Label-free and highly sensitive biomolecular detection using SERS and electrokinetic preconcentration†

Hansang Cho,[a] Brian Lee,[a] Gang L. Liu,[b] Ajay Agarwal[c] and Luke P. Lee*[a]

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In this paper, we present a method combining surface-enhanced Raman scattering (SERS) spectroscopy to detect biomolecules in a label-free way with an electrokinetic preconcentration technique (electrophoresis) to amplify biomolecular signals at low concentrations. A constant electric field is applied to charged biomolecules in solution, attracting them to an oppositely charged electrode, which is also used as a SERS substrate. Within 5 min, we observed that the SERS signal of 10 fM adenine was amplified to the level of the signal of non-preconcentrated 1 μM adenine (sensitivity improvement by 8 orders of magnitude) and the method was effective over a wide range of concentrations (10 fM to 1 μM). The signals were further amplified under stronger electric field and longer application: The increase of the signal intensity was observed to be 51 times at -0.6 V cm^{-1} after 25 min. The effectiveness of this method allows the creation of label-free, target-specific, and highly sensitive monitoring applications.

Introduction

Raman scattering has been investigated for its potential in molecular detection and analysis due to its many appealing benefits, including its label-free nature and potential for multiplex detection. Raman spectra provide unique molecular fingerprints composed of many narrow-band peaks corresponding to submolecular vibrational modes in each functional group making it possible to identify the species of the molecules and even monitor the dynamic change in the molecular structure. Due to its inherently weak signal intensity, however, its use in sensitive molecular detection was limited until the discovery of surface-enhanced Raman scattering (SERS), which dramatically enhances the weak Raman scattering signal of molecules as strong as fluorescence signals.[1,2] SERS signal enhancement occurs when the molecule comes within close proximity to a metal surface where local electromagnetic (EM) field enhancements are generated. Because the EM field enhancement is largely dependent on the structural features of the metal surface, much effort has been focused on creating novel designs and fabrication methods to develop effective SERS-active substrates.[3–8] In addition, other approaches to improve target signal intensities have focused on sample preconcentration methods[9,10] including surface-binding,[11,12] dielectrophoresis,[13–15] nanochannel/nanomembrane-based techniques[16,17] and opto-electric trapping.[18,19] These have been demonstrated to improve the detection limit by increasing the number of molecules in the enhancement region. However, these preconcentration methods often require the addition of structures or components to the device that makes adaptation for general purposes difficult. The ideal technique should provide speed and flexibility by introducing as few additional elements as possible to be compatible with a wide range of demands for a molecular detection system.

In this paper, we demonstrate an electrokinetic preconcentration method that is effective in improving the sensitivity of detection in a wide range of concentrations without the use of complicated equipment. We have observed that adenine was preconcentrated by up to 8 orders of magnitude after only 5 min of preconcentration. The improvement was effective over a wide range of initial concentrations (10 fM to 1 μM), and this method was implemented with only the addition of an electrode.

Theory

The intensity of the SERS signal can be estimated by considering N_{SERS}, the number of molecules involved in the SERS process, and the local EM field enhancement factor of the SERS substrate.[20,21] Therefore, increasing the value of N_{SERS} by attracting molecules to the SERS substrate will amplify the SERS signal proportionally to the preconcentration factor of the molecules.

The concept of sample preconcentration in this work is based on charged molecular movement along an electric field (electrophoresis) and physical adsorption to an oppositely charged electrode as shown in Fig. 1. This adsorption causes the molecule to be proximate to the surface of a SERS substrate while increasing the local concentration of the molecule near the surface of the substrate where the local EM field is effective. To analyze the concentration effect, we considered mass conservation and the electrokinetic velocity: $j = -D\nabla c = c \times u_{ek}$ and $u_{ek} = \mu_{ek} \times E$ under a simplified electric field ($E = V/d$),[22,23] where j, D, c, μ_{ek}, V, and d are the diffusion flux, the molecular diffusivity,

