Paper-Based Microfluidic Approach for Surface-Enhanced Raman Spectroscopy and Highly Reproducible Detection of Proteins beyond Picomolar Concentration

Arindam Saha* and Nikhil R. Jana*

Centre for Advanced Materials, Indian Association for the Cultivation of Science, Kolkata 700032, India

Ⓢ *Supporting Information*

ABSTRACT: Although microfluidic approach is widely used in various point of care diagnostics, its implementation in surface enhanced Raman spectroscopy (SERS)-based detection is challenging. This is because SERS signal depends on plasmonic nanoparticle aggregation induced generation of stable electromagnetic hot spots and in currently available microfluidic platform this condition is difficult to adapt. Here we show that SERS can be adapted using simple paper based microfluidic system where both the plasmonic nanomaterials and analyte are used in mobile phase. This approach allows analyte induced controlled particle aggregation and electromagnetic hot spot generation inside the microfluidic channel with the resultant SERS signal, which is highly reproducible and sensitive. This approach has been used for reproducible detection of protein in the pico to femtomolar concentration. Presented approach is simple, rapid, and cost-effective, and requires low sample volume. Method can be extended for SERS-based detection of other biomolecules.

KEYWORDS: *microfluidics, SERS, nanoparticle, functionalization, protein detection*
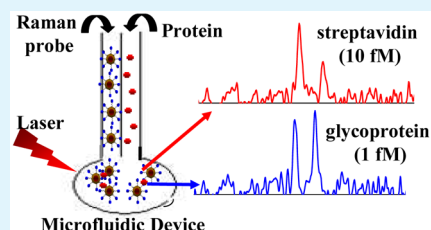
■ INTRODUCTION

Microfluidic approach-based chemical and biochemical analysis becomes very popular in the last two decades.[1−3] It deals with typical laboratory experiments in microscale reactors with the options of laminar flow of liquid, low sample volume, fast response time, high resolution and sensitivity, along with other advantages.[2−9] Because of these benefits, the microfluidic approach has been applied in various biomedical research[10] such as protein detection,[11−18] oligonucleotide detection,[19] mimicking organoids,[20,21] cell capture and isolation,[22,23] protein aggregation study,[24,25] single cell assay,[26] and quantitative detection of biologically active small molecules[27−30] or drugs.[31,32] However, fabrication of a low-cost microfluidic device is one of the major challenges to its widespread use in biomedical diagnostics. Conventional fabrication involved photolithography, etching, electron beam lithography, printing, and molding.[33,34] Theses steps involve sophisticated and costly technology along with expert human resources. Thus, researchers have focused on alternative paper-based microfluidics and naturally occurring microchannels that combine simple fabrication method along with the advantages of microfluidics.[34−42] Major examples include cellulose-based filter paper and membrane or silica based thin layer chromatography (TLC) plates.[34,36−38,40,41]

Variety of detection techniques have been integrated with microfluidic devices that includes colorimetry/fluorescence,[8,10−14,30,39] electrochemical detection,[17,18,27] and surface-enhanced Raman scattering (SERS)-based detection.[15,19,25,29,31,32,37,40,43−46] Among them, SERS-based diagnostic approaches are particularly important, because they give the vibrational fingerprint of individual molecules down to the single-molecule level.[47,48] Appearance of the SERS signal is linked with the presence of Raman active analyte molecules at the electromagnetic hot-spots, offered by the plasmonic nanomaterials.[47−52] SERS sensitivity strongly depends on the nature of plasmonic nanoparticles,[53−55] extent of particle aggregation in generating electromagnetic hot-spots[50,56,57] and effective localization of Raman probes in the hot-spots.[51,52,58,59] However, reproducibility of SERS signal is a serious concern that limits its routine detection applications.[51] SERS has been adapted in various types of microfluidic device and used for detection of drugs,[31,32] oligonucleotides,[19] biomolecules,[30] and proteins.[15] These microfluidic SERS approaches involve three majors limitations for widespread applications. First, microfluidic device used in most cases are complex and expensive.[15,19,25,29,31,32,35,38−45] Second, in most cases, either plasmonic nanomaterials or analyte is pre-embedded on microfluidic device and thus analyte induced particle aggregation and hot spot generation are restricted.[15,19,25,37,40,43,44,46] This results in poor SERS detection sensitivity.[15,19,31,37,40,44] Third, the signal reproducibility is poor because of the inefficient and uncontrolled particle aggregation process.[19,25,29,31,32,44−46]

Here we show that SERS can be adapted using a simple paper-based microfluidic system where both the plasmonic nanomaterials and analytes are used in the mobile phase.
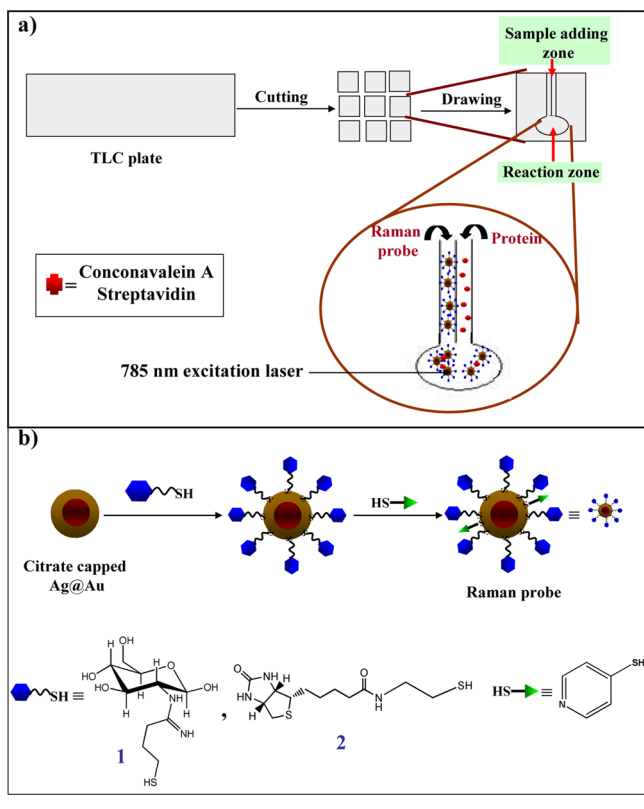
(Scheme 1) This approach allows analyte induced controlled particle aggregation and electromagnetic hot spot generation

