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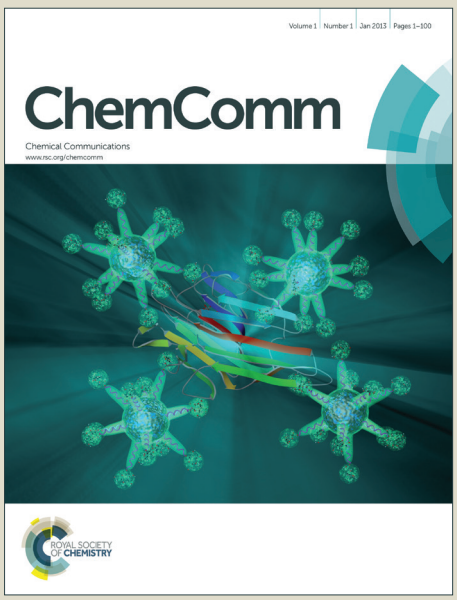

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Highly Sensitive DNA Methylation Analysis at CpG Resolution by Surface-enhanced Raman Scattering via Ligase Chain Reaction

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Sensitive and accurate DNA methylation analysis at CpG resolution was demonstrated with surface-enhanced Raman scattering (SERS) via ligase chain reaction (LCR). The method was sensitive to 10% changes in methylation and the accuracy of methylation estimates in cells and serum DNA validated with sequencing. The LCR/SERS approach may have broad applications as an alternative (epi)genetic detection method.

Single DNA base change such as aberrant DNA methylation epigenetic changes, where a cytosine (C) to thymine (T) base change occurs after bisulfite treatment of DNA,[1] single nucleotide polymorphism (SNP) and point mutation are common events in cancer.[2-3] The screening of such single DNA base change is clinically useful for disease diagnosis and the selection of the appropriate therapies eg. personalized medicine.[1-5] However, routine single DNA base change analysis relying on PCR alone is challenging and has many limitations.[6-9] Additionally, most single-base change detection methods still rely on fluorescence and mass spectroscopy, which are labor-intensive or instrumentally-expensive. Therefore, it is highly desirable to develop simple, cost effective and multiplexed assays with high sensitivity and specificity for routine diagnosis.

Surface enhanced Raman Scattering (SERS) is a physical phenomenon that happens on metal surfaces and has been demonstrated to be an attractive alternative to fluorescence-based approaches.[10-12] In particularly, SERS has a unique advantage over fluorescence in parallel detection of multiple analytes (multiplexing) due to the narrow and distinct bandwidths of vibrational Raman bands.[10-12] In contrast, the overlapping fluorescence emission spectra severely limit multiplexing. In addition, SERS also has comparable sensitivity yet improved photostability over flurophores.[10-12]

While detecting single DNA base mismatches by SERS is achievable via label-free methods coupled to intensive post-analysis,[13,14] the poor spectral reproducibility and the similarity of SERS signals from single and double stranded DNA limit their applications in routine medical diagnostics procedures.[13,14] A strategy to facilitate single DNA base detection via SERS could be a DNA ligase-based approaches. For instance, Erikson, *et. al.* reported single point mutation by SERS via a ligation detection reaction (LDR).[15-16] By labeling primers with silver nanoparticles and dyes, single-point mutation with the detection limit of 10-20 pM was achieved by SERS. Although highly specific, LDR is a linear amplification hence inherently limited in sensitivity and speed. The ligase chain reaction (LCR) however, may enhance sensitivity and speed over LDR due to its exponential amplification characteristics.[17] LCR has been applied to single DNA base change detection by fluorescence and electrochemical methods,[18-21] however, to the best of our knowledge, no one has reported multiplexed detection of single base changes via LCR coupled to a SERS readout nor has its application to DNA methylation at single CpG resolution been reported.

Herein, we demonstrate for the first time, a proof-of-concept multiplexed single DNA base change detection platform with SERS nanotags via LCR. We applied the LCR/SERS method to sensitive DNA methylation analysis at a single CpG site on synthetic DNA, a panel of breast cancer cell lines and a serum-derived DNA sample. In addition, methylation estimates also validated well with Next Generation Sequencing (NGS) thus demonstrating the high accuracy of our method.

