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Detection of Hepatitis B virus antigen from human blood: SERS immunoassay in a microfluidic system

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ABSTRACT

A highly sensitive immunoassay utilizing surface-enhanced Raman scattering (SERS) has been developed with a new Raman reporter and a unique SERS-active substrate incorporated into a microfluidic device. An appropriately designed Raman reporter, basic fuchsin (FC), gives strong SERS enhancement and has the ability to bind both the antibody and gold nanostructures. The fuchsin-labeled immuno-Au nanoflowers can form a sandwich structure with the antigen and the antibody immobilized on the SERS-active substrate based on Au–Ag coated GaN. Our experimental results indicate that this SERS-active substrate with its strong surface-enhancement factor, high stability and reproducibility plays a crucial role in improving the efficiency of SERS immunoassay. This SERS assay was applied to the detection of Hepatitis B virus antigen (HBsAg) in human blood plasma. A calibration curve was obtained by plotting the intensity of SERS signal of FC band at 1178 cm^{-1} versus the concentration of antigen. The low detection limit for Hepatitis B virus antigen was estimated to be 0.01IU/mL. The average relative standard deviation (RSD) of this method is less than 10%. This SERS immunoassay gives exact results over a broad linear range, reflecting clinically relevant HBsAg concentrations. It also exhibits high biological specificity for the detection of Hepatitis B virus antigen

1. Introduction

Surface-enhanced Raman spectroscopy (SERS), with its ability of nondestructive, ultrasensitive, reliable and fast detection of samples, has been extensively employed for analyzing complex biological molecules, ranging from DNA (Ke et al., 2005; Sanchez-Cortes et al., 2002) and peptides (Ortiz et al., 2004; Stewart and Fredericks, 1999) to whole proteins (Chowdhury et al., 2006) and cells (Huang et al., 2006). The most notable recent advances in SERS include innovative applications of biomolecular sensors for clinical diagnosis, biochemical, and environmental studies. In particular, a SERS immunoassay, which is based on a specific interaction between an antigen and a complementary antibody is a powerful analytical tool for clinical diagnosis (Driskell et al., 2007, 2005; Kim et al., 2006). Conventional immunoassays include radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). SERS-based immunosensors generally rely on the SERS tags consisting of Raman reporters, active substrates (silver or gold nanostructures), and conjugated antibodies (Grubisha et al., 2003; Han et al., 2008a, 2008b). Typically, the SERS-based immunoassays were carried out according to a standard sandwich protocol of ELISA. For example, an antibody immobilized on a solid substrate reacts with an antigen, which binds to another antibody labeled with peroxidase. If this immunocomplex is subjected to the reaction with *o*-phenylenediamine and hydrogen peroxide, azoaniline is generated, giving a strong SERS signal. Some authors (Dou et al., 1997; Xu et al., 2004) utilized the immunoreaction between immunogold colloids modified by Raman-active probe molecules, e.g. 4-mercaptobenzoic acid, and antigens, which were captured by antibody-assembled chips. Application of SERS in immunosensing included also a successful detection of the thyroid stimulating hormone (THS) (Rohr et al., 1989), monitoring the immunocomplex formation between mouse IgG and goat anti-mouse IgG (Zhang et al., 2008) or detection of membrane-bound enzymes within cells (Hawi et al., 1998). The strength of SERS-based detection lies in the combination of its high sensitivity and facility for multiplexing. Multiple analyses can be performed by using nanoparticles coated with different Raman reporter molecules and by immobilizing mixtures of different antibodies. Additionally, Raman responses are much less susceptible to photobleaching than fluorescence, allowing extended signal averaging in order to lower detection limits (Goluck et al., 2006).

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Previous SERS immunoassays were usually based on extrinsic Raman labels consisting of gold nanoparticles that were coated with co-adsorbed Raman reporter and antibody (Song et al., 2009; Yoon et al., 2010). This strategy has several disadvantages. First, weakly adsorbed antibodies may desorb from one metal particle and re-adsorb on another. This may lead to a spectral signature related to non-specific adsorption and hence hamper multiplex analysis. Second, problems appear with the particles aggregation, which may also be caused by the desorption of antibodies. In order to solve these problems, an alternative approach was investigated in the present study. Our Raman reporter molecule was strategically designed to chemisorb on a thiolate layer on a gold nanoparticle, to provide unique and strong Raman spectrum, and to covalently bind the antibody via a terminal amino group. This group can react with the carboxylic group of a protein activated earlier by EDC/NHS standard procedure to form an amide linkage (EDC=1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and NHS=hydroxysulfosuccinimide; see Supplementary material for more details). The added advantages of our method reflect the use of gold-coated GaN substrate as the immune-solid platform. The SERS-active solid substrate plays an important role in the sensitivity of SERS-based immunoassay. It was found that the enhanced electromagnetic field is not only excited around the gold or silver nanoparticles, but is also generated on the SERS-active substrate (Kusnezow and Hoheisel, 2003; Song et al., 2009). In the past, most substrates in SERS-based immunoassays were prepared without SERS activity. Among them, glass slides were used most widely as solid SERS supports (Zhang et al., 2007; Cao et al., 2003). We have developed a novel SERS-active substrates based on Au or Au–Ag coated GaN with bunched nanopillars (Kamińska et al., 2011; Weyher et al., 2012). The resultant SERS platforms exhibit very strong surface-enhancement factor (up to 1×10^7), high stability (up to three months under ambient conditions) and high reproducibility, which could be used in the design of efficient SERS-active platforms for analytical applications. Moreover, the incorporation of our solid SERS platform into a microfluidic device offers a significantly larger active surface for immune reactions and hence improved performance of the immunoassay.

