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ORIGINAL PAPER

Direct determination of the tumor marker AFP via silver nanoparticle enhanced SERS and AFP-modified gold nanoparticles as capturing substrate

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Abstract

The authors describe a rapid and direct SERS-based immunoassay for the determination of AFP, an important marker for diagnosis of hepatocellular carcinoma. Silver nanoparticles (AgNPs; 36 nm i.d.) serve as a support to immobilize antibody and as a SERS intensifier, and AFP-modified gold nanoparticles are employed as capturing substrate. Direct and quantitative detection of AFP is accomplished with a limit detection as low as 5 ng·mL^{-1}. Compared to assays based on the use of metal nanoparticles, the use of gold-silver nanoparticle heterodimers as an active SERS substrate can save costs because only a single antibody is required. Moreover, the high selectivity and good linear relationship of detecting AFP in fetal bovine serum indicates its potential applicability for the direct analysis of clinical samples.

Keywords Gold–silver nanoparticle heterodimers · Surface enhanced Raman spectroscopy · AFP antigen detection · Fetal bovine serum

Introduction

Alpha fetoprotein (AFP), a plasma protein originated by the yolk sac and the liver in fetal development, is one of the most confessed cancer biomarkers in clinical diagnosis and treatment [1]. Elevated AFP concentrations in plasma are connected to liver cancer and digestive tract cancer, while rising concentration in amniotic fluid may indicate severe congenital fetal defects such as spina bifida and anencephaly [2]. The average concentration of AFP in healthy human serum is approximate 25 ng•mL^{-1} [3]. Therefore, a direct method with a detection limit of less than 25 ng•mL^{-1} should be developed.

Various techniques of detecting AFP have been studied before, such as enzyme-linked immunosorbent assay (ELISA) [4], immunoradiometric assay (IRMA) [5], electrochemical method [6], quartz crystal microbalance (QCM) [7] and fluoroimmunoassay [8]. Although many detecting methods have been developed, some drawbacks still exist such as the detection was less sensitive and flexible in terms of design and application and fail to provide quantitative data with broad linear range. As a method drawing widely attractions and interests, surface-enhanced Raman scattering (SERS) technology can be utilized for providing signals with abundant information in biological sensing with the advantage of not destroying biological samples [9]. As is well known, the electromagnetism enhancement effect was the dominant contributor to the SERS activity of the noble metal nanostructures so that the morphology, size, dielectric constant of metal and the refractive index of around dielectric can strongly influence the SERS spectroscopic signatures [10]. Among various metal nanoparticles, Au nanoparticles (AuNPs) with great chemical stability [11] as well as good biocompatibility [11, 12] and Ag nanoparticles (AgNPs) with strong surface plasma resonance have been generally applied for the highly selective and sensitive detection of molecules with SERS [13, 14].

We report an ultrasensitive SERS-based immunoassay structure based on AuNPs and AgNPs. The 4-MBA-labeled Au or Ag nanoparticles was combined with AFP antigen and antibody, respectively. With the addition of BSA, the residual vacant portion was blocked and the immune structure was

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achieved by the high specific affinity of antigen–antibody immune recognition. This SERS-based biosensor allows the quantitative detection of AFP antigen to be performed directly in the aqueous phase while the absence of secondary antibody further simplifies the detection process. Moreover, the large detection range and specific performance in phosphate buffer, as well as quantitative detection in practical samples, were demonstrated by this approach, which indicates the SERS-based immunoassay is a promising technique for tumor markers detection in clinical diagnosis.