The principle of this platform for multiplexed detection of a single DNA base change and its application to DNA methylation analysis is illustrated in Scheme 1. In this approach (Scheme 1A), genomic DNA was first treated with bisulfite, in which methylated C remained inert while unmethylated C were converted to uracils and subsequently T after PCR.[22-23] Following PCR amplification of a sequence containing a CpG of interest, LCR was then applied to identify and exponentially amplify the C/T base change (as illustrated in Scheme S1, ESI) which, in turn, represented the

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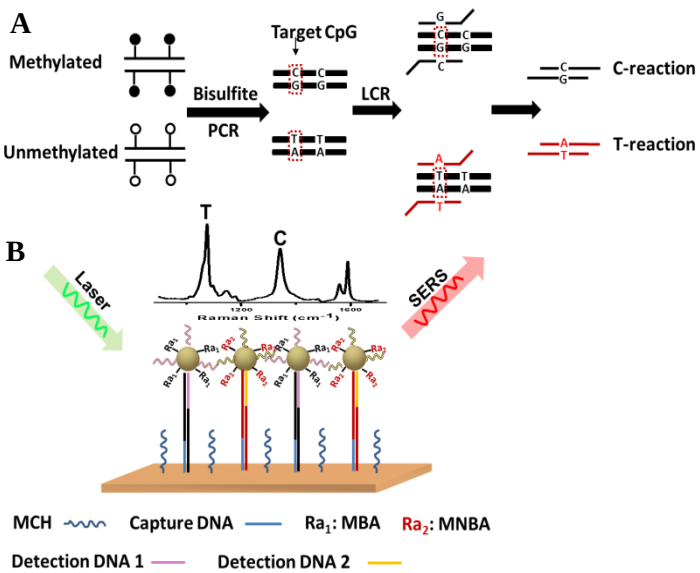
methylation state of the CpG of interest. The use of a PCR/LCR system has the potential benefit of improving the specificity, sensitivity and speed of a methylation assay.[24] The LCR products also had on one end, a universal barcode sequence for capture onto the SERS array, while the other end encodes for a methylation state-specific barcode to enable methylation-specific SERS nanotag labeling (Scheme 1B). Detailed experimental procedures and DNA oligonucleotides sequences used in this study (Table S1) are provided in the accompanying ESI.

The specificity of the optimized LCR reactions was first evaluated with gel electrophoresis (Figure S1, ESI). A clear band indicated a positive LCR reaction while no bands were observed for the no-template (NoT) and unmatched (UM) primer controls, thus indicating the high specificity of LCR. To enable a SERS-based evaluation (Scheme 1B), LCR products were then hybridized to the universal capture DNA probes pre-immobilized on gold surface through Au-S bond. To avoid non-specific physical adsorption of LCR products, the gold surface was blocked with 6-mercaptohexanol (MCH) prior to use. Finally, to identify the captured LCR products and eventually methylation levels, methylation state-specific SERS nanotags (made by modifying gold nanoparticles with methylation-state-specific detection DNA probes and Raman reporters) were further hybridized to the captured LCR.

To realize a quantitative SERS/LCR methylation assay, a 2-plex LCR reaction (simultaneous C and T-specific LCR reactions for methylated and unmethylated respectively) was performed, captured on the gold array, SERS nanotagged and interrogated with a laser. SERS signals generated for the corresponding LCR products were used to estimate DNA methylation levels at a specific CpG by measuring the ratio between the different Raman intensities (I) associated with the C-reaction (methylated) and T-reaction (unmethylated), i.e. % Methylation = $I_C/(I_T + I_C)$.



Scheme 1. Schematic illustration for simultaneous C and T single DNA base change detection by LCR (A), and DNA methylation analysis via simultaneous SERS nanotags detection on a gold surface array (B).

Studies have shown that gold nanoparticles (AuNPs) with distinct optical properties and good stability can enhance Raman signals of reporter molecules chemisorbed on their surface.[25-28] Moreover, AuNPs with the core size of ~60-80 nm are most efficient for SERS at red and near-infrared (785 nm) excitation.[29-30] Therefore, to enable a robust and sensitive SERS assay, AuNPs with the size of ~60 nm was chosen for use as SERS nanotags in this study. The size and optical properties of our SERS nanotags were characterized using TEM and extinction spectroscopy. The TEM image in Fig. 1A reveals AuNPs with a typical diameter of about 60 nm. The localized surface plasmon resonance (LSPR) peak of the bare AuNPs occurs at ca. 534 nm (Fig. 1B). Upon formation of AuNPs-DNA-Ra conjugates, the LSPR peak of AuNPs exhibits a red shift to 540 and 542 nm, respectively, depending on the particular Raman reporter.[31] The red-shift of the LSPR of AuNPs clearly indicated the binding of oligonucleotides and Raman reporters on AuNPs surface due to the change of the surrounding refractive index. Surface coverage of DNA on AuNPs is crucial for the stability of the nanoparticles, which was estimated using a previously described method.[32-33] On average, there were approximately 2235 oligonucleotides strands on each 60 nm-AuNPs.

