Article

Journal of Nanoscience and Nanotechnology
Vol. 18, 1083–1088, 2018
www.aspbs.com/jnn

Immunoreaction-Mediated Aggregation of Gold Nanoparticles for Sensitive and Selective Assay of Hepatitis B Surface Antigen

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A rapid, sensitive and quantitative assay method for Hepatitis B surface antigen (HBsAg) is of paramount importance for the drug development and in the diagnosis of this disease. Here, we proposed a novel biosensor that sensitively and selectively screen Hepatitis B surface antigen. This strategy relies on the cross-linking aggregation of gold nanoparticles (AuNPs) that were decorated with Hepatitis B surface antibody (HBsAb) and Raman reporter 5-thio-nitrobenzoic acid (TNB) by Hepatitis B surface antigen. The immune reaction between HBsAb and HBsAg offers this strategy high specificity, and the use of AuNPs additionally allows a visual and homogeneous assay format, thus permitting improved simplicity and throughput of the assays. The selectivity and sensitivity in HBsAg assay were achieved with a wide linear response range from 0.5 ng/mL to 50 ng/mL and a detection limit of 0.2 ng/mL. The results indicated that this strategy can offer a simple, robust and convenient platform for HBsAg analysis and related biochemical studies with high sensitivity and selectivity.

Keywords: Hepatitis B Surface Antigen Assay, Gold Nanoparticles, Surface-Enhanced Raman Scattering.

1. INTRODUCTION

Hepatitis B virus (HBV) is the most prevalent viral infection worldwide, resulting in over one million deaths per year. Among the approximately two billion people that have been infected globally, about 350 million are chronic carriers of the virus.[1,2] Hepatitis B virus surface antigen is the important serological marker of acute and chronic HBV infection.[3,4] The sensitive and accurate detection of HBsAg is critical to the clinical diagnosis of HBV infection and the prevention of HBV transmitted disease.[5,6] Therefore, development novel detection method of HBsAg is still a challenging prospect in bio-analytical chemistry.

Current methods for detection of Hepatitis B surface antigen and Hepatitis B surface antibody such as polymerase chain reaction (PCR),[7–10] Enzyme-Linked Immunosorbent Assay (ELISA)[11–14] and Microparticle Enzyme Immunoassay (MEIA),[15,16] these methods are time-consuming, expensive and require advanced instrumentation. Hence, alternative cost-effective methods that employ simple/user-friendly instrumentation and are able to provide adequate sensitivity and accuracy would be ideal for this kind of analysis. In this context, biosensor technology coupled with the use of gold nanoparticles labeled Raman reporter and HBsAb offers much more benefits compared to traditional methods in terms of time of analysis, selectivity, sensitivity and simplicity.

Gold nanoparticles have gained attention in the last years due to the unique structural, electronic, magnetic, optical, and catalytic properties, which have made them a very attractive material for biosensor systems and bioassays. AuNPs have recently become one of most popular Surface-Enhanced Raman Scattering (SERS) substrates because of their superior characteristics, such as a controllable particle size distribution, easy synthesis, long-term stability, and good biocompatibility.[17–21] The use of AuNPs as versatile and efficient substrates

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for immobilization of antibodies or antigens showed not only the increase in number of adsorbed antibodies or antigens on the metal surface, but also the preservation of immunoactivity.[22–26] In this present study, the Raman reporter labeled immunogold nanoparticles (TNB-AuNP-HBsAb) was introduced as a SERS-based immunoassay. 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) dissociates into two 2-nitro-5-mercaptobenzoic acids (or 5-thio-nitrobenzoic acid, TNB) upon the interaction with AuNP through the anchorage of thiol groups.[27] The quantitative and qualitative detection of HBsAg was tested on this SERS-based immunoassay. The immune reaction between HBsAb and HBsAg is detected via the strong SERS signal of TNB. By using only single step labeling of the highly Raman-active reporter molecule DTNB on gold nanoparticle, our detection process is more simplified. This immunoassay can be applied to other analytes by simply replacing HBsAb with suitable antibodies. Interestingly, the TNB-AuNP-HBsAb is found to be stable for at least two weeks.