Scheme 1. (a) Fabrication of Microfluidic Chip from Silica Gel-Based TLC Plate; (b) Functionalization Strategy of Ag@Au Nanoparticle with Glucose and 4-Mercaptopyridine or Biotin and 4-Mercaptopyridine

inside the microfluidic channel with the resultant SERS signal which is highly reproducible and sensitive. We have fabricated the microfluidic device using silica gel-based TLC plate and used functionalized silver coated gold nanoparticle (Ag@Au nanoparticle) as plasmonic platform and protein as the analyte molecule. TLC plate provides the same function as of cellulose-based paper where micrometer-sized pores and channels inside silica would represent functional mimic of microfluidic device. Moreover, the TLC plate has solid support, which would provide additional advantage for device fabrication. Ag@Au nanoparticle is functionalized with 4-mercaptopyridine and glucose (or biotin) where 4-mercaptopyridine acts as Raman probe and glucose (or biotin) interacts with Con A (or streptavidin). Protein solution and functionalized Ag@Au nanoparticles are separately introduced into two different channels and the protein induced aggregation of the Ag@Au nanoparticles occurs inside the reaction zone (microfluidic interface). SERS signal of 4-mercaptopyridine is then used for protein detection by a benchtop Raman spectrometer. This approach offers reproducible detection of protein in pico to femtomolar concentration. Presented approach is simple, rapid, cost-effective and requires microliter sample volume.

■ EXPERIMENTAL SECTION

Reagents. HAuCl$_4$, iminothiolane hydrochloride, glucosamine hydrochloride, biotin-N-hydroxy succinimide, cysteamine hydrochloride, 4-mercapto pyridine, concanavalin A (from *Canavalia ensiformis*),

streptavidin (from *streptomyces avidinii*), and dialysis membrane (cellulose membrane, MWCO ~12 000 Da) were purchased from Sigma-Aldrich and used as received. AgNO$_3$, tri sodium citrate, N,N-dimethylformamide (DMF), ascorbic acid, and TLC plates (average pore diameter 9.5−11.5 μm, layer thickness ~175−225 μm) were purchased from Merck.

Fabrication of Microfluidic Device from Silica Gel-Based TLC Plate. TLC plates were first cut into 1.5 cm × 1.5 cm pieces. Then two channels of ~0.1 cm wide along with a reactor vessel (diameter ~0.4 cm) where two channels merged were drawn using a waterproof marker pen (Scheme 1a and the Supporting Information, Figure S1). The two channels and reactor vessel are surrounded by boundaries drawn by waterproof marker pen to prevent any water leakage. All the boundaries are finally covered with hydrophobic oil (octadecene) to avoid channel mixing.

Preparation of Thiol-Modified Glucose and Thiol-Modified Biotin. 0.1 mmol iminothiolane hydrochloride (~14 mg) and 0.12 mmol glucosamine hydrochloride (~26 mg) were mixed together in 2 mL of PBS buffer of pH 7.4 and stirred for 4 h. The thiol-modified glucose was characterized by HRMS and NMR spectroscopy.

For the preparation of thiol-modified biotin, 0.1 mmol biotin-N-hydroxy succinimide (~34 mg) dissolved in 1 mL DMF and 0.1 mmol cysteamine (~11 mg) dissolved in 1 mL of water were mixed together and stirred for ~4 h. The product obtained was characterized using HRMS spectroscopy.

Preparation of Ag@Au Nanoparticle. Colloidal Ag@Au nanoparticle was prepared by adapting reported method.[51,58] Principle involved deposition of Ag on the surface of 12 nm Au nanoparticles. First, 12 nm colloidal Au nanoparticles was prepared by the well-known citrate reduction method.[58] Next, this Au nanoparticle solution was used as seed to deposit Ag on their surface. Typically, 10 mL aqueous solution of Au nanoparticles was mixed with 50 mL ascorbic acid (0.1 M) solution. Next, 0.25 mL AgNO$_3$ (0.01 M) solution was added in a dropwise manner under the continuous stirring condition. The solution color changed from deep yellow to gray yellow.

Preparation of Ag@Au Nanoparticle Functionalized with 4-Mercapto Pyridine and Glucose or 4-Mercapto Pyridine and Biotin. About 5 mL of Ag@Au nanoparticle solution is mixed with 50 μL of thiol modified glucose or biotin solution (10^{-5} M) and stirred for 4 h. Next, 50 μL solution of 4-mercapto pyridine (1×10^{-7} M) was added to it and stirred for further 2 h. Finally, solution was dialyzed or centrifuged to remove any unbound reagents and used for SERS experiments.

Confirmation of Biochemical Activity of Ag@Au Nanoparticle after Glucose/Biotin Functionalization. For this purpose, 1 mL of glucose-functionalized Ag@Au nanoparticle is mixed with 100 μL of 10 μM Con A solution and kept for an hour, which leads to complete precipitation of the nanoparticles. Similarly, 1 mL of biotin-functionalized Ag@Au nanoparticle is mixed with 100 μL of 10 μM streptavidin solution and kept for an hour. The solution turns blue followed by visible precipitation.

SERS-Based Protein Detection Using Microfluidics Device. One microliter of functionalized Ag@Au nanoparticle was introduced in one channel and one microliter of protein solution (of nanomolar to femtomolar concentration) was added in other channel. The two solutions migrate to the reactor vessel almost instantaneously and mixed together. The SERS spectra were then recorded by exposing the reactor channel with 785 nm laser from ~2 cm distance and with 10 min integration time. Reproducibility of SERS signal was checked by measuring the SERS of 5 different sets using five different microfluidic devices and keeping all other parameters same.

SERS-Based Protein Detection in Microfluidic Device Using Preadsorbed Plasmonic Nanoparticles. In this control experiment microfluidic device was dipped into functionalized Ag@Au nanoparticle solution for 2 h. Then the device was dried in air and washed with water several times. Protein solution of different concentration was then added to these preadsorbed nanoparticles and SERS spectra were recorded.

