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Stability optimization of microbial surface-enhanced Raman spectroscopy detection with immunomagnetic separation beads

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Abstract. Immunomagnetic separation (IMS) beads with antibody coating are an interesting option for biosensing applications for the identification of biomolecules and biological cells, such as bacteria. The paramagnetic properties of the beads can be utilized with optical sensing by migrating and accumulating the beads and the bound analytes toward the focus depth of the detection system by an external magnetic field. The stability of microbial detection with IMS beads was studied by combining a flexible, inexpensive, and mass producible surface-enhanced Raman spectroscopy (SERS) platform with gold nanoparticle detection and antibody recognition by the IMS beads. *Listeria innocua* ATCC 33090 was used as a model sample and the effect of the IMS beads on the detected Raman signal was studied. The IMS beads were deposited into a hydrophobic sample well and accumulated toward the detection plane by a neodymium magnet. For the first time, it was shown that the spatial stability of the detection could be improved up to 35% by using IMS bead capture and sample well placing. The effect of a neodymium magnet under the SERS chip improved the temporal detection and significantly reduced the necessary time for sample stabilization for advanced laboratory testing. © 2017 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: [10.1117/1.OE.56.3.037102](https://doi.org/10.1117/1.OE.56.3.037102)]

Keywords: surface-enhanced Raman spectroscopy; immunomagnetic separation beads; optical detection; biological cells.

Paper 161981 received Dec. 22, 2016; accepted for publication Feb. 17, 2017; published online Mar. 7, 2017.

1 Introduction

Optical sensing has a long history in biological and molecular analysis.^{1,2} Typically, the photonics-based detection methods are either label-free or utilize a conjugated label, such as a fluorophore. Although the fluorophores can often enhance detection sensitivity, they can also complicate the analysis procedure. Surface-enhanced Raman spectroscopy (SERS) is a recent optical detection method, which can offer both identification of the analyte and enhanced sensitivity without the use of labels.³ Additionally, SERS can offer a multiplexed analysis and quick discrimination of analytes, such as biological cells through their specific Raman spectra at the current state. Immunomagnetic separation (IMS) beads are an interesting alternative for the trapping and separation of biological cells before cell analysis.^{4–13} IMS beads combine the ability of cell separation with an antibody-assisted identification of the captured cells. In comparison to the more conventionally used membrane filtration and centrifugation, the IMS beads can show higher specificity and separation efficiency.⁶ During the past two decades, IMS beads have been used efficiently for biological cell trapping in food pathogen analysis and in environmental monitoring.^{4–9,11–13} SERS is a vibrational spectroscopy technique, which can be used for the detection and recognition of

captured biological cells.^{14–30} In SERS, the Raman scattering of excitation light forms a spectrum containing information about the chemical structure and bonds of the biomolecules on the surface of the cells. Noble metals, such as gold and silver, enhance the detected signal through localized surface plasmon resonance and enable sensitive detection for near-infrared Raman.^{3,31,32} As reported by Li et al.,³³ by combining SERS nanoparticle detection with a structured SERS substrates, it is possible to increase the total plasmonic electromagnetic field, and consequently, SERS enhancement, through a weak coupling of local fields if the substrate and the nanoparticles are gathered in the near vicinity of each other. The use of IMS beads in combination with detection is usually conducted by removing the beads before the detection^{34,35} or by using custom-made sandwich assays with Raman labels for signal enhancement.^{36,37} There are, to our knowledge, hardly any studies focusing on the effect of IMS beads on the signal construction during SERS detection. Although SERS has been receiving a lot of attention in the last decade because it is label free and it can ultimately be used even for single-cell detection,^{32,38,39} the practical use of SERS has encountered challenges in the repeatability and stability of the measurements. Detection of biological cells in liquid samples is highly dependent on the fact that the cells have to be in close proximity of SERS-active colloids or

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Fig. 1 A schematic illustration of the roll-to-roll UV-nanoimprint method for structured SERS substrate fabrication. The SERS pattern is imprinted onto a PMMA web with UV sensitive lacquer. The lacquer is set with UV light exposure through a transparent polymer web.