Finally, we combined the advantages of our unique SERS-active substrate based on Au–Ag coated GaN with those of the labeling method and developed a novel SERS immunoassay to detect Hepatitis B virus antigen (HBsAg) in human blood or blood plasma within a microfluidic system. Hepatitis B virus (HBV) infection is a very common cause of chronic liver disease worldwide and the HBsAg is most frequently used to screen for the presence of this infection. HbsAg is a complex particle, about 20 nm in diameter,

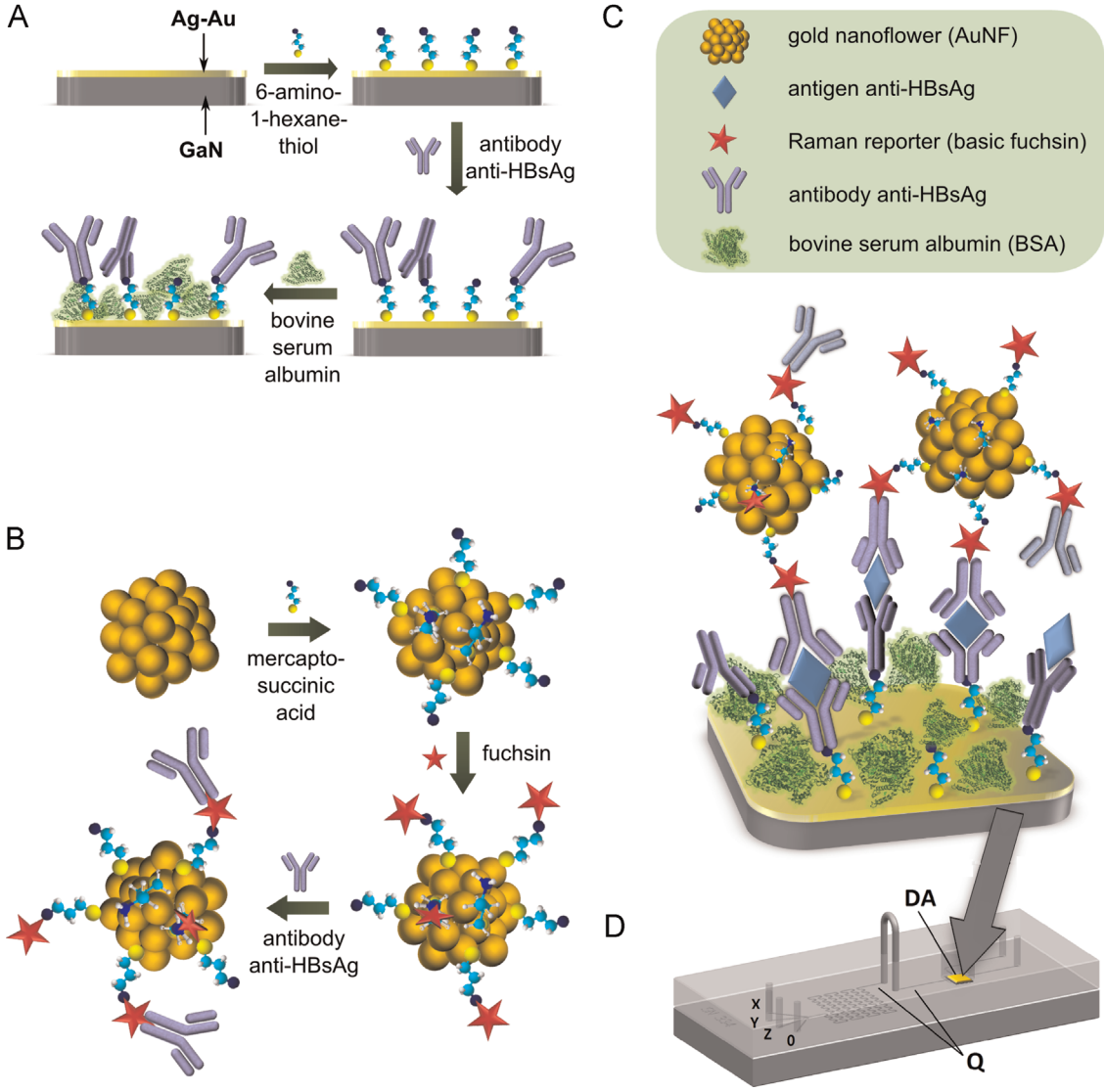

Fig. 1. Sequential steps for the formation of the SERS-based. immunoassay. (A) The capturing substrate preparation, (B) the Raman reporter-labeled immuno-Au nanoflowers synthesis, and (C) SERS detection of the sandwich interactions, (D) schematic illustration showing the integration of a microfluidic device with the SERS-active substrate based on Au–Ag coated GaN surface. DA, detection area chamber with GaN/Au–Ag SERS substrate.

composed of a protein, a carbohydrate, and a lipid (Skelly et al., 1979). It is the first detectable viral antigen appearing during infection.