Experimental section

Chemicals and apparatus

Alpha Fetoprotein (AFP), Alpha Fetoprotein antibody (anti-AFP) and carcino-embryonic antigen (CEA) were purchased from Linc-Bio Science Co. Ltd. (Shanghai, China, http://en.linc-bio.com/). Chloroauric acid ($HAuCl_4$) was from Sigma-Aldrich (Shanghai, China, https://www.sigmaaldrich.com/china-mainland.html) and bovine serum albumin (BSA) was from CNS bioservices Co. Ltd. (Beijing, China).Tannic acid was from J&K Scientic Co. Ltd. (Beijing, China, http://www.jkchemical.com/). L-ascorbic acid (AA) was purchased from Xilong Chemical Co. Ltd. (Guangzhou, China, http://www.xilongs.com) and folic acid (FA) and urine acid (UA) were acquired from Sangon Biotech Co. Ltd. (Shanghai, China, http://www.sangon.com/).Potassium chloride (KCl), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), sodium chloride (NaCl), potassium dihydrogen phosphate (KH_2PO_4), sodium citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$), silver nitrate ($AgNO_3$) and D-Glucose were obtained from Beijing Chemical Works (Beijing, China, http://www.beijingchemworks.com/).All solutions were prepared with Milli-Q water from a Millipore system.

The characterization was achieved by an UV − vis spectrophotometer (Evolution 60S, Thermo Fisher Scientific, Waltham, MA) and transmission electron microscopy (TEM) (JEM-2100, JEOL) during the experiment. Raman spectrometer (Jobin Yvon HR-800, France) was utilized to characterize the SERS intensity of the samples and detect AFP antigen, with a holographic grating of 600 g/mm, an air-cooled He − Ne laser of 632.8 nm, and an excitation of ~30 mW.

Preparation of 4-mercaptobenzoic acid labelled AuNPs and AgNPs

Gold nanoparticles and silver nanoparticles, which were employed as SERS substrates, were synthesized according to literatures [15, 16]. The detailed experiments were displayed in Electronic Supporting Material. The properties of the obtained AuNPs and AgNPs were characterized by UV-vis spectrophotometry together with TEM images.

4-mercaptobenzoic acid labelled nanoparticles were obtained as follows: 0.5 mL of 4-MBA (0.1 mM) aqueous solution was added to 4.5 mL of the purified nanoparticles with stirring for 1 h and left for 12 h at room temperature. Then, the unbound 4-MBA molecules were removed by centrifugation, and the result samples were redispersed with phosphate buffer.

Binding AFP antigen and antibody to metal nanoparticles

Firstly, the AFP antigen and anti-AFP in phosphate buffer (10 mM, pH 7.4) were added into gold nanoparticles and silver nanoparticles, respectively. These mixed solutions were allowed to react for 2 h at 37 °C to achieve the binding procedure. Then 1% BSA solution was added to block the non-specific binding sites for 1.5 h.Each step above was followed by the washing procedure with 10 mM phosphate buffer containing 5% Tween-80 solution (pH 7.4) and 10 mM phosphate buffer (pH 7.4).

The SERS measurement to detect AFP antigen

The coated AuNPs solution and AgNPs solution were mixed together for 2 h to assemble the AuNP–AgNP heterodimers with the specific reaction between antigen and antibody. The samples were analyzed by the presented SERS-based immunoassay.

Sensitivity and specificity

AFP, diluted to 0, 0.05, 0.1, 0.5, 1, 5 and 10 $\mu g{\bullet}mL^{-1}$ in phosphate buffer, was added to the labelled AuNPs, and this mixture solution reacted with anti-AFP coated AgNPs to assess the sensitivity of the immunoassay. AA, CEA, FA, Glu, UA and AFP at 1 $\mu g{\bullet}mL^{-1}$ in dilution buffer were used to measure the specificity of the immunoassay. The Limit of detection (LOD) was calculated in terms of 3 folds of standard deviation (SD) above the blank value.

Detection of AFP in fetal bovine serum (FBS) samples

To evaluate the impact of serum matrices on the detection of AFP antigen using SERS test, AFP antigen with known concentrations were exposed to fetal bovine serum (FBS) diluted solution. Briefly, the FBS stock solution was diluted with phosphate buffer to reduce the concentration of interfering substances and utilized to prepare the AFP antigen solution. And then this AFP antigen with FBS solution reacted with anti-AFP. The performance of detecting in FBS was investigated by SERS measurement in the same way.