SERS-Based Protein Detection in Solution. In this control experiment, 1 mL of functionalized Ag@Au nanoparticle is mixed with
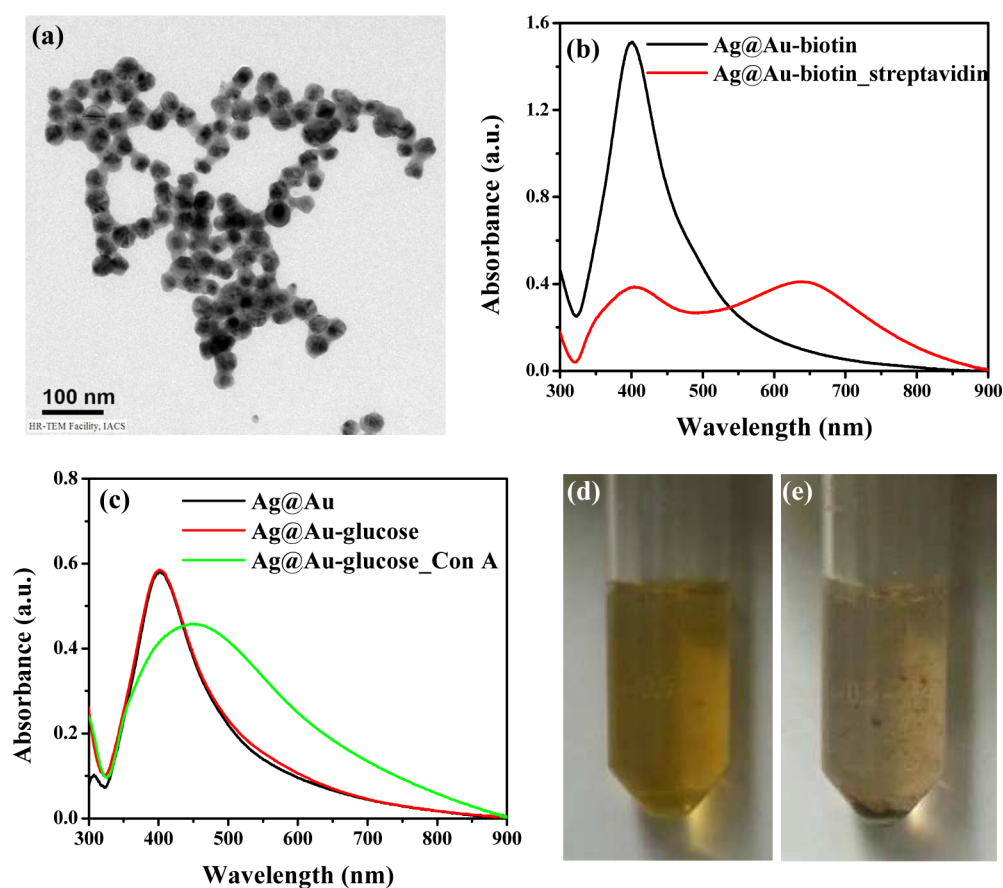
Figure 1. (a) TEM image of as-synthesized Ag@Au nanoparticles, (b) UV−visible absorption spectra of biotin functionalized Ag@Au nanoparticles before and after streptavidin addition, (c) UV−visible absorption spectra of Ag@Au nanoparticle and glucose functionalized Ag@Au nanoparticles before and after Con A addition, (d) digital image of glucose functionalized Ag@Au nanoparticles, and (e) digital image of glucose functionalized Ag@Au nanoparticles after Con A addition.

100 μL of protein solution of different concentration. The solutions were allowed to aggregate for 5 min to 5 h depending on the protein concentration and then the SERS spectra were measured.

Instrumentation. UV−visible absorption spectra were measured using a Shimadzu UV-2550 spectrophotometer. Diffuse reflectance spectra (DRS) were measured using a Shimadzu UV-3600 spectrophotometer using an integrating sphere and barium sulfate standard. SERS measurement was performed using an Agiltron R3000 Raman spectrometer with 785 nm excitation laser having 5 mW laser power and 10 s integration time. The FEI Technai G2 transmission electron microscope was used for transmission electron microscopic (TEM) image of samples.

RESULTS

Microfluidic Device Fabrication. Silica gel-based TLC plates are selected for microfluidics device fabrication as shown in Scheme 1 and Figure S1 in the Supporting Information. The micrometer-sized porous channels inside the silica gel matrix act as microfluidic channels. Hydrophobic marker pen has been used for marking and fabricating the device. The hydrophobic ink isolates the channels and prevents the mixing of solutions between channels. Typically, two separate channels are drawn on TLC plate which merges into a small round shaped region acting as reaction chamber. Depending on the experiment and reactants, number of channels can be controlled. The diameter of the channel depends upon the tip of the marker pen. Pointier tip can produce channels with lower width. In present case the diameter of the channels are typically ∼0.1−0.2 cm. All the

boundaries are carefully treated with hydrophobic octadecene solution, which further ensures no mixing between channels.