[a]*Biomolecular Nanotechnology Center, Berkeley Sensor & Actuator Center, Department of Bioengineering, University of California, Berkeley, California, 94720, USA. E-mail: lplee@berkeley.edu; Fax: +1 510 642 5835; Tel: +1 510 642 5855*
[b]*Department of Electrical and Computer Engineering, University of Illinois at Urbana-Champaign, Illinois, 61801, USA*
[c]*Institute of Microelectronics, Singapore*

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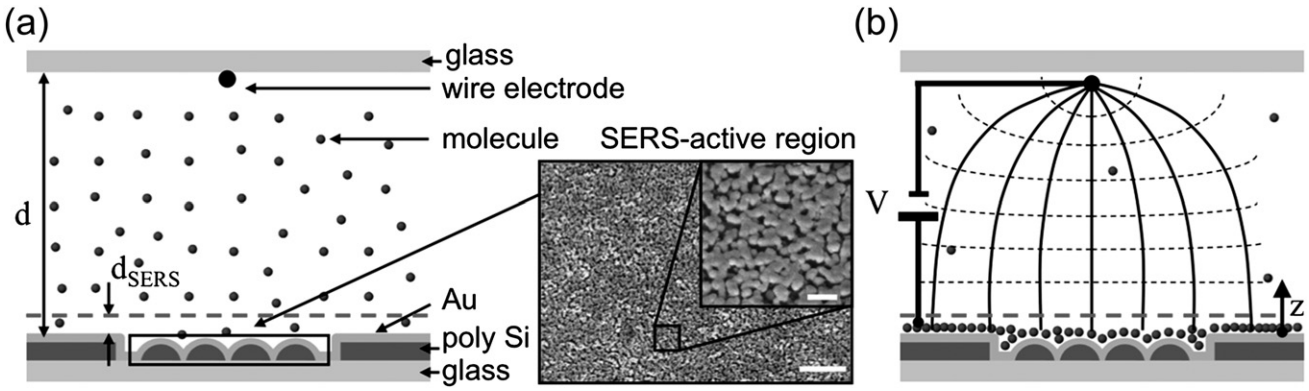

Fig. 1 Schematics of electrokinetic-based preconcentration method for signal amplification. (a) Biomolecules are uniformly dispersed in solution in the absence of an electric field. Inset shows the SEM images of a SERS-active region. Scale bars are 1 μm and 200 nm. (b) An electric field is applied between a top cylindrical wire electrode and a bottom plate Au-electrode including the SERS-active region. In the presence of an electric field gradient (dotted lines), most of the charged biomolecules are attracted along an electric field (solid lines) and then concentrated onto the oppositely charged electrode resulting in amplification of the SERS signal for the molecules.

the molecular concentration, the electrokinetic mobility of molecule, a constant voltage, and the distance between electrodes, respectively. Based on these equations, the local concentration is calculated with molecular properties and applied electrokinetic conditions as $c = c_o \times \exp(-\gamma \times z/d)$, where $\gamma = (\mu_{ek}/D) \times V$, $c_o = c_i \times \gamma/(1 - \exp(-\gamma))$ and c_i and z are the initial concentration before preconcentration and a distance from the SERS substrate. Preconcentration factor (PF) is defined as the ratio of the number of molecules within a distance of d_{SERS} from the SERS substrate before and after the preconcentration step as:

$$PF = \frac{N_{SERS}^{pre}}{N_{SERS}} = \frac{\lim_{z\to 0}(c(z) \times d_{SERS})}{(c_i \times d_{SERS})} = \frac{\gamma}{(1 - \exp(-\gamma))} \quad (1)$$

N_{SERS}^{pre} and d_{SERS} are the number of preconcentrated molecules and the distance from metal surface involved with the SERS process. According to the equation, PF increases with γ representing that more molecules are preconcentrated under higher value of V. PF can be estimated by experimentally measuring the SERS signal intensity before and after preconcentration steps assuming that the intensity is linearly proportional to the number of molecules within the distance of d_{SERS}.