2. Methods

2.1. General procedure

The performed immunoassay is based on a sandwich structure consisting of three layers (Fig. 1). The first layer is composed of immobilized Hepatitis B virus (anti-HBsAg) antibodies captured on the GaN/Au–Ag surface via the 6-amino-1-hexanethiol (AHT) layer. The second layer is the complementary Hepatitis B virus surface (HBsAg) antigen captured by anti-HBsAg on the GaN/Au–Ag surface. The third layer consists of fuchsin-labeled immuno-Au nanoflowers (antibody–FC–AuNFs). The microfluidic SERS-device (Fig. 1) allows us to perform and control the reactions at each step of the immunocomplexes formation. Fig. 1D shows a detailed layout of this microfluidic chip. The inlets X, Y, Z,O are used for injection via the microchannel Q of the following reagents: Bovine serum albumin (BSA) in PBS buffer solution, Hepatitis B virus surface (HBsAg) antigen in human blood plasma (infection marker), Raman reporter-labeled immuno-Au-nanoflowers (nanoprobes), PBS buffer solution, respectively, into the detection area of the microfluidic device (DA in Fig. 1). The detection area (DA) is the chamber in the shape of a square containing the SERS-active substrate. This is the place where sandwich immunocomplexes are created and detected via Raman technique. Hepatitis B virus surface (HBsAg) antigen (infection marker) is identified in this chamber by the appearance of SERS spectrum of basic fuchsin (FC).

3. Experimental

3.1. Reagents

Hepatitis B virus surface antigen (HBsAg), Hepatitis B virus monoclonar antibody (anti-HBsAg) and Akt blocking peptide were obtained from Labjot Company. Basic fuchsin (FC), mercaptosuccinic acid, L-ascorbic acid, gold (III) chloride trihydrate, N-hydroxysulfosuccinimide sodium salt (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 6-amino-1-hexanethiol (AHT), bovine blood plasma albumin (BSA), (3-aminopropyl)trimethoxysilane (APS), phosphate-buffered saline (PBS) packs (10 mM, pH=7.2), and mercaptosuccinic acid (MSA) were obtained from Sigma.

3.2. Fabrication of a microfluidic chip

Fig. 1 shows a layout of the microfluidic chip fabricated in polycarbonate (PC) and integrated with SERS-active substrate based on Au–Ag coated GaN. To develop our microfluidic device we micromachined it with a computer numerical-controlled (CNC) milling machine (ErgWind, type MFG4025P) in a 5 mm polycarbonate slab (Bayer). We used MasterCAM software to design the setup. The channels have 200 or 400 μm width and 350 μm depth. To join milled and plain PC slabs we pressed them together at high temperature (T=130 °C). The detection area chamber (DA) has a square shape with dimensions of $3.5 \times 3.5\ mm^2$ and is 1.5 mm deep. A high precision syringe pump system (Harvard Apparatus Pump Series, MA, USA) was used for automated control of flow. Standard HPLC capillaries with an inner diameter of 0.5 mm were used for interconnection of the chip at the syringe pump. The SERS-active substrate, in the shape of a square with dimensions of $3 \times 3\ mm^2$ was placed into the detection area chamber (DA). This detection point is open to the air and the recorded SERS signals are not affected by the material of the microfluidic device. Additionally, polycarbonate surfaces were oxidized using UV-generated oxygen plasma to make them hydrophilic in order to reduce non-specific adsorption of various kinds of biomolecules and other reagents from tested samples.

3.3. Capturing substrate preparation

3.3.1. GaN/Au–Ag substrate

The GaN/Au–Ag SERS substrate was synthesized according to previously published procedures (Kamińska et al., 2011; Weyher et al., 2012). GaN epitaxial layers grown on sapphire by MOCVD method were subjected to photo-etching in KSO-D solution (0.02 M KOH+0.02 M $K_2S_2O_8$). The photo-etching in alkaline solutions (both in KOH and in KOH/$K_2S_2O_8$) is normally used for revealing and analyzing dislocations and electrically active defects in GaN and in SiC. Due to effective recombination of photo-generated carriers (electron–hole pairs) on dislocations, the protruding pillars are formed around these linear defects and after long etching form “sheafs” (Fig. S4). A metallic layer was deposited on photo-etched GaN samples by sputtering, using Quorum Q150R S sputter coater. An alloy of gold with silver (70–30% by weight) was used as target. The thickness of the deposited layer measured on a flat surface was 90 nm. The coated samples were immersed in 65% nitric acid (Sigma-Aldrich) for 24 h in order to perform dealloying and to obtain porous structure (Dixon et al., 2007). Subsequently, the substrates were washed in deionized water and dried in a stream of nitrogen and placed in the microfluidic chip (chamber DA in Fig. 1).

In the next step, the substrate was modified with 0.1 mM ethanolic solution of 6-amino-1-hexanethiol for 5 h to form an amino-terminated linkage monolayer. Then, the thiol-modified surfaces were completed by injecting the mixture of 5 μL of 100 μg/ml Hepatitis B virus monoclonal antibody (anti-HBsAg) and the activation solution (0.2 M EDC/0.05 M NHS; mixture in deionized water) in the ratio of 5:1 into the detection area (chamber DA in Fig. 1D). After 1 h, the remaining active surface was blocked by injecting 5 μL of 2% BSA in PBS buffer solution (pH 7.2) via inlet X (Fig. 1D). In the next step, the sample was rinsed twice with 5 mL of 10 mM PBS buffer solution via the same inlet O. Finally, the antibody-immobilized substrate was stored at 4 °C for future use.