surfaces, and that both the cells and the SERS active agents have to be within the focus depth of the detection system.⁴⁰ Detecting a sample droplet on a surface can show low SERS signal reproducibility and stability due to microcurrents generated by the evaporation of the liquid and shrinkage of the droplet.²⁹ During the evaporation, enhanced with the focussed laser beam, the microcurrents move the cells toward the edges of the droplet, where the signal can be significantly higher than in the middle of the droplet. This phenomenon is referred to as the coffee ring effect.⁴¹ The flow of cells to the edges of the droplet can be suppressed by the use of IMS beads, which are heavier objects and do not flow as easily along the microcurrents, thus showing higher stability in respect to their location. Herein, we have studied the positive effect of IMS beads on the spatial and temporal stability of cell detection with SERS. The use of a magnetic field to accumulate paramagnetic IMS beads with gold nanoparticles near the surface of the SERS substrate was analyzed. *Listeria innocua* ATCC 33090 was used as a model sample for biological cell capture.

2 Experimental Methods

2.1 Gold Nanoparticle Synthesis

The Frens method⁴² was used for the synthesis of gold nanocolloids (AuNPs) with a median size of 85 nm as determined from transmission electron microscopy images. In brief, a 0.01% (wt/vol) HAuCl_4 aqueous solution was heated up to boiling point while continuously stirring the solution. 1% (wt/vol) trisodium citrate solution was dropwise added

to the HAuCl_4 aqueous solution. This low viscous solution boiled for ~45 min until the color of the solution changed to dark-red. The resulting AuNP solution was centrifuged at 3500 rpm for 5 min to remove the supernatant sequentially. The concentration of the final AuNP solution was ~5500 mg/L, which was diluted in a 1:5 ratio in deionized water before experiments.

2.2 Surface-Enhanced Raman Spectroscopy Substrate Fabrication

The structured SERS substrates were fabricated with a high throughput method by using UV-nanoimprint lithography with roll-to-roll equipment, as shown in Fig. 1.^{17,43} A poly(methyl methacrylate) (PMMA) polymer sheet was coated with a UV-curable lacquer by reverse gravure technique. The lacquer was patterned with embossing reel and UV light was used to cure the structures through the PMMA web. The rolls were die-cut into smaller sensor strips, which were coated with a 240-nm gold layer by evaporation.

2.3 PDMS Well Integration

Hydrophobic sample wells with a diameter of 1 mm were cut into 1-mm thick polydimethylsiloxane (PDMS) foil. The wells were bonded to the gold layer by physical adsorption. The hydrophobicity of the PDMS around the wells allowed for the sample amount to be larger than the well volume. A neodymium magnet was placed underneath the wells and the SERS substrate to force the IMS beads near the sensing surface and accumulate the bacteria cells. Figure 2 depicts a

Fig. 2 SERS substrate with an integrated hydrophobic sample well, IMS beads with *L. innocua* ATCC 33090 cells and gold nanoparticles for signal enhancement around the microbe cells. A neodymium magnet placed below the chip for optimal signal collection through the rapid descending of the IMS beads onto the SERS substrate. The arrows show the direction of the IMS bead motion.

situation where the *L. innocua* ATCC 33090 cells are bound by the IMS beads and the sample is placed into a sample well on a SERS substrate. The SERS chip with the well is positioned on top of a neodymium magnet for the studies of rapid IMS bead migration.

2.4 Cultivation of *L. innocua* and Capture of Cells with Immunomagnetic Separation Beads

The bacteria, *L. innocua* ATCC 33090, was grown in Listeria Express Enrichment (LEE) Broth (Labema, Lab M Limited, pH 7.2 ± 0.2) at 35°C for 20 h. Spectrophotometry (Dynamica HALO DB-20S) was used to determine the concentration of the bacteria, which was then diluted into the used concentrations (10^6 to 10^9 CFU/ml) in LEE Broth. Dynabeads® anti-Listeria [Life Technologies (Invitrogen) 71006] with the standard IMS protocol was used for IMS capture of bacteria with a Dynal Magnetic Particle Concentrator DynaMag™-2 (Invitrogen Dynal). Briefly, 1 ml of bacterial culture was incubated at room temperature in a microcentrifuge tube containing 20 μl of Dynabeads® anti-Listeria (Dynal) for 10 min with continuous mixing. Dynal MPC-M magnetic field was used to concentrate the IMS beads. After the supernatant was removed, a washing buffer (0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4 with 0.05% Tween 20) was used for sample washing. The beads were again concentrated to remove the supernatant and the IMS beads with the bacteria were resuspended into 100 ml of washing buffer for SERS detection.