2. EXPERIMENTAL SECTION

2.1. Materials and Reagents

Hepatitis B surface antigen and polyclonal Hepatitis B surface antibody from goat were purchased from Luoyang Baitaike Biotechnology Co., Ltd. (Henan, China). Raman-active dyes 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). Hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$) and trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) were purchased from Sigma Aldrich Corporation (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. (Beijing, China). All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.3 MΩ.

2.2. Preparation of Raman Reporter Labeled Immunogold Nanoparticles

AuNPs (∼30 nm) were synthesized by citrate reduction of $HAuCl_4$ according to a documented protocol,[28, 29] briefly described as follows: 3 ml 1% (w/w) sodium citrate was added rapidly under vigorous stirring in 200 mL boiling solution of 0.01% (w/w) $HAuCl_4$. Within several minutes, the color of the solution changed from blank to wine red. The solution was heated under reflux for another 20 min to ensure complete reduction, and it was then slowly cooled to room temperature. The concentration of AuNPs was determined to be ∼0.3 nM based on the extinction coefficient of 3.0×10^9 $M^{-1}cm^{-1}$ at 528 nm for AuNPs (∼30 nm) in a 60 μL quartz cuvette using a UV-2450 UV-visible absorption spectrophotometer (Shimadzu, Japan). The AuNPs were stored at 4 °C for future use.

SERS probe molecules, such as tetramethylrhodamine-isothiocyanate (TMRITC), 3,3′-diethylthiodicarbocyanine iodide (DTDC), 4-mercaptobenzoic acid (MBA), thiophenol (TP), 2-naphthalenethiol (NT), etc., have been successfully introduced to increase sensitivity and resolution of SERS-based immunoassay. In this work, DTNB, a highly Raman-active molecule, was employed as SERS-reporter co-immobilized on immunogold nanoparticles via a two steps process according to documented protocol[30] with any briefly. In the first step, 50 μL DTNB solution (12 μM in Na_2HPO_4 50 mM) was added to 1.0 mL AuNP solution (0.3 nM in borate buffer (BB) 2 mM, pH 8.5). The resultant mixture was allowed to react at 25 °C for 2 h under continuous stirring. DTNB upon interaction with AuNPs was bisected into two TNB, which was spontaneously immobilized onto the surface of gold nanoparticle through the thiol group. After centrifugation at 10,000 rpm at 4 °C for 15 min, the TNB-labeled AuNP (TNB-AuNP) was thoroughly rinsed by centrifugation and resuspended in 1 mL BB (2 mM, pH 8.5). In the second step, 10 μL HBsAb solution (3.5 mg/mL) was slowly added to 1mL suspension of TNB-AuNP. The amount of antibody used was more than the minimum amount required for coating a monolayer of HBsAb on the surfaces of gold nanoparticles. The mixture was incubated at 37 °C for 1 h, allowing the adsorption of HBsAb onto TNB-AuNP through thiol groups as well as ionic, hydrophilic, and hydrophobic interactions. After centrifugation at 10,000 rpm for 15 min, free antibodies were removed and 1 mL of Bovine serum albumin (BSA) (1% in 2 mM BB, pH 8.5) was added into the residual complexes to block the unmodified portion of the AuNP surface. The HBsAb-TNB-AuNP were then separated from the mixture solution, thoroughly rinsed by centrifugation, and resuspended in 1 mL BSA (1% in 10 mM Tris-HCl, 10 mM NaCl and 1.5 M $MgCl_2$, pH 7.9) solution. The TNB-AuNP-HBsAb was typically stable for two weeks under 4 °C storage.

2.3. SERS Detection of HBsAg

A varied HBsAg sample was added to 10 μL aliquot of TNB-AuNP-HBsAb (∼0.6 nM) and diluted to 20 μL final volume solution followed by incubation at 37 °C for 30 min to perform the immunoreaction. The resulting mixture was immediately subjected to SERS measurements. The Raman spectra were recorded on a silicon wafer using a Laboram 010 confocal Raman System (Horiba JobinYvon, France) equipped with 632 nm HeNe laser. The SERS spectra were acquired using a laser beam exposure time of 10 s and an accumulation of 1 scan. The concentration of TNB-AuNP-HBsAb used for SERS detection was ∼0.3 nM.