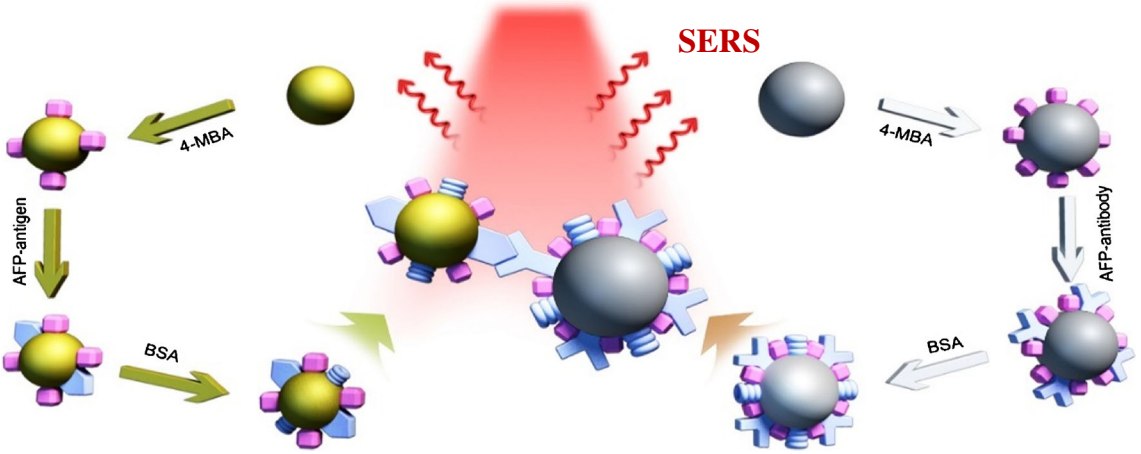

Fig. 1 The scheme diagram of the SERS detecting platform for AFP antigen

Results and discussion

Principle of the SERS bioassay

Figure 1 depicts the principle of the SERS detecting platform for AFP antigen utilizing the specific interaction between antigen and antibody. Briefly, the first step is binding 4-mercaptobenzoic acid (4-MBA) with metal nanoparticles by the thiol group of 4-MBA. The reason of utilizing 4-MBA molecule as a SERS detection probe is from the fact that the use of Raman labels avoids complicating background signals originating from the biological system [17]. TEM images were used to confirm the fact that morphology of metal nanoparticles had no obvious change before and after 4-MBA adsorption. The result was shown in Fig. 2. Gold or silver nanoparticles with large diameters (>30–50 nm) have low optical losses and allow a large number of electrons to participate in the local plasmon resonances, which can lead to a strong enhancement of light intensity. In addition, small gold nanoparticles have more stable properties to be applied to biomedical therapeutic fields [9, 18] and gold nanoparticles with small diameter are photo stable and ease in bioconjugation [11]. Based on the above theories, this paper selected 15 nm gold nanoparticles as AFP antigen loaded substrate and 36 nm silver nanoparticles coated antibody. The next step is the procedure of AFP antigen and anti-AFP conjugated to the gold and silver surface, respectively. Anti-AFP attached and immobilized on the silver