2.5 Surface-Enhanced Raman Spectroscopy Experiments and Postprocessing of Data

An in-house built Raman system, coupled with an optical microscope (Olympus BX-51) and a 785-nm excitation laser wavelength, was used for spectral recording. For signal collection, a laser power of 40 mW was used with 20 $\times$ objective magnification. The sample volume in the experiments for spatial stability without a PDMS well was 6 μl . The signal integration time was 5 s without averaging for the 10^9 CFU/ml samples and 10 s for the 10^8 CFU/ml samples. A sample volume of 5 μl was used with the IMS bound microbe samples mixed with gold nanoparticles (AuNPs) in hydrophobic (PDMS) sample wells. The mixing ratio for microbe samples and AuNPs before dispensing was 2.5 parts AuNP solution (AuNP concentration 1000 mg/l in H_2O) to 10 parts microbe sample solution. The AuNPs are mixed with gold colloids before the stability testing for 30 s. For consistency, the amount of gold nanoparticles in a sample droplet with each concentration was kept constant at 3.6×10^7 particles in a 5- μl droplet volume. The relation between AuNP amount and the bacteria cell amount in one droplet ranges from 10 particles/cell with a higher cell concentration of 10^9 cfu/ml to 10^3 particles/cell with a lower cell concentration of 10^6 cfu/ml. Therefore, the amount of nanoparticles adsorbing to one bacteria cell can differ with different concentrations, although the particle density in the droplet volume is constant. MATLAB (release 2015a, Mathworks Inc.) and PLS toolbox (version 2.0, Eigenvector Research Inc., Manson, Washington) were used for a simple baseline correction (linear fitting) and transfer of the data into Origin Pro (version 9.4, OriginLab Corp.). Origin Pro was used for data postprocessing and plotting.

Fig. 3 SERS spectrum of IMS bound *L. innocua* with a concentration of 10^8 CFU/ml, 5-s integration time and 40-mW excitation laser power. Savitsky–Golay function was used for spectrum smoothing.

3 Results and Discussion

The SERS spectrum of the model bacteria *L. innocua* ATCC 33090, measured with the combination of AuNP and a roll-to-roll produced SERS substrate, is presented in Fig. 3. The *L. innocua* ATCC 33090 cells exhibit a strong Raman peak at a Raman shift of 737 cm^{-1} . This main peak, related to a glycosidic ring mode, will be utilized when analyzing the effect of the IMS beads on the signal construction and stability. The measured spectrum is in good agreement with similar spectra previously presented by Luo et al. and Kairyte et al.^{16,29}

The difference in the stability of the SERS signal as a deposited SERS droplet on a surface without and with IMS bead capture is presented in Figs. 4(a) and 4(b), respectively. The 44% average deviation of the signal intensity of the main Raman peak at 737 cm^{-1} in the case of Fig. 4(a) is too large even for semiquantitative detection. By using IMS beads, as shown in Fig. 4(b), the average deviation of the signal intensity of the main Raman peak at 737 cm^{-1} diminishes to 13%, thus yielding a better signal stability and repeatability. However, detecting a SERS signal from a droplet can be affected by many factors. Repetitively pipetting a droplet with a constant shape and a similar footprint is difficult. The lens like shape of the droplet can cause challenges with correct focus depth and the efficient capture of the scattered Raman signal. By placing the sample droplet into a hydrophobic PDMS well, the repeatability of the detection can be enhanced. As can be seen from Fig. 4(c), the use of the PDMS well also lowers the average deviation of the signal intensity of the main Raman peak at 737 cm^{-1} .

Although the spatial stability of the signal can be increased with the combination of IMS bead capture and an integrated sample well, the temporal stability is still low. Figures 5(a)–5(c) show the temporal growth of the SERS signal of the main Raman peak at 737 cm^{-1} as a function of time. The signal growth requires at least a 10- to 20-min waiting time for the signal to reach the maximal peak height. This is the time from the appliance of the sample into the well, until the majority of the IMS beads slowly descend to the SERS substrate and to the focus depth of the Raman system. The waiting time adds variance into the detected signal height and delays the signal recording. However, it does increase the intensity of the Raman bands originating from the analyte. Figure 5(d) shows the intensity relation of 10^6 to 10^8 CFU/ml sample concentrations of IMS captured

Fig. 4 (a) Deviation of *L. innocua* spectrum measured from a 10^9 CFU/ml droplet. Result of eight spectra measured from 1 mm^2 area. (b) *L. innocua* spectrum with microbes bound to IMS beads from a 10^8 CFU/ml droplet. Result of 10 spectra measured 1 mm^2 . (c) *L. innocua* spectrum with microbes bound to IMS beads from a 10^6 CFU/ml sample in a PDMS well. Result of nine spectra measured 1 mm^2 .