3.3.2. Aminosilane substrate

The silicon wafer was cleaned by sequential ultrasonication in methanol, acetone and isopropanol for 15 min each. The wafer was then silanized according to the protocol described elsewhere (Goss et al., 1991). Briefly, cleaned, dried pieces of silicon were immersed in a mixture of water, isopropanol and (3-aminopropyl)trimethoxysilane (APS) and brought to reflux for 10 min. Next, each wafer was extracted with tweezers, rinsed with isopropanol and blown dry with Ar. This process was repeated twice. Finally, Hepatitis B virus (anti-HBsAg) antibodies were immobilized on these (3-aminopropyl)trimethoxysilane-modified silicon wafers using the EDC/NHS standard method for coupling amines to carboxylic acids outlined above.

3.4. Preparation of the Raman reporter-labeled immuno-Au nanoflowers (anti-HBsAg/AuNFs-Raman reporter)

For the SERS immunoassay, basic fuchsin adsorbed on gold nanoflowers was used as Raman reporter. Fuchsin-labeled immuno-Au nanoflowers (anti-HBsAg/Au-Raman reporter) were prepared in three steps. First, gold nanoflowers were obtained. The route for synthesis of gold nanoflowers (AuNFs) was modified compared to that previously reported (Boca et al., 2011). Water

solution of ascorbic acid (40 mL, 20 mM) was cooled to about 0 °C. Then water solution of tetrachloroauric acid (200 μL, 10 mM) was injected into the stirred ascorbic acid solution. The mixture rapidly changed color to blue. After 1 h of stirring in an ice bath, the mixture was allowed to warm slowly to room temperature.

The average diameter of gold nanoflowers was about 20–40 nm according to the SEM images (Fig. S3). In the second step, the Raman reporter-labeled Au nanoflowers were fabricated. Raman reporter molecules were immobilized on the prepared nanoflowers according to the following procedure. To the above-mentioned reaction mixture, water solution of mercaptosuccinic acid (MSA), (100 μL, 1 M) was added. After 6 h the mixture was cooled to about 0 °C and EDC (27 mg, 0.14 mM) was added, followed by addition of catalytic amount of NHS and solution of basic fuchsin (500 μL, 200 mM) in DMF. MSA–fuchsin coated AuNFs were collected by centrifugation (5000 rpm, 20 min) and next purified by six-fold dissolution in DMF and centrifugation procedure. Finally, the purified AuNFs were dried and suspended in 1 mL of water. In the third step, Raman reporter-labeled immuno-Au-nanoflowers (Fig. 1B) were prepared by immobilizing the antibody (anti-HBsAg) on the Raman reporter Au nanoflowers using EDC and NHS coupling reagents.

In the first step, 5 μl of 40 μg/ml anti-HBsAg (in a PBS buffer, pH 7.7) were mixed with 10 μL of solution containing Raman reporter-labeled Au nanoflowers and then the coupling reagents (0.2 M EDC/0.05 M NHS; in the ratio of 5:1, mixture in deionized water) were added to initiate the conjugation reaction at 4 °C for 4 h. The Raman reporter-labeled immuno-Au-nanoflowers were separated from solution by centrifugation at 20,000 rpm for 10 min. Finally, the suspension of the Raman reporter-labeled immuno-Au-nanoflowers was blocked by adding 2.5 μL of 2% BSA in PBS buffer solution. After 2 h, the mixture was centrifuged again for 10 min at 20,000 rpm and then re-suspended in 1 mL PBS solution. The prepared Raman reporter-labeled immuno-Au-nanoflowers were stored at 4 °C for future use.

In our experiments we used human blood samples from 10 healthy volunteers available by courtesy of Regional Blood Center. The samples underwent morphological analyses prior to use and revealed no abnormalities. All plasma and serum samples were evaluated for HBsAg antigen by using commercial Architect HBsAg test, relative to 2nd HBsAg WHO international standards, in order to confirm no evidence of HBV infection. For each of the ten healthy unvaccinated volunteers the level of HBsAg was < 0.05 IU/mL. The value < 0.05 IU/mL is considered as a negative result for HBsAg test for unvaccinated persons (Scheiblauer et al., 2010).

For the determination of analytical sensitivity of our SERS-based immunoassay, the samples of HBsAg antigen in human blood plasma or serum with different concentrations were prepared in ng/mL and next converted to IU/mL, considering, that 0.2 ng/mL is equivalent to 0.05 IU/mL of HBsAg according to the previously reported calculations (Hadziyannis 2013; Deguchi et al., 2004).

3.5. Immunoassay protocol

The SERS-based immunoassay was carried out as presented in Fig. 1. Hepatitis B Virus (anti-HBsAg) antibodies were immobilized on the GaN/Au–Ag surface via 6-amino-1-hexanethiol (AHT) layer. Then, PBS buffer solution (pH 7.2) was added via inlet X (Fig. 1D) to remove physically unadsorbed antibodies. In the next step, Hepatitis B virus surface (HBsAg) antigen in human blood plasma (infection marker) and Raman reporter-labeled immuno-Au-nanoflowers (nanoprobes) were sequentially injected into inlets Y and Z (Fig. 1D), respectively, and attached to capturing GaN/Au–Ag surface for the formation of sandwich immunocomplex. Usually, the (HBsAg) antigen or Akt blocking peptides in human blood plasma at the appropriate concentration were injected into inlet Y at the rate of 1.5 μL min^{-1} and Raman reporter-labeled immuno-Au-nanoflowers were injected into the inlet Z at the rate of 2.5 μ L min^{-1}. After 3 min, the flows were stopped for 30 min and all reagents were incubated in the detection area of the microfluidic device (chamber DA in Fig. 1D) to generate sandwich immunocomplex. At the end, PBS buffer solution (pH 7.2) was again injected via inlet X (Fig. 1D) to remove physically unadsorbed antigens and other reagents.