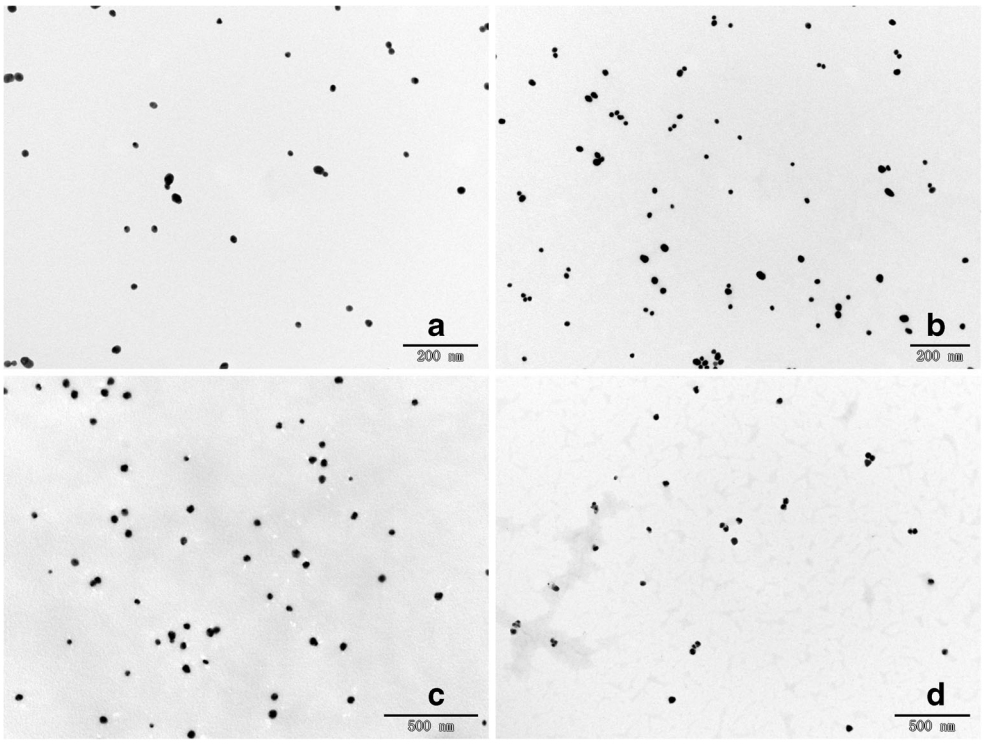

Fig. 2 TEM images of metal nanoparticles before and after modification. **a** and **c** was pure AuNPs and AgNPs. **b** and **d** was 4-MBA functionalized AuNPs and AgNPs

nanoparticle surface via strong ionic interactions in addition to the Au-S bonds, which was proved by the change of characteristic peak wavelength of AgNPs (As shown in Fig. S1). And then BSA was used to block non-specific sites. Based on our previous work, the SERS signal of 4-MBA loaded on gold nanoparticles with 15 nm diameter would not present an obvious SERS signal [19]. Therefore, the signal enhancement originated mainly from electromagnetic effect attributed to the localized surface plasmon of 36 nm AgNPs and the surface plasmon coupling of AuNPs and AgNPs. In addition, it is similar that strong Raman enhancement can't be achieved when the Raman reporter molecule (4-MBA) is attached to AgNPs. The specific conjunction between antigen and antibody can be proved by the obvious signal change, as shown in Fig. S2. At last, different concentrations of AFP was mixed with AuNPs and captured by anti-AFP immobilized AgNPs, generating different SERS signals corresponding to different concentrations for readout.

Optimization of the method

Considering the main factors of reaction environment and the sensitivity of our presented SERS platform, the following parameters were optimized: (a) temperature; (b) sample pH value; (c) diameter of AgNPs; (d) concentration of 4-MBA. Respective data and Figures are given in the Electronic Supporting Material (Fig. S3 and Fig. S4). As more suitable for biological reaction and the high Raman scattering cross sections, the following experimental conditions were found to give best results: (a) Best temperature: 37 °C; (b) Best sample pH value: 7.4; (c) Best concentration of 4-MBA: 10^{-4} M; (d) Best diameter of substrate: 36 nm.