2.4. Transmission Electron Microscopy and Dynamic Light Scattering Analysis

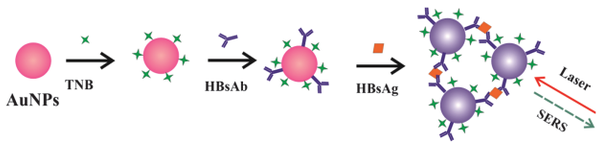
The Transmission electron microscopy images were obtained on a field emission high resolution 2100F TEM (JEOL, Japan) at an acceleration voltage of 200 kV.

The sample films for TEM analysis were formed by dropping the diluted solutions of AuNPs suspension on a holey carbon mesh grids (400 meshs) and air dried at room temperature. Dynamic light scattering analysis (DLS) measurements were used to determine the hydrodynamic sizes of AuNPs and the aggregates using a Malvern Zetasizer 3000 HS particle size analyzer (Malvern Instruments, UK).

3. RESULTS AND DISCUSSION

3.1. Analytical Principle of HBsAg Based SERS Biosensor

Our biosensor strategy relies on aggregation of gold nanoparticles for SERS transduction, as illustrated in Scheme 1. AuNPs are decorated through mixed self-assembly with a thiol-containing Raman-active dye (TNB) and Hepatitis B surface antibody. In the presence of HBsAg, the immune reaction between HBsAb and HBsAg can trigger a network-like assembly of the TNB-AuNP-HBsAb, subsequently inducing enhance the SERS signal of TNB with a visualized color change. Note that the assembly of TNB-AuNP-HBsAb is achieved in the presence of the HBsAg. The strict immunoreaction of HBsAb and HBsAg shows that this strategy is highly specific and simplified by using only single step labeling of the highly Raman-active reporter molecule TNB on gold nanoparticles. This immunoassay can be applied to other analytes by simply replacing HBsAb with suitable antibodies.



Scheme 1. Illustration of surface-enhanced Raman scattering based hepatitis B surface antigen assay.

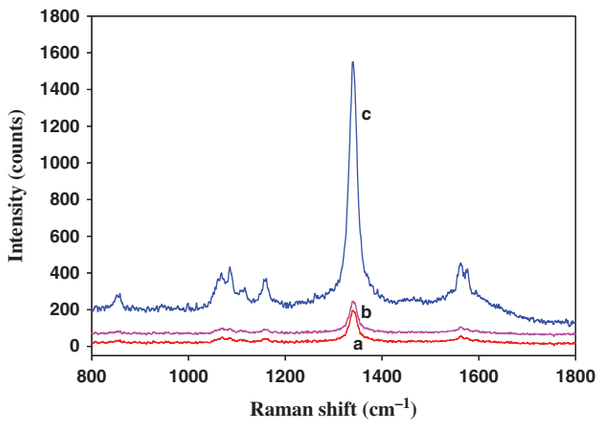


Figure 1. Typical SERS responses in Hepatitis B surface antigen assay: TNB-AuNP-HBsAb (a), TNB-AuNP incubated with HBsAg (b); TNB-AuNP-HBsAb incubated with HBsAg (c). The concentrations of TNB-AuNP-HBsAb, TNB-AuNP, HBsAg are ~0.3 nM, ~0.3 nM, 50 ng/mL.

3.2. Surface-Enhanced Raman Scattering Characteristics of the Biosensor

Figure 1 shows the SERS signals of our biosensor using 5,5-dithio-bis (2-nitrobenzoicacid) (DTNB) reporter for HBsAg assay. The TNB-AuNP-HBsAb merely displayed a very weak SERS signal (a curve) with the area of Raman peak at 1334 cm^{-1} ~130 counts/cm. When the TNB-AuNP-HBsAb were incubated for 30 min with HBsAg, the strong SERS signal was obtained with the valid of Raman peak ~1300 counts/cm (c curve). The weak SERS