Fig. 5 SERS signal growth of IMS bound *L. innocua* samples with different concentrations in a hydrophobic sample well as a function of time. As the heavy IMS beads slowly descent to the surface of the structured SERS substrate, the signal intensity grows. (a) 10^6 CFU/ml *L. innocua* sample, (b) 10^7 CFU/ml *L. innocua* sample, and (c) 10^8 CFU/ml *L. innocua* sample. (d) Relation of the intensity of the main Raman peak at 737 cm^{-1} as a function of sample concentration after allowing the beads to descend for 10 min. Savitsky–Golay function was used for spectrum smoothing.

L. innocua ATCC 33090 cells detected after allowing the beads to slowly migrate toward the SERS substrate for 10 min. The limit of detection (LoD) with the method is clearly smaller than the lowest detected concentration 10^6 cfu/ml, possibly near 10^5 cfu/ml or lower. According to our previous study without magnet stabilization, an LoD of 1.4×10^4 cfu/ml can be achieved.¹⁷

By employing the paramagnetic property of the IMS beads, the beads rapidly accumulate onto the SERS

substrate. From Figs. 6(a) and 6(b), it can be seen how a neodymium magnet located underneath the SERS substrate functions as an IMS bead attractor. The overall quality of the SERS peaks is increased with the magnetically stabilized samples. Minor peaks of the spectrum can be identified more clearly.

The saturation in the rise of signal intensity is reached within 2 min or less after depositing the sample. The changes in SERS signal intensity with and without the magnet as

Fig. 6 SERS signal of IMS bound *L. innocua* samples (a) without and (b) with a neodymium magnet placed underneath the SERS chip. The neodymium magnet placed underneath the SERS chip clearly forces the beads to descend faster to the bottom of the hydrophobic sample well. Sample concentration: 10^6 CFU/ml. Savitsky–Golay function was used for spectrum smoothing.

Fig. 7 The change in relative SERS signal intensity of IMS bound *L. innocua* samples as a function of time detected without and with a neodymium magnet placed underneath the SERS chip. Sample concentration: 10^6 CFU/ml.

a function of time are presented in Fig. 7. When the magnetic field is applied, the signal intensity changes only 4.9% after 2 min. The slope of the signal after 2 min is attributed to the local heating of the measurement spot and the degrading effect of the heating to the bacteria cells and IMS beads. Whereas, without the magnetic field, the signal intensity is growing at a constant rate of 2.5%/min up to 10 min and continues to rise further as time progresses even up to 20 min.

In the present study, the effect of IMS beads on the SERS signal stability of bacteria cells was analyzed. The IMS beads stabilized both the temporal and the spatial variance, and accumulated the bacteria cells evenly into the focus depth and to the focal point of the laser beam. The results show the superiority of bacteria cell detection employing IMS capture in comparison to biological cell detection with free cells.

4 Conclusions

The spatial and temporal stability of SERS detection of bacteria cells in solution phase was studied with IMS bead capture and hydrophobic PDMS sample wells. We

demonstrate significant improvements of the spatial signal stability by 35% when the bacteria cells were captured by the heavier IMS beads, which were placed into the restricted area of the sample well. The temporal stability was affected by the descending time of the IMS bead inside the sample well. A sample stabilization time of 10 to 20 min was required to achieve saturated SERS signal intensity when no magnetic field was present. However, by using a neodymium magnet as an IMS bead attractor underneath the SERS substrate, the lag time for sample stabilization could be reduced to below 2 min. Besides producing reproducible and more reliable SERS results, this method enables quicker and straightforward laboratory testing.

Acknowledgments

The funding for this project was provided, in part, by TEKES (the Finnish Funding Agency for Technology and Innovation) and University of Oulu Graduate School through Infotech Oulu Doctoral Program. Academy of Finland joined in funding the work through research projects (Foulsens Grant No. 292253 and M-SPEC Grant No. 284907). We thank Tiina Väyrynen (National Resources Institute Finland) for the work with the bacterial sample preparation.

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