Materials and methods

The SERS substrate was fabricated on a whole quartz wafer through a single batch process to minimize sample variation affecting the SERS effect (see ESI, Fig. S1†).[24] In summary, 300 nm of polysilicon (polySi) were deposited on a 4 inch quartz wafer of 500 μm thickness. Photoresist window (a circular opening of 3 mm in diameter) was patterned using standard optical lithography. PolySi on the window was roughened with the oxygen gas etching process at an oxygen flow rate of 3 cm^3 min^{-1} for 30 s, which resulted in a pillar-like nanostructure. Au 40 nm in thickness was deposited on the wafer, which created a ball-like structure on the top of the pillar-like nanostructure for a SERS-active region (SEM images in Fig. 1a, inset) and a uniform thin layer as the plate electrode. The wafer was cut into pieces of about 1 cm × 0.5 cm for experiments.

Adenine powder (Sigma-Aldrich, Milwaukee, WI) was dissolved in 50 mM disodium phosphate buffer (Na_2HPO_3) at pH 7.4, adjusted with H_3PO_4. The SERS substrate was fixed to the bottom of a plastic chamber with double adhesive tape. A gauge wire (Page Digital, Inc., Duarte, CA) was connected to the Au plate surface (working electrode). A reference electrode made of a Ag/AgCl pellet, 1 mm in diameter, embedded on a 0.25 mm diameter wire (E205, Warner Instruments, Hamden, CT) and a ground electrode made of a cylindrical Ag/AgCl wire were immersed in the buffer and fixed on the top of the chamber. The plate and the cylindrical electrodes were separated by an average distance of 1 cm. The applied electric field was generated by a potentiostat (CHI750A, CH Instrument, Austin, TX). Fresh adenine solution, 600 μL in volume, was introduced into the plastic chamber and covered with a glass cover slide to prevent evaporation during measurement.

As for the quantitative analysis while avoiding spatial variation of SERS effect and molecular attraction, we measured SERS signals at the same spot for each experiment while preconcentrating molecules through time, then we normalized the measured signals with a signal taken from the same spot before the preconcentration technique had been applied. The solution was maintained for more than 5 min before applying an electric field to stabilize the conditions. SERS spectra were measured with a fully integrated Raman system (R2001S, Raman Systems Inc., Austin, TX).

Results and discussion

For a proof-of-concept, the SERS signal was continually measured over time while maintaining the electric field between the electrodes with adenine at a concentration of 10 nM, at which adenine could be barely detected without any preconcentration (Fig. 2). Adenine is assumed to carry a net positive charge in pH 7.4 buffer solution. Placed in the chamber with an applied electric field, the positively charged adenine molecules are attracted to the negatively charged plate electrode, which doubles as a SERS substrate.[25] Adenine-specific peaks appeared discernibly from the background within a few minutes and the SERS intensity

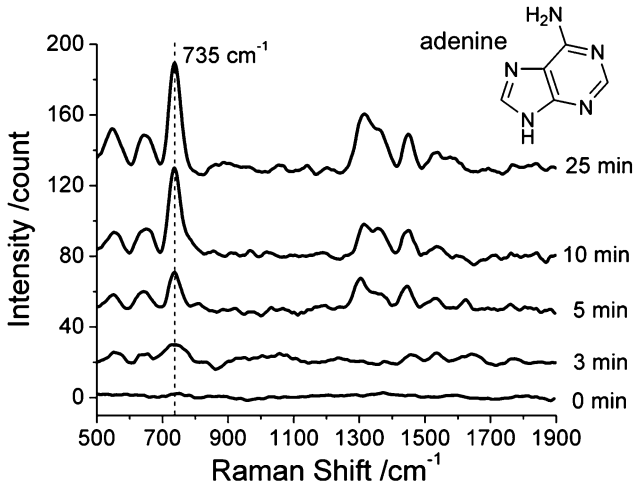

Fig. 2 SERS signal amplification of adenine by electrokinetic preconcentration. A weak SERS signal of 10 nM adenine was observed before electrokinetic preconcentration (0 min). Under a constant electric field of -0.6 V cm^{-1}, the SERS signal was significantly amplified within a few minutes and reached a maximum value at 25 min. The SERS spectra were measured with a 785 nm excitation laser of 90 mW and an integration time of 10 s.