Selection of Plasmonic Nanoparticle and Their Functionalization. Ag@Au nanoparticle of ∼25−30 nm size has been selected as plasmonic nanoparticle for SERS study as this nanoparticle is shown to produce high SERS sensitivity.[51] Ag@Au nanoparticle has been synthesized using reported method.[51,58] UV−visible absorption spectrum of this nanoparticle is dominated by the Ag plasmon having a broad peak at around 420 nm. (Figure 1) This nanoparticle is then functionalized with a Raman probe and affinity molecule that binds with specific protein. We have selected 4-mercaptopyridine as Raman probe that produces strong SERS signal with the Ag@Au nanoparticle. Two different affinity molecules such as glucose and biotin have been selected as they selectively bind with Con A and Streptavidin, respectively. The 4-mercaptopyridine has a thiol group that has strong binding affinity to silver surface and thus it adsorbs on the surface of Ag@Au nanoparticle. As glucose and biotin do not interact strongly with silver surface, they have been modified with thiol groups (see the Supporting Information, Scheme S1). Thiol-modified glucose is synthesized by reacting glucosamine with iminothiolane hydrochloride. In this reaction, primary amine group on glucosamine reacts with the iminothiolane, producing a thiolated glucose molecule. The product formation is confirmed by HRMS and NMR measurement (see the Supporting Information, Figure S2−S4). Similarly, thiolated biotin is
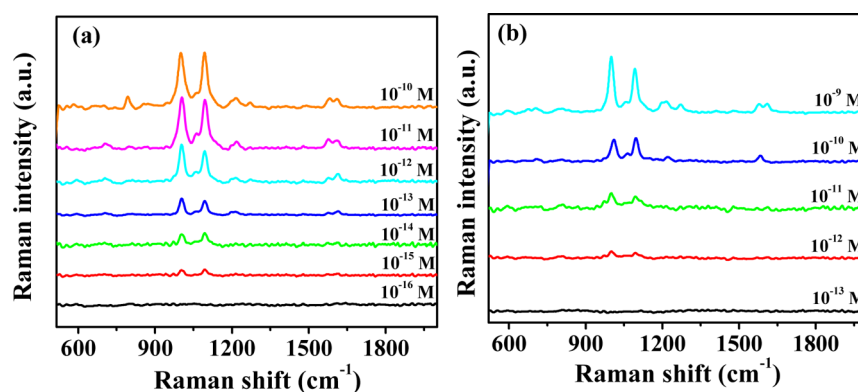
Figure 2. Comparative sensitivity of SERS signal of 4-mercaptopyridine under different concentration of Con A using (a) paper-based microfluidics and (b) a conventional solution-based approach. In the microfluidic approach, the SERS spectra are measured within 5 min of adding Con A. In the solution-based approach, the SERS spectra are measured 2 h after adding Con A.
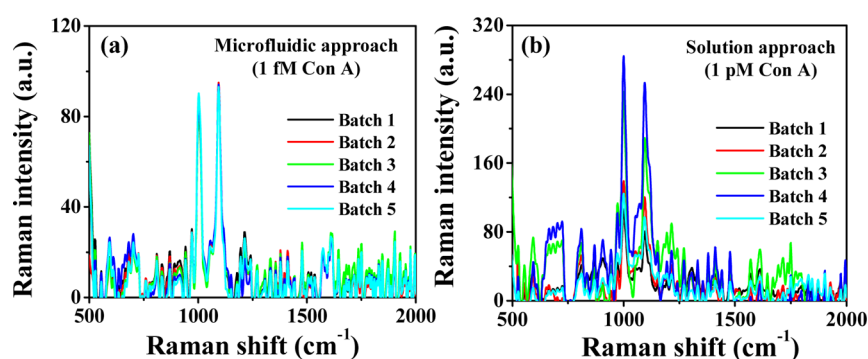

Figure 3. Comparative study of signal reproducibility in SERS based Con A detection using (a) paper-based microfluidics versus (b) conventional solution-based approach at their minimum detectable concentrations. Con A concentration was 1×10^{-15} M and 1×10^{-12} M in microfluidic and solution approach, respectively. In the microfluidic approach, the SERS spectra are measured within 5 min of adding Con A and in the solution-based approach, the SERS spectra are measured 2 h after adding Con A.

synthesized by reacting biotin-NHS ester with amine group of the cysteamine.

Functionalization of Ag@Au nanoparticle involves simple incubation with thiolated molecules that leads to their adsorption on the nanoparticle surface. In the preparation of glucose and 4-mercaptopyridine functionalized nanoparticle, the Ag@Au nanoparticle is first mixed with thiolated glucose followed by incubation with 4-mercaptopyridine. Similarly, biotin and 4-mercaptopyridine functionalization have been performed by first incubating the Ag@Au nanoparticle with thiolated biotin followed by incubation with 4-mercaptopyridine. The concentration of the thiolated glucose/biotin and 4-mercaptopyridine have been maintained as ~1×10^{-7} M and ~1×10^{-9} M, respectively, for best result having particle with minimum particle aggregation. Finally the solution is dialyzed for 4−5 h to remove any unbound glucose/biotin or 4-MPy molecules from the nanoparticle solution. The functionalization of nanoparticle with glucose and biotin has been confirmed via biochemical interaction studies with Con A and streptavidin, respectively. Addition of protein leads to the aggregation of functional Ag@Au nanoparticle along with a red shift in UV−visible spectra due to the multivalent interaction between protein and glucose/biotin. (Figure 1)

High Sensitivity, Reproducibility and Rapid Detection of Protein via Microfluidic SERS. The principle for SERS-based protein detection involves the aggregation of Ag@Au nanoparticle in the presence of protein. The proteins Con A and streptavidin have four binding sites for glucose and biotin,

respectively. As a result addition of protein to the solution of functional nanoparticle leads to cross-linking between nanoparticles. This particle aggregation processes leads to the formation of electromagnetic hot-spots between particles. As the nanoparticles are also functionalized with Raman probe (i.e., 4-mercaptopyridine), a significant number of Raman probes are trapped at the electromagnetic hot-spots that results in the appearance of SERS signal from Raman probe.

In a typical procedure, functional Ag@Au nanoparticle solution is introduced in one of the microfluidic channel and protein solution in other microfluidic channel. The solutions in both the channels move through the microchannels by capillary action and mix together in reaction chamber. Protein-induced aggregation of functionalized Ag@Au nanoparticles occur in a controlled manner in the microfluidic reaction chamber and produces the SERS signal of 4-mercaptopyridine. Results of SERS based detection of two proteins are summarized in Figures 2−5 and Figures S5−S9 in the Supporting Information. Protein detection sensitivity has been observed down to femtomolar concentration with a detectable vibrational fingerprint of 4-mercaptopyridine. For example detection sensitivities for Con A and streptavidin are 1×10^{-15} M and 1×10^{-14} M, respectively (Figures 2 and 5). In contrast, the conventional solution based approach shows relatively poor sensitivity and the reasonable signal is observed only above the picomolar protein concentration (Figures 2 and 5). Control experiment has been performed by preadsorbing functional nanoparticle in the microfluidic reaction chamber and then adding the protein
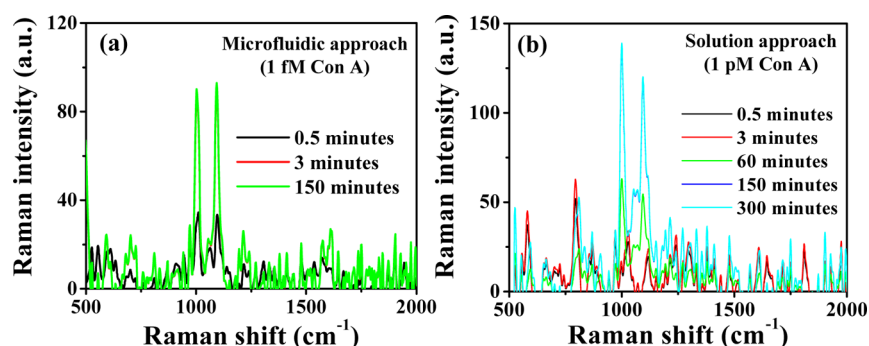
Figure 4. Comparative time dependence of SERS signal intensity using (a) paper-based microfluidics versus (b) conventional solution based approach. In the microfluidic approach the SERS spectra are measured using 1×10^{-15} M Con A and in solution-based approach the SERS spectra are measured using 1×10^{-12} M Con A.
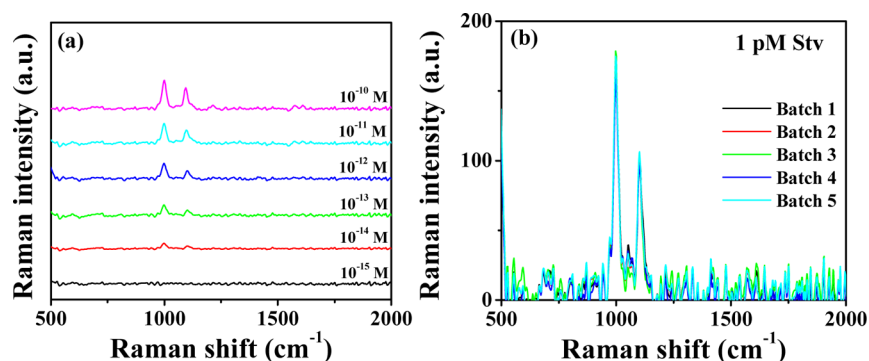