3.6. SERS measurements

SERS measurements were performed using a Renishaw inVia Raman system equipped with a 300-mW diode laser emitting a 785 nm line used as the excitation source. The light from the laser was passed through a line filter and focused on a sample mounted on an X–Y–Z translation stage with a 10 × microscope objective. The Raman-scattered light was collected by the same objective through a holographic notch filter to block out Rayleigh scattering. A 1800 groove/mm grating was used to provide a spectral resolution of 5 cm^{-1}. The Raman scattering signal was recorded by a 1024 × 256 pixel RenCam CCD detector. The beam diameter was approximately 5 μm. Typically, the normal Raman spectra were acquired for 20 min; for SERS experiments the spectra were acquired for 30–60 s with the laser power measured at the sample being 5 mW.

4. Results and discussion

4.1. Capturing substrate and fuchsin-labeled immuno-Au nanoflowers characterization

Hepatitis B virus (anti-HBsAg) antibodies were immobilized on the GaN/Au–Ag surface via 6-amino-1-hexanethiol (AHT) layer using EDC/NHS standard coupling chemistry. The formation of the gold-bound thiolates and their subsequent coupling to antibodies was confirmed by Raman spectroscopy (Fig. S1). The successful binding of basic fuchsin and (anti-HBsAg) antibodies to the gold nanoflowers was also demonstrated by UV experiments (Fig. S2). In the next step, the SERS activity of fuchsin-labeled immuno-Au nanoflowers was examined. Fig. 2 displays the normal Raman (Fig. 2a and b) and SERS spectra of fuchsin basic (Fig. 2c). The nanoflowers sample was prepared by dropping a small amount of labeled AuNFs solution onto a glass slide. As can be seen, the normal Raman signal of FC without enhancement by AuNFs was very weak (Fig. 2a and b). The powder spectrum of FC (Fig. 2a) is dominated by bands at 738, 844, 913, 1179, 1386 and 1594 cm^{-1}. Even weaker bands appear in 10^{-4} M water solution of FC at 736, 842, 915, 1179, 1384 and 1594 cm^{-1}. SERS signal of FC was obviously very intensive, as shown in Fig. 3c. All of the bands present in the normal Raman spectrum are present in the SERS spectrum of FC after chemisorption of basic fuchsin onto gold nanoflowers. Some bands undergo a small shift in position upon adsorption onto AuNFs. The SERS spectrum of FC (Fig. 2c) is dominated by bands at 1178, 1384, and 1592 cm^{-1} due to ring C–H bending modes, N-phenyl stretching, and ring C–C stretching modes, respectively (Moskovits and Suh, 1984).

The immobilization of FC onto gold nanoflowers results in significant enhancement (Fig. 2c). Similarly, a strong characteristic SERS signal of FC (Fig. 2d) was obtained for FC-labeled immuno-Au-nanoflowers (after the antibody immobilization), which indicates that the basic fuchsin can work as a good Raman reporter for SERS immunoassay.

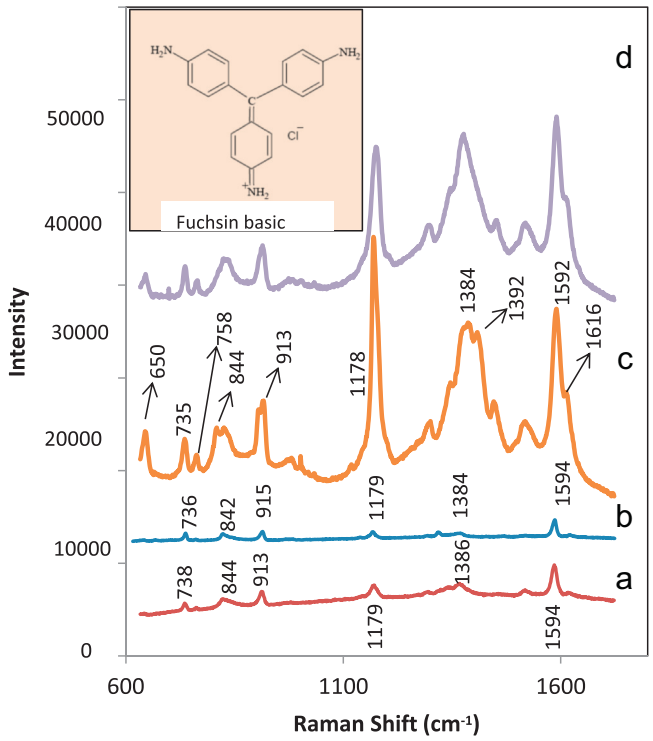

Fig. 2. Raman spectra of the Raman reporter: (a) normal Raman spectrum of FC powder, (b) normal Raman spectrum of FC solution in water (10^{-4} M), (c) SERS spectrum of gold nanoflowers following the reaction with FC, and (d) SERS spectrum of fuchsin-labeled immuno-Au nanoflowers (antibody–FC–AuNFs).