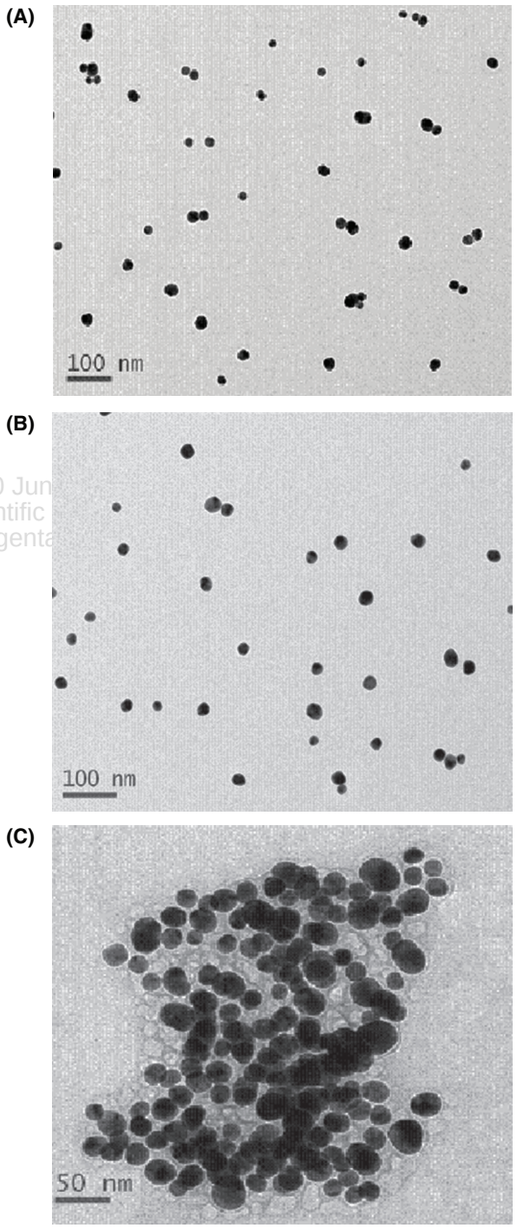


Figure 2. TEM images for TNB-AuNP-HBsAb (A); TNB-AuNP incubated with HBsAg (B); and TNB-AuNPs-HBsAb incubated with HBsAg (C). The concentrations of TNB-AuNP-HBsAb, TNB-AuNP, HBsAg are ~0.3 nM, ~0.3 nM, 10 ng/mL.

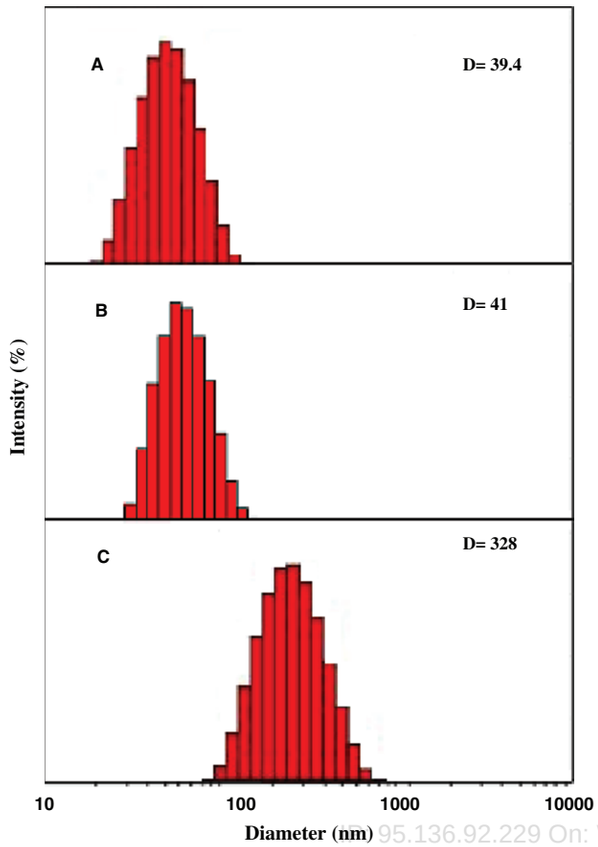


Figure 3. Hydrodynamic sizes of AuNPs determined by DLS analysis. (A) TNB-AuNP-HBsAb; (B) TNB-AuNP incubated with HBsAg and (C) TNB-AuNP-HBsAb incubated with HBsAg. The concentrations of TNB-AuNP-HBsAb, TNB-AuNP, HBsAg are ~0.3 nM, ~0.3 nM, 10 ng/mL.