Figure 5. (a) Sensitivity and (b) reproducibility of streptavidin (Stv) detection in our microfluidic platform using biotin and 4-MPy-functionalized Ag@Au nanoparticle. Reproducibility has been tested using 1×10^{-12} M streptavidin (Stv) solution.

solution. Results shows that SERS signal was much lower with the low protein detection sensitivity, suggesting that controlled aggregation in the microfluidic channel plays important role for high sensitivity (see the Supporting Information, Figure S6). Other control experiments are also performed to confirm that particle aggregation and SERS signal generation is actually due to the analyte (see the Supporting Information, Figure S6). Aggregate formation between Ag@Au nanoparticles inside the microfluidic system has been studied via diffuse reflectance spectroscopy (DRS). Typically, bare Ag@Au nanoparticles and functionalized (by 4-MPy and glucose) Ag@Au nanoparticles are mixed with Con A solution inside the microfluidic device and then studied by DRS (see the Supporting Information, Figure S8). It is expected that Con A would induce aggregation of functionalized Ag@Au nanoparticles but not the bare Ag@Au nanoparticles. It is observed that the reflectance minima have been broadened and slightly red-shifted for functionalized Ag@Au nanoparticles as compared to bare Ag@Au nanoparticles, which suggest the formation of aggregates (see the Supporting Information, Figure S8).

The most significant advantage of this approach is the high reproducibility of SERS signal. Five identical batches of microfluidic chip have been used for the detection of same concentration of protein. It has been observed that SERS signal is highly reproducible between identical batches. For example SERS signal intensity for 1×10^{-15} M Con A or 1×10^{-12} M streptavidin in five identical batches varies within 10% error. (Figures 3 and 5) Similar testing has also been performed with conventional solution based approach. However, in this case SERS signal fluctuates significantly from batch to batch. For example, SERS signal intensity for 1×10^{-12} M Con A in five

identical batches varies drastically (Figure 3 and Figure S5 in the Supporting Information).

The time dependence of SERS signal intensity has been studied by measuring the SERS spectra in the reaction zone at different time interval. (Figure 4 and Figure S5 in the Supporting Information) It has been found that the SERS signal intensity reaches a maximum value within 3−4 min, irrespective of protein concentration. However, in solution-based approach it takes much longer time for observing a detectable signal, typically minutes to hours depending the protein concentration and for detection of lowest detectable concentration (picomolar protein concentration) it takes 2−3 h to produce detectable SERS signal. For example SERS signal intensity for 1×10^{-15} M Con A detection reaches maxima within 3 min in microfluidic approach. In contrast, SERS signal for 1×10^{-12} M Con A detection slowly increases up to 300 min (Figure 4 and Figure S5 in the Supporting Information) This result suggests that microfluidic channels enhances the rate of protein-induced nanoparticle aggregation and thus decreases the detection time.

■ DISCUSSION

The presented SERS-based protein detection approach is unique for two specific reasons. First, this approach uses simple and low-cost paper-based natural microfluidic platform for SERS-based detection that requires low sample volume and short detection time. Second, this approach provides high reproducibility of protein detection up to the femtomolar concentration, which is generally uncommon in SERS-based approach. The origin of theses advantages is due to unique detection scheme, right choice of functional nanoparticle and

use of microfluidic platform. Ag@Au nanoparticle of ∼25−30 nm size and 4-mercaptopyridine as Raman probe are well-studied and proven to generate high SERS signal.[51,58] High SERS sensitivity of this system arises because of the strong Ag plasmon band along with strong aggregation tendency that leads to generation of electromagnetic hot-spots. The detection scheme is unique in the sense that protein induces the nanoparticles aggregation that in turn switches on the SERS signal by generating the electromagnetic hot-spots. We have intentionally used this type condition as it would offer maximum positioning of Raman probe at electromagnetic hot spots.[51] Advantage of microfluidic platform is that the porous microchannels inside the silica gel matrix controls the extent of nanoparticle aggregation and diameter of the channels restricts the formation of larger aggregates.[36] In addition, the aggregates are stabilized by physical attachment/trapping inside the microchannels. It is known that smaller aggregate, particularly nanoparticle dimers and trimers, produces more SERS signals.[51,60] It is known from earlier study that smaller aggregation (dimer/trimer) produces higher SERS intensity since it provides more number of effective hot-spots.[60] In larger aggregate, some of the hot-spots get screened leading to lowering of the numbers of effective hot-spots.[60] Thus, restricted aggregation contributes to the enhanced SERS signal and stabilization of aggregates contributes to the enhanced signal reproducibility. In contrast larger and uncontrolled aggregates are formed in solution based approach that leads to poor signal reproducibility. Control experiment shows that if the nanoparticles are pre-embedded inside the microfluidic platform, the detection sensitivity is drastically reduced. Other advantage of the presented microfluidic platform is that the external pressure is not required for the fluids flow, since the capillary action can provides the necessary pressure. Thus, nanoparticle and protein solution are mixed in the reaction chamber in less than a minute and this rapid mixing leads to quicker appearance of SERS signal.

