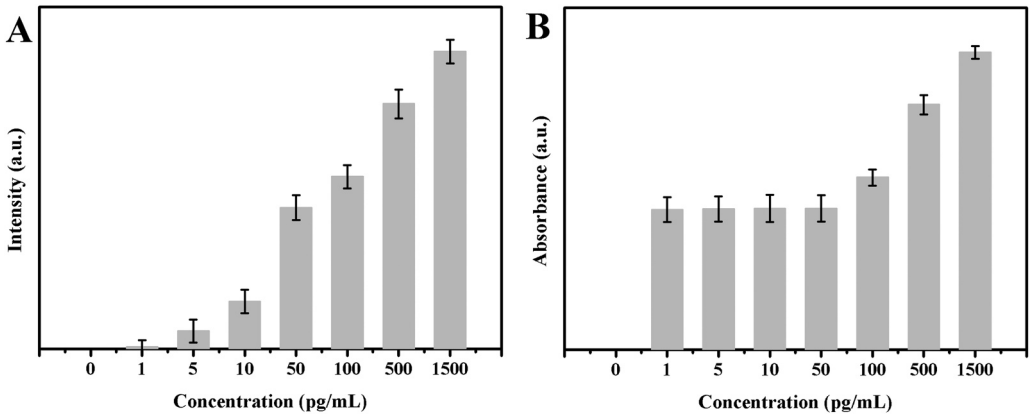


Fig. 8. Comparison of the assay results for different concentrations of MMP-9 using (A) the reference (SERS assay) method and (B) the proposed (commercially available ELISA Assay Kit) method. The error bars indicate the standard deviations calculated from five measurements.

Table 1
Assay results of clinical serum samples using the SERS and the ELISA methods.

Analyte	MMP-9	
Number	ELISA (ng/mL)	SERS-based assay (ng/mL)
1	1.05 ± 0.037	1.03 ± 0.035
2	3.73 ± 0.070	3.42 ± 0.055
3	8.52 ± 0.130	8.65 ± 0.061
4	2.01 ± 0.076	2.02 ± 0.041
5	4.81 ± 0.050	4.51 ± 0.045
6	9.53 ± 0.085	9.60 ± 0.043
7	1.12 ± 0.042	1.10 ± 0.034
8	5.71 ± 0.047	5.55 ± 0.056
9	6.33 ± 0.064	6.41 ± 0.054
10	7.73 ± 0.044	7.72 ± 0.042

3.4. MMP-9 detection

The concentration of MMP-9 plays an important role in diagnosis and prognosis of stroke. The levels of MMP-9 are higher liver cancer from 40 ng/mL to 100 ng/mL. To testify the feasibility of the MMP-9 SERS method in practical analysis, we employed the SERS method to measure different concentrations of MMP-9 spiked into normal human whole blood. The SERS intensities at 1332 cm^{-1} of different MMP-9 concentrations in cell extract are plotted in Fig. 7. Normal human whole blood is chosen as the control group, shows a quite weak intensity of ~4700 counts. As the concentration of MMP-9 increase in human whole blood, the Raman intensity of the SERS method significantly intensified. All the above observations indicate that the SERS method offers a method to detect MMP-9 in human whole blood.

3.5. Clinical application

To investigate the potential clinical application of our SERS assay, we compared its analytical performance with that of a commercially available ELISA assay kit (Quant-iT™ PicoGreens). Eight different concentrations of MMP-9, ranging from 0 to 1500 pg/mL, were prepared and tested. As shown in Fig. 8, the minimum detectable concentration of MMP-9 using the commercial assay kit is 50 pg/mL; this is much higher than the minimum detection limit measured by our SERS assay (1 pg/mL). Furthermore, it is also possible to carry out a highly sensitive quantitative assay of MMP-9 in the lower concentration range. This means that our SERS assay is suitable for the early diagnosis of MMP-9 infections in the clinical laboratory.

The SERS assay is easy to operate, which enables clinical blood plasma samples to be measured rapidly with a portable Raman spectrometer. The clinical impact of such an inexpensive and rapid bedside diagnostic tool stands to change practice in the diagnosis of stroke in both the clinical and research arenas through early, easy, and accurate detection of true stroke and will reduce the diagnostic radiation exposure, increase the accuracy of the diagnosis, decrease costs, allow for earlier interventions aimed at mitigating both short and long term sequelae, and improve the quality of stroke clinical trials.

The feasibility of the prepared immunosensor for possibly clinical application is investigated by analyzing human whole blood samples from students of Affiliated Hospital of Taishan Medical University. The immunoassay for human serum samples is investigated by analyzing three real samples in comparison with ELISA technique. Each human serum sample is analyzed for ten times. Table 1 demonstrated that there is no signification difference between the results given by the two methods. As a result, the prepared immunosensor could be reasonably applied in the clinical determination of MMP-9.

4. Conclusion

In summary, a new SERS method MMP-9 detection method is successfully presented here, which is quite sensitive and straightforward. The feasibility of this protocol is confirmed by using human whole blood lines as the MMP-9 positive samples and normal human cell as the negative control. The SERS method detection limited is much lower than those of previously published methods. Compare PCR technology, the SERS method provides excellent reliability and simplicity. Since MMP-9 is a vital biomarker in stroke development, this sensitive and efficacious SERS method promising potential for MMP-9 based diagnosis and treatment of stroke diseases.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.02.040.

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