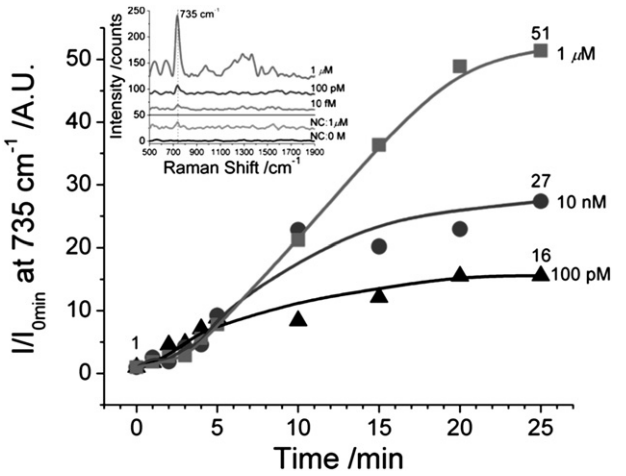

Fig. 4 Kinetics of electrokinetic preconcentration with varying initial concentrations. The SERS signals of adenine increased with time and initial concentrations and reached saturation limit about after 25 min. Inset shows signals of non-preconcentrated adenine (NC: 0 M and NC: 1 μM) and the signals amplified for 5 min over a wide range of the initial concentrations (from 10 fM to 1 μM). The intensity of 10 fM adenine was amplified as high as that of non-preconcentrated 1 μM adenine (NC: 1 μM). The experimental conditions were the same as in Fig. 2.

increased with time. Signal amplification sloped off at 25 min, indicating proximity to the saturation limit under the conditions. The baselines of the spectra were removed for clear comparisons.

To further understand the kinetics of the electrokinetic preconcentration, the most significant Raman shift at 735 cm^{-1}, assigned to NH_2 deformation, was normalized and compared at varying electric fields and initial concentrations of adenine as in Fig. 3 and 4, respectively. The normalization was done by subtracting the baseline from the time-dependent spectra and dividing the value of the height of the distinctive adenine peaks at

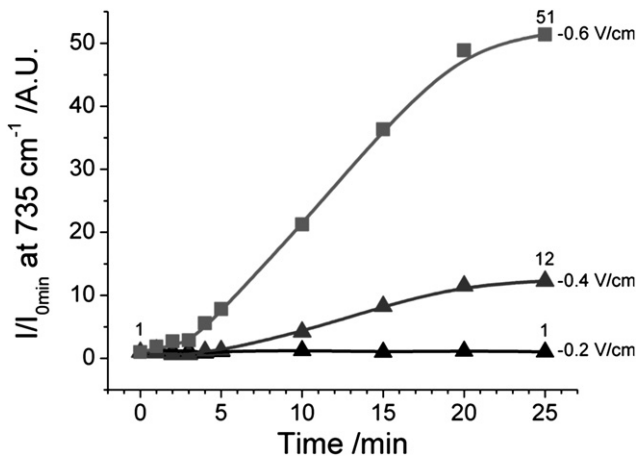

Fig. 3 Kinetics of electrokinetic preconcentration with varying electric fields. The intensity of the SERS signal at 735 cm^{-1} for 1 μM adenine was amplified by 51 times after the electrokinetic preconcentration with an applied electric field of -0.6 V cm^{-1} for 25 min. Signal amplification was not observed in the case of a weak electric field of -0.2 V cm^{-1}. The amplified signals were normalized using the measured signal intensity before the preconcentration (I_{0min}). The experimental conditions were the same as in Fig. 2 except for the magnitude of the applied electric field.