Conventional protein detection methods like ELISA, Western blotting and flow cytometry suffer from long processing time and require costly reagents. In contrast SERS based protein detection can be a powerful tool in diagnostic.[61−63] However, reproducibility is serious problem in this approach. Although microfluidic platform has been introduced in SERS they have low sensitivity, poor reproducibility and requires complex device fabrication.[15,29] These limitations are linked with preadsorption of plasmonic nanoparticles that restrict particle aggregation and thus restricts electromagnetic hot-spot generation.[15,29] In contrast, our microfluidic approach uses mobile phase for both plasmonic nanoparticle and analyte and allows analyte induced nanoparticle aggregation inside microchannels. This approach offers highly sensitive and reproducible SERS signal along with all the advantages of microfluidic approach. This concept and approach can be extended in detecting other proteins or biomolecules.

CONCLUSION

In conclusion, we report an ultrasensitive and reproducible protein detection strategy using SERS based approach where a paper based simple microfluidic platform has been applied. Our approach is simple, rapid and requires very low sample volume. The SERS signals are recorded in a portable table-top Raman instrument. This detection strategy allows femtomolar protein detection with high reproducibility. This result is very significant as it can be extended in detecting various

biomolecules down to single molecule level and in point-of-care diagnostics with high sensitivity.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Table summarizing the SERS-based biomedical detection approach in microfluidic platform, synthesis strategy and characterization details of thiolated glucose and biotin, and results of different control experiments. This material is available free of charge via Internet at http://pubs.acs.org

AUTHOR INFORMATION

Corresponding Authors
*E-mail: arindamchemiitkgp@gmail.com.
*E-mail: camnrj@iacs.res.in.

Notes
The authors declare no competing financial interest.

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REFERENCES

(1) Manz, A.; Harrison, D. J.; Verpoorte, E. M. J.; Fettinger, J. C.; Paulus, A.; Lüdi, H.; Widmer, H. M. Planar Chips Technology for Miniaturization and Integration of Separation Techniques into Monitoring Systems - Capillary Electrophoresis on a Chip. *J. Chromatogr.* **1992**, *593*, 253−258.

(2) Figeys, D.; Pinto, D. Lab-on-a-Chip: A Revolution in Biological and Medical Sciences. *Anal. Chem.* **2000**, *72*, 330A−335A.

(3) Whitesides, G. M. The Origins and the Future of Microfluidics. *Nature* **2006**, *442*, 368−373.

(4) Atencia, J.; Beebe, D. J. Controlled Microfluidic Interfaces. *Nature* **2005**, *437*, 648−655.

(5) Gunther, A.; Jensen, K. F. Multiphase Microfluidics: From Flow Characteristics to Chemical and Materials Synthesis. *Lab Chip* **2006**, *6*, 1487−1503.

(6) Haeberle, S.; Zengerle, R. Microfluidic Platforms for Lab-on-a-Chip Applications. *Lab Chip* **2007**, *7*, 1094−1110.

(7) Velve-Casquillas, G.; Berre, M. L.; Piel, M.; Tran, P. T. Microfluidic Tools for Cell Biological Research. *Nano Today* **2010**, *5*, 28−47.

(8) Nge, P. N.; Rogers, C. I.; Woolley, A. T. Advances in Microfluidic Materials, Functions, Integration, and Applications. *Chem. Rev.* **2013**, *113*, 2550−2583.

(9) Elvira, K. S.; Solvas, X. C.; Wootton, R. C. R; DeMello, A. J. The Past, Present and Potential for Microfluidic Reactor Technology in Chemical Synthesis. *Nat. Chem.* **2013**, *5*, 905−915.

(10) Sackmann, E. K.; Fulton, A. L.; Beebe, D. J. The Present and Future Role of Microfluidics in Biomedical Research. *Nature* **2014**, *507*, 181−189.

(11) Hu, M.; Yan, J.; He, Y.; Lu, H.; Weng, L.; Song, S.; Fan, C.; Wang, L. Ultrasensitive, Multiplexed Detection of Cancer Biomarkers Directly in Serum by Using a Quantum Dot-Based Microfluidic Protein Chip. *ACS Nano* **2010**, *4*, 488−494.

(12) Wang, J.; Onoshima, D.; Aki, M.; Okamoto, Y.; Kaji, N.; Tokeshi, M.; Baba, Y. Label-Free Detection of DNA-Binding Proteins Based on Microfluidic Solid-State Molecular Beacon Sensor. *Anal. Chem.* **2011**, *83*, 3528−3532.

(13) Lee, W. B.; Chen, Y. H.; Lin, H.; Shiesh, S. C.; Lee, G. B. An Integrated Microfluidic System for Fast, Automatic Detection of C-Reactive Protein. *Sens. Actuators B* **2011**, *157*, 710−721.

(14) Alino, V. J.; Sim, P. H.; Choy, W. T.; Fraser, A.; Yang, K. L. Detecting Proteins in Microfluidic Channels Decorated with Liquid Crystal Sensing Dots. *Langmuir* **2012**, *28*, 17571−17577.

(15) Galarreta, B. C.; Tabatabaei, M.; Guieu, V.; Peyrin, E.; Lagugné-Labarthet, F. Microfluidic Channel with Embedded SERS 2D Platform for the Aptamer Detection of Ochratoxin A. *Anal. Bioanal. Chem.* **2013**, *405*, 1613−1621.

(16) Tekin, H. C.; Gijs, M. A. M. Ultrasensitive Protein Detection: A Case for Microfluidic Magnetic Bead-Based Assays. *Lab Chip* **2013**, *13*, 4711−4739.

(17) Wang, Y.; Ye, Z.; Ping, J.; Jing, S.; Ying, Y. Development of an Aptamer-Based Impedimetric Bioassay Using Microfluidic System and Magnetic Separation for Protein Detection. *Biosens. Bioelectron.* **2014**, *59*, 106−111.

(18) Mok, J.; Mindrinos, M. N.; Davis, R. W.; Javanmard, M. Digital Microfluidic Assay for Protein Detection. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 2110−2115.

(19) Strelau, K. K.; Kretschmer, R.; Möller, R.; Fritzsche, W.; Popp, J. SERS as Tool for the Analysis of DNA-Chips in a Microfluidic Platform. *Anal. Bioanal. Chem.* **2010**, *396*, 1381−1384.