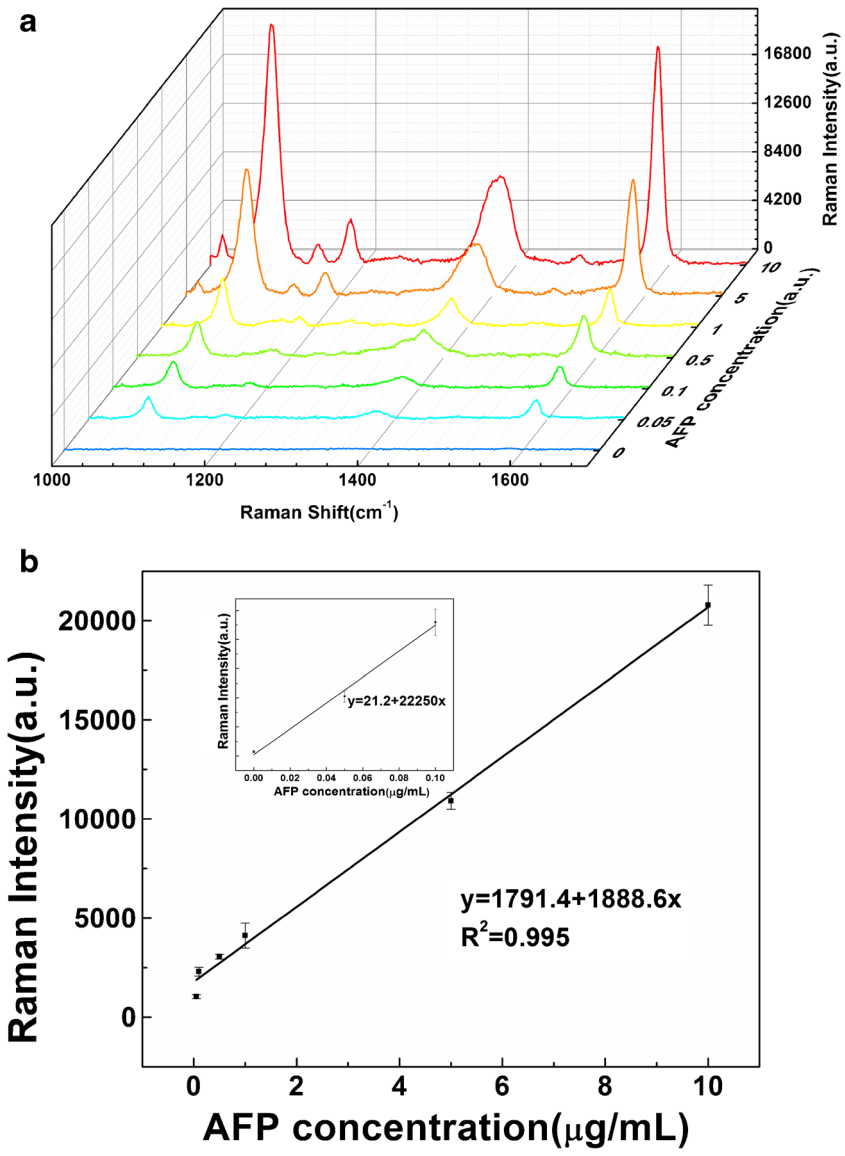

Fig. 3 A was SERS spectra of this presented biosensor detecting different concentrations of antigen. AFP concentrations of 10 μg/ml, 5 μg/ml, 1 μg/ml, 500 ng/ml, 100 ng/ml, 50 ng/ml and a blank sample were detected by this method. B was plots of the Raman intensity of the band at 1079 cm^{-1} versus the antigen concentration

Sensitive and quantitative detection of AFP antigen

The sensitive and quantitative detection of AFP antigen was conducted with SERS. From the section of Principle of the SERS Bioassay, It is acknowleged that Raman signal from 4-MBA would be enhanced when the AFP antigen was mixed with anti-AFP. There are two sharp characteristic peaks of 4-MBA in 1080 and 1590 cm^{-1} [20], which belonged to ν(C-C) ring-breathing modes. We chose the signal intensity of 1079 cm^{-1} as the quantitative analysis standard. The SERS spectra of 4-MBA on metal nanoparticles under different AFP antigen concentrations were shown in Fig. 3a. Figure 3b depicted the linear relationship between concentrations of AFP antigen and the normalized Raman intensity. The obtained calibration curve indicated an excellent linear range from 0.05 to 10 μg•$mL^{-1}$and exhibited a good correlation coefficient (R^2) of 0.995. The limit of detection (LOD) was calculated to be 5 ng•mL^{-1}.

Specificity

To prove that the enhanced AFP detection is specific, the response of some tumor markers and interfering species on this sensor was compared with AFP antigen at 1.0 μg•mL^{-1}. Based on antigen–antibody immunoreaction, there was obvious signal enhancement only in the presence of AFP antigen, while less signal change was observed to the additions of ascorbic acid (AA), carcino embryonic antigen (CEA), folic acid (FA), glucose (Glu) and uric acid (UA) in the same experimental conditions, which is shown in Fig. 4. The above result indicated the specificity of the presented AFP sensor was satisfactory.