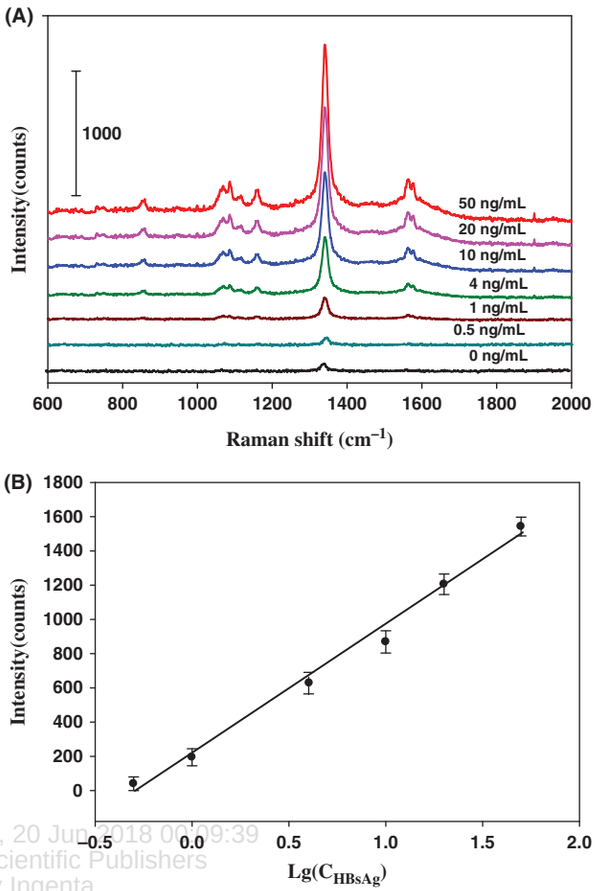


Figure 4. (A) SERS signals in response to HBsAg of varying concentrations. Each spectrum is offset by 200 counts. (B) Plot of SERS peak intensities at 1334 cm^{-1} versus logarithm HBsAg concentrations. Concentration of TNB-AuNP-HBsAb is ~0.3 nM. The error bars are standard deviations across four repetitive assays.

signal of ~140 counts/cm was also obtained when the TNB-AuNP incubated for 30 min with HBsAg. It was suggested that the network-like assembly of the TNB-AuNP is not conducted (b curve). Taken together, the network-like assembly TNB-AuNP-HBsAb was highly specific for HBsAg assay, implying the strategy may provide a selective platform for Hepatitis B surface antigen assay.

3.3. Transmission Electron Microscopy and Dynamic Light Scattering Characterization of the Biosensing Strategy for HBsAg Assay

Because the AuNP aggregate usually displayed a large hydrodynamic diameter. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) analysis could provide straightforward evidences for the assembly of AuNPs. Hence, DLS and TEM were performed to inspect the assembly of AuNPs in the assays in order to further verify the mechanism of developed biosensing strategy.

As shown in Figures 2(A) and 3(A) for the TNB-AuNP-HBsAb, an average hydrodynamic diameter of ~39.4 nm was observed. This diameter value was larger than the core size (~30 nm) of AuNPs because of the additional TNB and HBsAb hydration layers. After incubating the substrate TNB-AuNP-HBsAb with HBsAg, a substantial increase in the average hydrodynamic diameter (~328 nm) was observed for the AuNPs, giving an immediate evidence for the assembly of the TNB-AuNP-HBsAb into large aggregates (Figs. 2(C) and 3(C)). On the other hand, in the control experiment where AuNPs decorated only TNB without antibody incubated with HBsAg, we obtained an average of hydrodynamic diameter of ~41 nm for AuNPs after incubated with HBsAg (Figs. 2(B) and 3(B)).

These results were very consistent with those obtained with the Surface-enhanced Raman scattering measurements, which further confirmed that the network-like assembly of the TNB-AuNP-HBsAb by HBsAg may provide a selective platform for Hepatitis B surface antigen assay.