(20) Morina, F.; Nishimura, N.; Griscom, L.; LePioufle, B.; Fujita, H.; Takamura, Y.; Tamiya, E. Constraining the Connectivity of Neuronal Networks Cultured on Microelectrode Arrays with Microfluidic Techniques: A Step Towards Neuron-Based Functional Chips. *Biosens. Bioelectron.* **2006**, *21*, 1093−1100.

(21) Khademhosseini, A.; Eng, G.; Yeh, J.; Kucharczyk, P. A.; Langer, R.; Vunjak-Novakovic, G.; Radisic, M. Microfluidic Patterning for Fabrication of Contractile Cardiac Organoids. *Biomed. Microdevices* **2007**, *9*, 149−157.

(22) He, M.; Edgar, J. C.; Jeffries, G. D. M.; Lorenz, R. M.; Shelby, J. P.; Chiu, D. T. Selective Encapsulation of Single Cells and Subcellular Organelles into Picoliter- and Femtoliter-Volume Droplets. *Anal. Chem.* **2005**, *77*, 1539−1544.

(23) Nagrath, S.; Sequist, L. V.; Maheswaran, S.; Bell, D. W.; Irimia, D.; Ulkus, L.; Smith, M. R.; Kwak, E. L.; Digumarthy, S.; Muzikansky, A.; Ryan, P.; Balis, U. J.; Tompkins, R. G.; Haber, D. A.; Toner, M. Isolation of Rare Circulating Tumour Cells in Cancer Patients by Microchip Technology. *Nature* **2007**, *450*, 1235−1239.

(24) Meier, M.; Darling, J. K.; Choi, S. H.; Norstrom, E. M.; Sisodia, S. S.; Ismagilov, R. F. Plug-Based Microfluidics with Defined Surface Chemistry to Miniaturize and Control Aggregation of Amyloidogenic Peptides. *Angew. Chem., Int. Ed.* **2009**, *48*, 1487−1489.

(25) Choi, I.; Huh, Y. S.; Erickson, D. Size-Selective Concentration and Label-Free Characterization of Protein Aggregates Using a Raman Active Nanofluidic Device. *Lab Chip* **2011**, *11*, 632−638.

(26) McClain, M. A.; Culbertson, C. T.; Jacobson, S. C.; Allbritton, N. L.; Sims, C. E.; Ramsey, J. M. Microfluidic Devices for the High-Throughput Chemical Analysis of Cells. *Anal. Chem.* **2003**, *75*, 5646−5655.

(27) Srinivasan, V.; Pamula, V. K.; Fair, R. B. An Integrated Digital Microfluidic Lab-on-a-Chip for Clinical Diagnostics on Human Physiological Fluids. *Lab Chip* **2004**, *4*, 310−315.

(28) Jokerst, J. V.; Jacobson, J. W.; Bhagwandin, B. D.; Floriano, P. N.; Christodoulides, N.; McDevitt, J. T. Programmable Nano-Bio-Chip Sensors: Analytical Meets Clinical. *Anal. Chem.* **2010**, *82*, 1571−1579.

(29) Zhou, J.; Ren, K.; Zhao, Y.; Dai, W.; Wu, H. Convenient Formation of Nanoparticle Aggregates on Microfluidic Chips for Highly Sensitive SERS Detection of Biomolecules. *Anal. Bioanal. Chem.* **2012**, *402*, 1601−1609.

(30) Zhang, R. Q.; Liu, S. L.; Zhao, W.; Zhang, W. P.; Yu, X.; Li, Y.; Li, A. J.; Pang, D. W.; Zhang, Z. L. A Simple Point-of-Care Microfluidic Immunomagnetic Fluorescence Assay for Pathogens. *Anal. Chem.* **2013**, *85*, 2645−2651.

(31) Ackermann, K. R.; Henkel, T.; Popp, J. Quantitative Online Detection of Low-Concentrated Drugs via a SERS Microfluidic System. *ChemPhysChem* **2007**, *8*, 2665−2670.

(32) Andreou, C.; Hoonejani, M. R.; Barmi, M. R.; Moskovits, M.; Meinhart, C. D. Rapid Detection of Drugs of Abuse in Saliva Using Surface Enhanced Raman Spectroscopy and Microfluidics. *ACS Nano* **2013**, *7*, 7157−7164.

(33) Mcdonald, J. C.; Duffy, D. C.; Anderson, J. R.; Chiu, D. T.; Wu, H.; Schueller, O. J. A.; Whitesides, G. M. Fabrication of Microfluidic Systems in Poly (Dimethylsiloxane). *Electrophoresis* **2000**, *21*, 27−40.

(34) Ren, K.; Zhou, J.; Wu, H. Materials for Microfluidic Chip Fabrication. *Acc. Chem. Res.* **2013**, *46*, 2396−2406.

(35) Martinez, A. W.; Phillips, S. T.; Carrilho, E.; Thomas, S. W., III; Sindi, H.; Whitesides, G. M. Simple Telemedicine for Developing Regions: Camera Phones and Paper-Based Microfluidic Devices for Real-Time, Off-Site Diagnosis. *Anal. Chem.* **2008**, *80*, 3699−3707.

(36) Fang, X.; Chen, H.; Jiang, X.; Kong, J. Microfluidic Devices Constructed by a Marker Pen on a Silica Gel Plate for Multiplex Assays. *Anal. Chem.* **2011**, *83*, 3596−3599.

(37) Lee, C. H.; Hankus, M. E.; Tian, L.; Pellegrino, P. M.; Singamaneni, S. Highly Sensitive Surface Enhanced Raman Scattering Substrates Based on Filter Paper Loaded with Plasmonic Nanostructures. *Anal. Chem.* **2011**, *83*, 8953−8958.

(38) Li, X.; Ballerini, D. R.; Shen, W. A Perspective on Paper-Based Microfluidics: Current Status and Future Trends. *Biomicrofluidics* **2012**, *6*, 011301.

(39) Schilling, K. M.; Lepore, A. L.; Kurian, J. A.; Martinez, A. W. Fully Enclosed Microfluidic Paper-Based Analytical Devices. *Anal. Chem.* **2012**, *84*, 1579−1585.

(40) Qu, L.; Song, Q.; Li, Y.; Peng, M. P.; Li, D. W.; Chen, L. X.; Fossey, J. S.; Long, Y. T. Fabrication of Bimetallic Microfluidic Surface-Enhanced Raman Scattering Sensors on Paper by Screen Printing. *Anal. Chim. Acta* **2013**, *792*, 86−92.