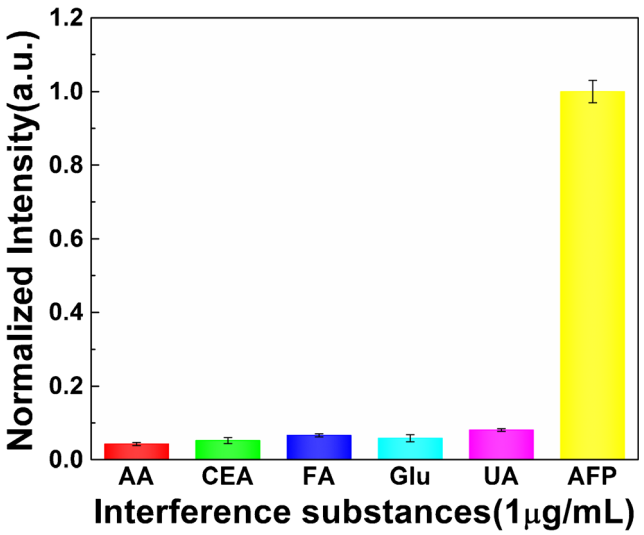

Fig. 4 Selectivity assay toward AFP antigen against other interferences, including AA, CEA, FA, Glu, UA. The control is the Raman intensity in the presence of different targets

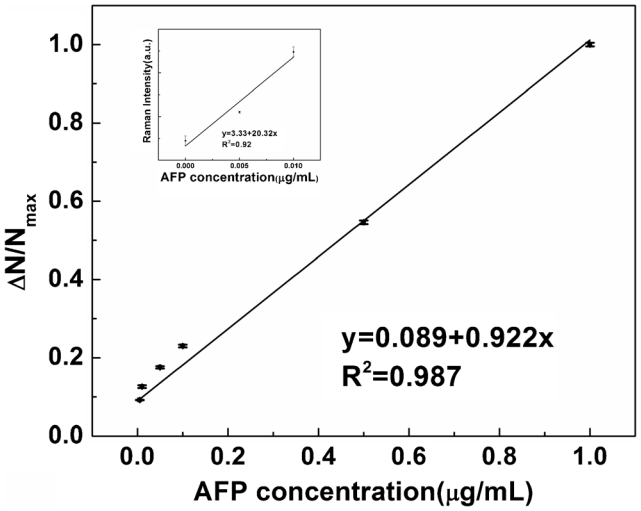

Fig. 5 Raman relative intensity at 1079 cm^{-1} varies with different concentrations of AFP under the optimized conditions. The inset of B shows the limit of detection of AFP

AFP detection in real samples

To demonstrate the feasibility of this sensing strategy for clinical applications, fetal bovine serum (FBS) samples spiked with different concentrations of AFP ranging between 0.005 and 1 μg•mL^{-1} was monitored using the presented sensor. Fetal bovine serum was a complicated environment, so this stock solution was spiked with phosphate buffer to evade disturbance. As shown in Fig. 5, there was also linear relationship between the signal response and concentrations of AFP antigen. More importantly, lower LOD of 2 ng•mL^{-1} was obtained in FBS compared with the calculated result in phosphate buffer. The possible reason was that the mobility of AFP molecules was hindered by serum protein in the FBS and few AFP antigen can reach to the sensor surface [21]. Even though, this phenomenon can show that this presented SERS sensor has a promising future for the rapid and sensitive detection of tumor biomarkers in clinical samples. We display a comparison between our method and others aiming at some specific features for detection of AFP in Table S1, and the result indicates that our presented method is the same or easier to obtain than other spectroscopic detection methods. Although the electrochemistry immunoassay has higher sensitivity for detecting AFP, the spectroscopic method can be more convenient in practical application.

Conclusion

To sum up, SERS immunosensor utilizing the advantage of different sized metal nanoparticles as SERS substrate are presented. The fabricated substrates enable quantitative analysis of AFP within the range from 0.05 to 10 μg•mL^{-1}. AuNPs coupled with AgNPs by the specific reaction between antigen

and antibody can be used to efficiently enhance SERS effect, achieving higher sensitivities (LODs: 5 ng•mL^{-1}) and selectivity. In addition, this work simplified the detection procedure by using only one antibody and detecting directly to detect in fetal bovine serum, which provides a promising application of detecting tumor marker in a direct and fast way without complicated experimental treatment.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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