3.4. Hepatitis B Surface Antigen Detection

Figure 4(A), a dramatic increase in the SERS intensity was observed with the increasing concentrations of HBsAg.

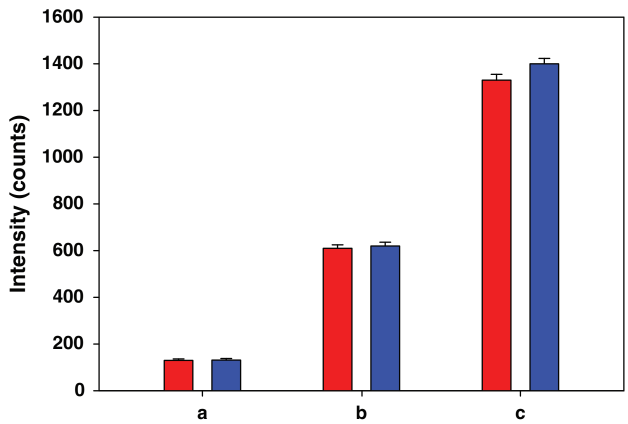


Figure 5. Bar plot of corresponding SERS intensities responses at 1334 cm^{-1} of HBsAg analysis in 10-fold-diluted fetal bovine serum (blue) and BSA (1% in 10 mM Tris-HCl, 10 mM NaCl and 1.5 M $MgCl_2$, pH 7.9) (red): containing TNB-AuNP-HBsAb ~0.3 nM in the absence (a) or presence of 4 (b) or 40 (c) ng/mL HBsAg. Error bars are standard deviations across four repetitive experiments.

A plot of the SERS peak intensities at 1334 cm^{-1} versus logarithmic value of the HBsAg concentration revealed a dynamic correlation between the peak intensities and the HBsAg concentrations in the range from 0.5 to 50 ng/mL (Fig. 4(B)) with a detection limit of 0.2 ng/mL according to 3s rule. The relative standard deviations of peak intensities at 1334 cm^{-1} were 3.8%, 2.9%, 3.2% and 4.3% in four repetitive assays of 1 ng/mL, 2 ng/mL, 4 ng/mL and 10 ng/mL HBsAg. Therefore, we might conclude that the developed strategy held great potential for quantitative assay of HBsAg with high selectivity and reproducibility.

Additionally, further experiments using fetal bovine serum as the reaction substrate were performed to demonstrate the specificity of the developed strategy under complex experiment conditions. Serum usually contains many biomolecules and some small molecules, such as H_2O_2, dopamine, ethanol and CO, which may interfere with the assay of HBsAg. As shown in Figure 5, it could be found that when the same concentration of HBsAg added, there was no significant difference between the intensities of the samples containing fetal bovine serum as the substrate and that of the samples with BSA (1% in 10 mM Tris-HCl, 10 mM NaCl and 1.5 M $MgCl_2$, pH 7.9) as the substrate. These results exhibited that even under a complex experiment conditions, the developed sensing strategy still had desirable selectivity for HBsAg.

4. CONCLUSIONS

We have demonstrated a novel method for the quantitative detection of HBsAg using SERS-based immunoassay. The TNB-labeled immunogold nanoparticle can form a structure network-like assembly with the antigen and antibody labeled-AuNP. Based on this method, a calibration curve was obtained to detect the concentration of HBsAg in the range from 0.5 ng/mL to 50 ng/mL with the detection limit of 0.2 ng/mL. This study represented the first example of using SERS-based immunoassay for HBsAg in a homogeneous format. Compared with conventional HBsAg assay methods, this strategy showed high specificity, and simplified. Furthermore, the homogeneous assay format, single step labeling of the highly Raman-active reporter molecule TNB on gold nanoparticle as well as this immunoassay can be applied to other analytes by simply replacing HBsAb with suitable antibodies. In view of these advantages, the developed HBsAg screening strategy was expected to provide an intrinsically robust, highly specific assay platform for HBsAg analysis and related biochemical studies.

Conflicts of Interest

The authors declare no conflict of interest.

Acknowledgments: The authors would like to thank Industrial University of Ho Chi Minh City, Hunan University, and Dong Nai Technology University for financial supports.

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Received: 16 September 2016. Accepted: 19 October 2016.