(41) Betz, J. F.; Yu, W. W.; Cheng, Y.; White, I. M.; Rubloff, G. W. Simple SERS Substrates: Powerful, Portable, and Full of Potential. *Phys. Chem. Chem. Phys.* **2014**, *16*, 2224−2239.

(42) Warren, A. D.; Kwong, G. A.; Wood, D. K.; Lin, K. Y.; Bhatia, S. N. Point-of-Care Diagnostics for Noncommunicable Diseases Using Synthetic Urinary Biomarkers and Paper Microfluidics. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 3671−3676.

(43) Chen, L.; Choo, J. Recent Advances in Surface-Enhanced Raman Scattering Detection Technology for Microfluidic Chips. *Electrophoresis* **2008**, *29*, 1815−1828.

(44) Tong, L.; Righini, M.; Gonzalez, M. U.; Quidant, R.; Kall, M. Optical Aggregation of Metal Nanoparticles in a Microfluidic Channel for Surface-Enhanced Raman Scattering Analysis. *Lab Chip* **2009**, *9*, 193−195.

(45) Han, B.; Choi, N.; Kim, K. H.; Lim, D. W.; Choo, J. Application of Silver-Coated Magnetic Microspheres to a SERS-Based Optofluidic Sensor. *J. Phys. Chem. C* **2011**, *115*, 6290−6296.

(46) Li, Q.; Li, B.; Wang, Y. Surface-Enhanced Raman Scattering Microfluidic Sensor. *RSC Adv.* **2013**, *3*, 13015−13026.

(47) Kneip, K.; Wang, Y.; Kneipp, H.; Perelman, L. T.; Itzkan, I.; Dasari, R.; Feld, M. S. Single Molecule Detection Using Surface-Enhanced Raman Scattering (SERS). *Phys. Rev. Lett.* **1997**, *78*, 1667−1670.

(48) Nie, S. M.; Emery, S. R. Probing Single Molecules and Single Nanoparticles by Surface-Enhanced Raman Scattering. *Science* **1997**, *275*, 1102−1106.

(49) Michaels, A. M.; Jiang, J.; Brus, L. Ag Nanocrystal Junctions as the Site for Surface-Enhanced Raman Scattering of Single Rhodamine 6G Molecules. *J. Phys. Chem. B* **2000**, *104*, 11965−11971.

(50) Lim, D. K.; Jeon, K. S.; Hwang, J. H.; Kim, H.; Kwon, S.; Suh, Y. D.; Nam, J. M. Highly Uniform and Reproducible Surface-Enhanced Raman Scattering from DNA-Tailorable Nanoparticles with 1-nm Interior Gap. *Nat. Nanotechnol.* **2011**, *6*, 452−460.

(51) Saha, A.; Palmal, S.; Jana, N. R. Highly Reproducible and Sensitive Surface-Enhanced Raman Scattering from Colloidal Plasmonic Nanoparticle via Stabilization of Hot Spots in Graphene Oxide Liquid Crystal. *Nanoscale* **2012**, *4*, 6649−6657.

(52) Saha, A.; Jana, N. R. Detection of Cellular Glutathione and Oxidized Glutathione Using Magnetic-Plasmonic Nanocomposite-Based "Turn-Off" Surface Enhanced Raman Scattering. *Anal. Chem.* **2013**, *85*, 9221−9228.

(53) Manimaran, M.; Jana, N. R. Detection of Protein Molecules by Surface-Enhanced Raman Spectroscopy-Based Immunoassay Using 2−5 nm Gold Nanoparticle Labels. *J. Raman Spectrosc.* **2007**, *38*, 1326−1331.

(54) Mulvihill, M. J.; Ling, X. Y.; Henzie, J.; Yang, P. Anisotropic Etching of Silver Nanoparticles for Plasmonic Structures Capable of Single-Particle SERS. *J. Am. Chem. Soc.* **2010**, *132*, 268−274.

(55) Zhu, Z.; Meng, H.; Liu, W.; Liu, X.; Gong, J.; Qiu, X.; Jiang, L.; Wang, D.; Tang, Z. Superstructures and SERS Properties of Gold Nanocrystals with Different Shapes. *Angew. Chem., Int. Ed.* **2011**, *50*, 1593−1596.

(56) Chen, T.; Wang, H.; Chen, G.; Wang, Y.; Feng, Y.; Teo, S. W.; Wu, T.; Chen, H. G. Hotspot-Induced Transformation of Surface-Enhanced Raman Scattering Fingerprints. *ACS Nano* **2010**, *4*, 3087−3094.

(57) Yokota, Y.; Ueno, K.; Misawa, H. Essential Nanogap Effects on Surface-Enhanced Raman Scattering Signals from Closely Spaced Gold Nanoparticles. *Chem. Commun.* **2011**, *47*, 3505−3507.

(58) Jana, N. R. Silver Coated Gold Nanoparticles as New Surface Enhanced Raman Substrate at Low Analyte Concentration. *Analyst* **2003**, *128*, 954−956.

(59) Jana, N. R.; Pal, T. Anisotropic Metal Nanoparticles for Use as Surface-Enhanced Raman Substrates. *Adv. Mater.* **2007**, *19*, 1761−1765.

(60) Wustholz, K. L.; Henry, A.-I.; McMahon, J. M.; Freeman, R. G.; Valley, N.; Piotti, M. E.; Natan, M. J.; Schatz, G. C.; Van Duyne, R. P. Structure-Activity Relationships in Gold Nanoparticle Dimers and Trimers for Surface-Enhanced Raman Spectroscopy. *J. Am. Chem. Soc.* **2010**, *132*, 10903−10910.

(61) Charles Cao, Y.; Jin, R.; Nam, J. M.; Shad Thaxton, C.; Mirkin, C. A. Raman Dye-Labelled Nanoparticle Probes for Proteins. *J. Am. Chem. Soc.* **2003**, *125*, 14676−14677.

(62) Wang, Y.; Wei, H.; Li, B.; Ren, W.; Guo, S.; Dong, S.; Wang, E. SERS Opens a New Way in Aptasensor for Protein Recognition with High Sensitivity and Selectivity. *Chem. Commun.* **2007**, 5220−5222.

(63) Zengin, A.; Tamer, U.; Caykara, T. A SERS-Based Sandwich Assay for Ultrasensitive and Selective Detection of Alzheimer's Tau Protein. *Biomacromolecules* **2013**, *14*, 3001−3009.