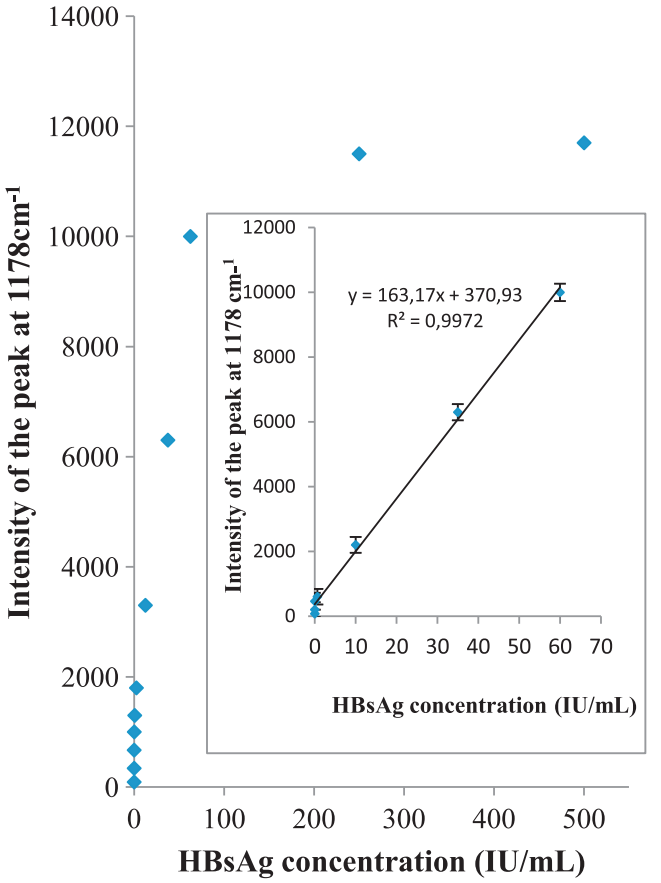

Fig. 3. The relationship between the intensity of the marker band at 1178 cm^{-1} versus the concentration of antigen in the range from 0 to 625 IU/mL. The inserted figure shows the plot of the intensity of the marker band at 1178 cm^{-1} versus the concentration of antigen in the range from 0 to 60 IU/mL The data were collected by averaging six measurements.

4.2. SERS immunoassay detection of Hepatitis B virus antigen (HBsAg)

The results of our SERS detection of (HBsAg) antigen in human blood plasma are shown in Fig. 3 and S5. Samples of HBsAg antigen in human blood plasma with different concentrations in international unit per mL (IU/mL) which are traceable to the World Health Organization (WHO) HBsAg international standard were prepared. A dilution series was created in the range of 0.0, 0.00125, 0.0125, 0.125, 0.125, 0.25, 2.5, 12.5, 35, 60, 250, and 625 IU/mL. The concentration range was chosen to cover clinically relevant HBsAg antigen concentrations. Fig. S5a–h presents the SERS spectra for selected concentrations after completion of the immunoassay protocol outlined above. Various concentrations of the antigen were applied using a microfluidic system. In order to increase the density of immunocomplexes, the SERS spectra were measured under steady state condition in the reaction chamber. We checked that under the continuous flow conditions, the intensities of SERS signals were too low to perform the quantitative analysis. Therefore, in our microfluidic chip we combined the continuous flow of reagents with the static flow (the incubation step). The intensity of the fingerprint spectra of the Raman reporter increases linearly with increasing the concentration of HBsAg antigen, indicating the potential of our immunoassay protocol for its use in the quantitative detection of antigens in the real fluid sample. Fig. 3 demonstrates the relationship between the intensity of the marker band at 1178 cm^{-1} versus the concentration of antigen over the whole tested concentration range. The inset in Fig. 3 illustrates a calibration curve obtained by plotting the intensity of the SERS signal of basic fuchsin (Raman reporter) marker band at 1178 cm^{-1} versus the concentration of the antigen in the range from 0.0 to 60.0 IU/ml. The error bars indicate the standard deviations from six measurements of different spots for each concentration. The blank spectrum was obtained for the blank plasma sample without adding HBsAg antigen. Without the complementary antigens, the Raman reporter-labeled immuno-Au-nanoflowers were easily removed from substrate by the washing process. A weak peak at 1178 cm^{-1} indicated that there was still some nonspecific binding between Raman reporter and substrate. In the linear region the calibration curve was fitted as $y=163.17x+370.93$ and the correlation coefficient (R^2) was 0.997. For the linear calibration curve, it was assumed that the SERS intensity at 1178 cm^{-1} (y) is linearly related to the concentration of HBsAg antigen (x). In addition, the low detection limit (LOD) was estimated using the signal-to-noise method (Shrivastava and Gupta, 2011). A signal-to-noise ratio (S/N) of three is generally accepted for estimating LOD. Based on these data, the detection limit for HBsAg antigen in human blood plasma was conservatively estimated as 0.01 IU/ml. The current lower limit for detection specification for previously reported HBsAg assays, used to test whole blood and blood components samples, ranges from 0.03 to 0.62 IU/mL. Our assay demonstrates a capability to detect HBsAg antibody at an even lower level. The assay gave exact results over a broad linear range (0.0125–60 IU/mL) reflecting clinically relevant HBsAg titers (0.125–25 IU/mL) depending on the stage of disease, age, gender, viral genotype (Mukherjee et al., 2010).