The molecular structures of the Raman reporters and the corresponding SERS fingerprint are shown in Figure 1C. The characteristic Raman peak of 4-mercapto-3-nitro benzoic acid (MNBA) at 1334 cm^{-1} was assigned to the symmetric nitro stretching vibration,[34-35] while the peaks at ca. 1080 and ca. 1580 cm^{-1} of both MNBA and 4-mercaptobenzoic acid (MBA) arose from phenyl ring modes.[34-35] The SERS spectrum of the binary mixture from the two Raman reporters (MNBA and MBA) clearly shows the distinct fingerprints from the corresponding SERS nanotags, which can be used for identification of the individual targets.

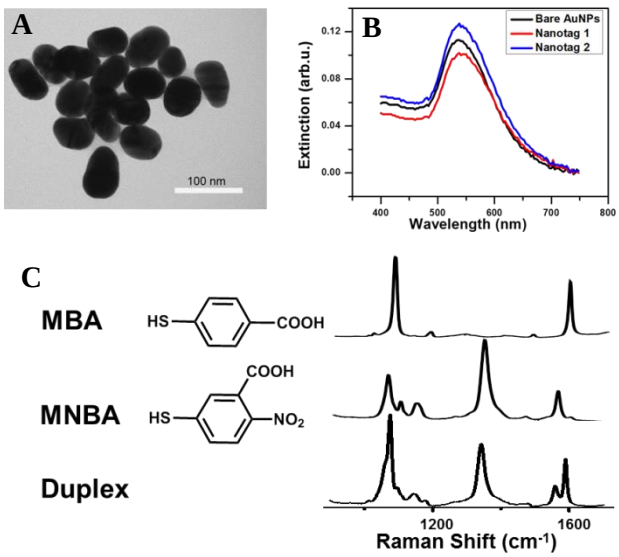


Fig. 1. TEM image of AuNPs (A), extinction spectra of bare AuNPs and SERS nanotags (B), and molecular structure of Raman reporters with corresponding SERS spectra (C).

We first evaluated the feasibility of the SERS/LCR assay by applying individual C or T LCR reactions products derived from various concentrations of synthetic template inputs onto gold surface. As evident from the optical image of the gold surface assay, the binding of SERS nanotags can already be observed by the naked eye on the gold surface (Fig.S2A, ESI) with good specificity. A clean and bright surface was observed with no-template control (NoT), un-matched target (UM) and with false SERS nanotags. With increasing concentration of targets, successively more SERS nanotags selectively bound onto the gold surface, which lead to the formation of "SERS dots" of high nanotags densities, thus generating high Raman intensities. SEM images further indicated the binding of SERS nanotags on the gold surface with high specificity as

demonstrated in Figure S2 (ESI). No nanoparticles were observed on the gold surface with UM control (ESI, Fig. S2B). In contrast, an abundance of AuNPs appeared on the gold surface at even very low (5 pM) initial input concentration (ESI, Fig. S2C). These results not only demonstrated the potential for good assay sensitivity, it also suggested a possible naked eye evaluation assay for LCR/SERS-based applications. Fig. 2A shows the typical SERS spectra obtained from different concentrations of input synthetic targets and negative controls. The distinct SERS peak located at 1334 cm^{-1} was observed corresponding to MNBA for the C-reaction thus indicating the presence of the LCR product. Figure 2B clearly shows the positive relationship between SERS intensity and target concentration. In contrast, control experiments performed with no template input (NoT), unmatched target (UM) and false SERS nanotags (non-specific hybridization control), no particles and therefore only background Raman signals (Fig. S3, ESI and Figure 2A) were observed. The detection limit was determined to be 0.5 pM based on the signal-to-noise ratio being three-times higher than the background (No template control, NoT) with relative standard deviation (RSD) of 6.4% over 5 independent tests (20 SERS spectra were typically collected for each of the 5 independent tests; Fig. S3, ESI), demonstrating good assay reproducibility. This level of sensitivity was 10-fold better than previous LDR/SERS approaches and comparable to fluorescence and electrochemical-based LCR methods.[15-16, 18-21].