0 min (no preconcentration). As expected, the signal amplification was more effective at the higher electric fields: 51-fold at -0.6 V cm^{-1}, 12-fold at -0.4 V cm^{-1}. However, signal amplification was not observed at weak field: -0.2 V cm^{-1} probably because the weak field could not extend into the solution. Over -0.8 V cm^{-1}, the signal increased rapidly but disappeared within 10 min, as a result of the damage to the thin gold layer.

To estimate the extent of signal amplification, adenine solution at varying concentrations was subjected to the electric field of -0.6 V cm^{-1} (Fig. 4). The inset of Fig. 4 shows spectra measured in different conditions for comparison. As for negative control (NC), we measured the signals from the buffer solution in the absence of adenine ('NC: 0 M') under an electric field of -0.6 V/cm after 5 minutes of preconcentration and 1 μM adenine without application of the electric field ('NC: 1 μM'). There was no discernible change in spectra with time in the control experiments ('NC: 0 M' and 'NC: 1 μM'). However, this method can amplify the signal of 10 fM to a similar level to the signal from unconcentrated 1 μM ('NC: 1 μM') (improved sensitivity by a 10^8-fold). Also, the intensity of the signal for preconcentrated 1 μM adenine for 5 min became 10 times higher than that of non-preconcentrated 1 μM adenine, indicating that the electrokinetic preconcentration was a predominant factor for signal amplification above any other factors including time. The baselines were removed for clear comparison.

Fig. 4 shows that the signals at all three tested adenine concentrations were steadily amplified until 25 min and reached a saturation limit proportional to the initial concentration. Up to 5 min, preconcentration factors increased at the same rate through the wide range of initial concentrations as we estimated. However, the preconcentration factors were saturated proportionally to the initial concentrations. Different saturation levels are presumably caused by a modeling error from the assumption of the simplified electric field or the statistical nature of preconcentration.

The movement of charged molecules may have been affected by both electrophoresis (EP) and dielectrophoresis (DEP) because the electric field was not perfectly homogeneous. However, we observed that EP was the dominant force because the molecules moved to the plate electrode rather than to the cylindrical electrode, where the stronger electric field gradient would be created for positive DEP.[26–28] Furthermore, we reversed the direction of the electric field after preconcentrating adenine (see ESI, Fig. S2†). A decrease in signal was observed, indicating that a positive charge on the electrode repelled and displaced the positively charged adenine. This also showed the negligible effect of negative DEP, which would attract molecules down the electric field gradient to the plate electrode, where the weaker electric field gradient would be created. The conditions for SERS measurement were the same as described in Fig. 2.

Although a uniform electric field is ideal for characterizing electrokinetic preconcentration, we encountered technical issues and limitations that prevented us from creating a true uniform field. Once these issues are resolved, future experiments will examine signal amplification under a uniform electric field. However, our current setup sufficiently demonstrates the proof-of-concept for this method and optimization of conditions such as the magnitude of the field and the distance between electrodes can further improve the sensitivity of SERS detection.

Conclusions

Using electrokinetic-based sample preconcentration, we have accomplished additional SERS signal amplification of adenine with further potential to improve the sensitivity and shorten the time length of electric field application. In its application to real-world samples, any charged molecules might be attracted to an oppositely charged electrode, which implies that this method would have the potential for broad applications: a wide variety of other charged biomolecules and cancer markers present in solution or secreted from cancer cells at extremely low concentrations including DNA, RNA, peptides, charged phospholipids (*e.g.* lysophosphatidic acid), and proteins (*e.g.* serum albumin, growth factors) for label-free biomolecular analysis, cancer diagnostics, and water monitoring. The only potential concern on the use of the biological solutions may be surface contamination or coverage on a SERS-active region by other strongly charged biomolecules, which may decrease the strength of an electric field or SERS effect. However, this issue can be resolved by combining electrokinetic preconcentration method with sample preparation techniques.

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