In clinical practice the presence of HBsAg antigen is monitored in whole blood, serum or plasma. In this study we showed that our SERS-based immunoassay could be used for the detection of HBsAg antigen in human serum or plasma samples. Fig. S6 presents the SERS spectra for two selected concentrations of HBsAg antigen (0.6 and 12.0 IU/mL) in human blood plasma (Fig. S6a and b) and serum (Fig. S6a′ and b′) after completion of the immunoassay protocol described in Section 3.5. There is no

significant difference in the intensity of FC band at 1178 cm^{-1} in assays results of serum and plasma samples with the same concentration of antigen. Such blood immunoassay may be of value when rapid screening of an important and easily accessible biological fluid, such as plasma and serum is required.

The reproducibility of the presented SERS immunoassay towards the HBsAg antigen detection was also investigated. Fig. S7 (Supplementary Materials) shows 15 individual readings from 1 to 15 randomly selected spots for three different immunoassays; each assay was subjected to a different concentration of HBsAg antigen in the blood sample (0.125, 0.60, and 12.5 IU/mL). To get a statistically valid result, the marker band of Raman reporter at 1178 cm^{-1} was chosen to calculate the relative standard deviation (RSD). The corresponding relative standard deviations were 10.0%, 12.0%, and 9%. The relative average standard deviation (RSD) of this method is less than 10%, which is comparable to that of conventional ELISA assays.

4.3. Immunologic specificity detection

Taking into account that fuchsin-labeled immuno-Au nanoflowers may adsorb onto the SERS-active substrate without any immune recognition and thus generate false positive signals, their immune specificity was examined. The specific HBsAg antigen and unspecific Akt blocking peptide for anti-HBsAg antibodies were employed in the same immunoassay protocol as described in Fig. 1. The Akt blocking peptide specifically binds only the Akt (pan) Rabitt mAb antibody.

The experimental results are presented in Fig. S8. A strong characteristic SERS signal of FC was generated only in the case of specific recognition between fuchsin-labeled (anti-HBsAg)-gold nanoflowers and HBsAg antigen (Fig. S8b), while no signal (or an extremely low one) was recorded for unspecific Akt blocking peptide (Fig. S8a). This indicates that, in spite of the rigorous washing process, a small number of fuchsin-labeled immuno-Au nanoflowers may adsorb onto the SERS-active substrate without any immune recognition. Nevertheless, the difference in the intensity of SERS signals obtained under specific and unspecific recognition regimes clearly demonstrates high specificity of our immunoassay.

Our experiments successfully demonstrate the detection of Hepatitis B virus antigen HBsAg in real fluid sample using basic fuchsin-labeled immuno-Au nanoflowers. We confirmed also that the solid SERS substrate can enhance the Raman signal of blood, but it does not capture or bind blood components very strongly. Fig. 4a shows the SERS spectrum from a human blood plasma placed onto GaN/Au–Ag surface. Blood plasma was separated from the blood by centrifugation at 5000 rpm for 15 min.; 15 μL of this sample was injected onto a GaN/Au–Ag substrate placed in chamber DA (Fig. 1). The SERS spectra were acquired after 10 min. As seen in Fig. 4a, the vibrational transitions are observed at 652, 1021, 1141, 1233, 1265, 1343, 1588, and 1665 cm^{-1}. These vibrational features arise from a variety of components: proteins, carbohydrates, lipoproteins, metabolites, and other small organic molecules, such as amino and nucleic acid, vitamins, steroids (Premasiri et al., 2012). After washing the SERS platform with water, a strong characteristic SERS signal of blood plasma disappeared (spectrum not shown), and in the next step, after the injection of fuchsin-labeled gold nanoflowers into the chamber, the typical bands of fuchsin appeared (Fig. 4b). This observation confirms that chemical modification of SERS platform for the covalent binding of an antibody (Fig. S1) and/or other blood components is the crucial step for the successful antigen–antibody SERS detection.

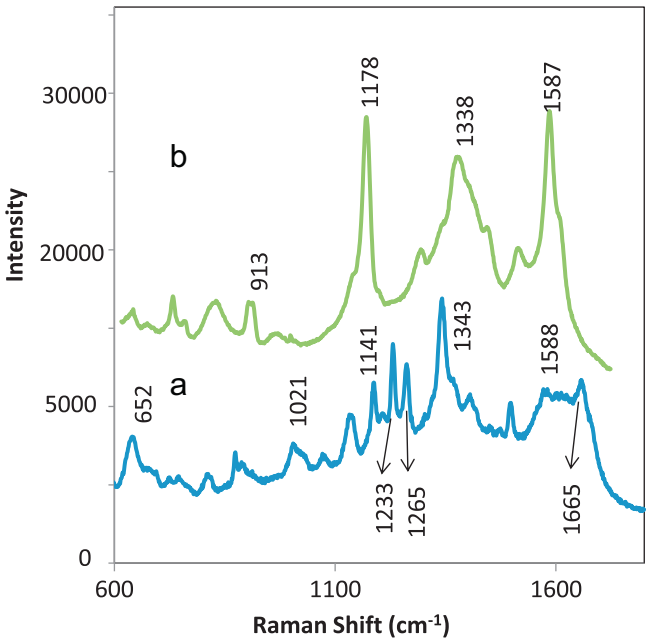

Fig. 4. SERS spectrum from a human blood plasma placed onto (a) a GaN/Au–Ag. surface and (b) after injection of the fuchsin-labeled gold nanoflowers.

4.4. The role of GaN/Au–Ag solid-active surface in the SERS-based immunoassay sensitivity

Typically, the classical SERS immunoassays have been previously performed on a solid-immune substrates without SERS activity (Cui et al., 2006; Brosseau et al., 2011). These biochemically modified substrates serve just for antigen immobilization at the bottom of the sandwich structure of the SERS-immunoassay. In this case, the enhancement effect and sensitivity of an immunoassay depend mainly on the surface plasmon resonance of the gold or silver nanoparticles labeled with antibodies and Raman reporter molecules. In our novel SERS immunoassay, the Au–Ag coated GaN substrate was introduced as the immune solid platform.

In the present study, we compared the efficiency of this immunoassay based on a SERS-active substrate with that of an inactive one. The SERS-inactive immune substrate was prepared on a (3-aminopropyl)trimethoxysilane-modified silicon slide. These two immune substrates were employed in the same immunoassay protocol as described in Fig. 1. The HBsAg antigen in human blood plasma with seven concentrations (0.0, 0.0125, 0.125, 0.60, 12.5, 35.0 and 60.0 IU/mL) was tested. The detection results are shown in Fig. 5.

Quantitative analysis was performed by measuring the SERS intensity of the marker band at 1178 cm^{-1} versus the concentration of the antigen. The LOD for HBsAg antigen was estimated using the signal-to-noise method mentioned above (Shrivastava and Gupta, 2011). It can be seen that the sensitivity of the SERS immunoassay performed on the active GaN/Au–Ag solid surface is much higher than that based on the SERS-inactive substrate. The detection limit for HBsAg antigen on the SERS-active surface is as low as 0.01 IU/mL, whereas on the inactive surface it increased to 0.625 IU/mL. Although detailed understanding of this extra-enhancement requires further explanations, and it is a rather complex phenomenon, it is very likely due to the large electromagnetic field between plasmonically active metal structures (Matthews et al., 1999). Such sandwich configuration helps to obtain even higher enhancement, as the adsorbates are surrounded by metallic structures from multiple directions. In summary, the Au–Ag coated GaN surface plays an important role in the efficiency of SERS-based immunoassay.

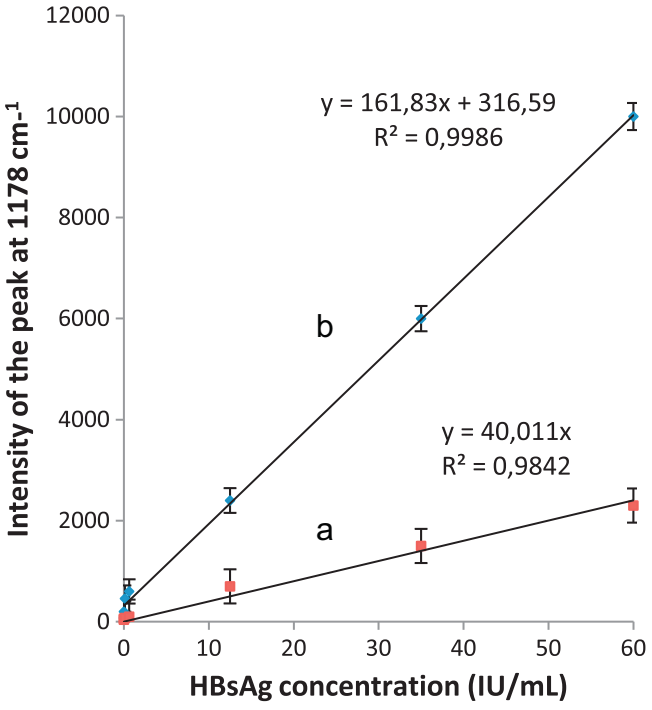

Fig. 5. The relationship between the intensity of the marker band at 1178 cm^{-1} versus the concentration of the antigen for immunoassay based on SERS-inactive (a) and active (b) solid surfaces. The data were collected by averaging six measurements. The error bars indicate the standard deviations from six measurements at different spots for each concentration.

5. Conclusions

An efficient SERS immunoassay with high selectivity and sensitivity for the quantitative detection of HBsAg antigen in blood plasma at a level of 0.01 IU/mL was demonstrated with unique fuchsin-labeled immuno-Au nanoflowers and a SERS-active immune substrate.

The immune reaction between HBsAg antigen and anti HBsAg antibody was detected via strong SERS signal of basic fuchsin (Raman reporter). The experimental results indicated that the immunoassay carried out on Au–Ag coated GaN SERS-substrate was more sensitive than the one based on a (3-aminopropyl)trimethoxysilane-modified silicon slide. The low detection limit for HBsAg antigen on the inactive SERS surface increased to 0.625 IU/mL. The average relative standard deviation (RSD) of this method is less than 10%, which is comparable to that of classic ELISA methods. Attempts to improve this immunoassay, now underway, are focusing on reducing nonspecific binding and expediting the time of detection. In the future our strategy can be extended to detection of HBsAg in blood plasma from clinical samples of patients with Hepatitis B infection. It is believed that the proposed SERS immunoassay can be also used for the detection of other important biomarkers and for point-of-care analysis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.10.082.

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