Likewise, with the T-reaction, which uses MBA-modified SERS nanotags instead, had similar assay performance to the C-reaction (Fig 2C and 2D). As expected, the characteristic Raman peaks at 1080 and 1580 cm^{-1} arose from phenyl ring mode of MBA. As low as 0.5 pM T-base target could be detected with very high specificity and reproducibility (RSD=1.8% with n=5; Figure 2D), Together, these data underscored the high specificity and sensitivity of the LCR/SERS approach and suggests its viability as an alternative to fluorescence and electrochemical-based methods.

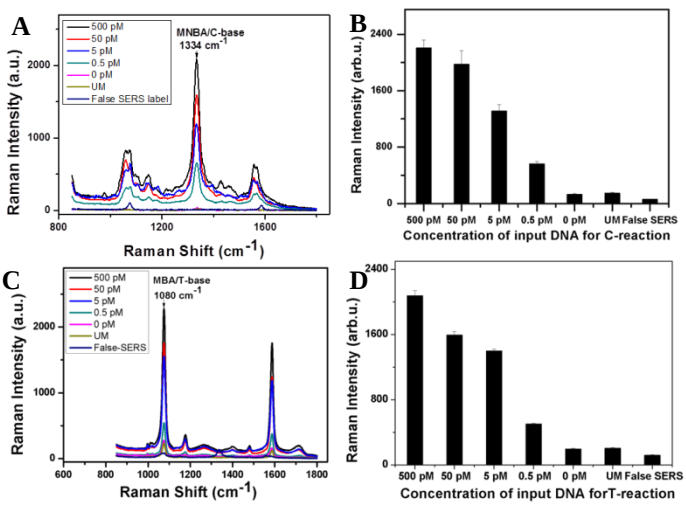


Fig.2. Concentration-dependent raw SERS spectra (A, C) with non-cognate probe controls and (B, D) the corresponding concentration-dependent response derived from SERS peaks in (A) and (C) for C and T-reactions respectively. Error bars represent ±SD, n = 5.

Due to the unique multiplexing capability of SERS, we next turned our attention to the simultaneous detection of the C and T-reactions for quantitative methylation analysis. As indicated in Scheme 1B, multiplexed LCR products may be immobilized onto a gold surface followed by simultaneous SERS nanotags interrogation. As a-proof of-concept, Figure 3A shows the typical SERS spectra (normalized to the peak at 1080 cm^{-1}) for a 2-plex LCR detection in which C:T targets were mixed in ratios 1:0; 3:1; 1:1. 1:3; 1:9 and 0:1. These titrations represented samples at 100%, 75%, 50%, 25%, 10% and 0% methylation. With an increasing ratios of C to T, i.e, %methylation, the SERS intensity of MNBA (SERS nanotags to C-reaction) at 1334 cm^{-1} increased. Since DNA methylation is based on C to T base change after bisulfite treatment and owing to the specificity of LCR, the SERS intensity (I) ratio of $I_{C\text{-}base}/I_{T\text{-}base}+I_{C\text{-}base}$ may be used to estimate the degree DNA methylation at a single CpG. As indicated in Figure 3B, the signal from the C-reaction increased with increasing methylation and as low as 10% differences in DNA methylation could be detected (RSD of 3.99 % over 5 independent tests). Overall, these results indicated that LCR/SERS assay was a feasible multiplex detection method for single DNA base changes and one potential application was DNA methylation analysis at single CpG resolution.

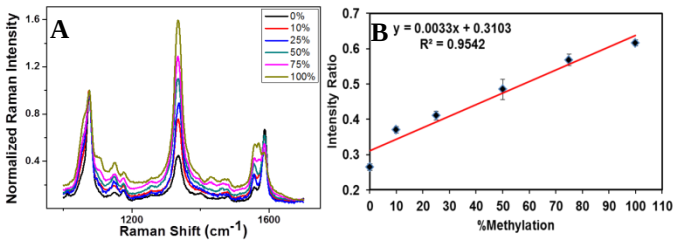


Fig.3. Typical SERS spectra for duplex point mutation detection (A) and SERS intensity ratio response to methylation level (B). Error bars represent ±SD, n = 5.

Next, to demonstrate a complex biological application, we applied the LCR/SERS assay to detect the methylation state at CpG12 of the miR200b P2 promoter, a potential biomarker of breast cancer metastasis.[36-38] LCR/SERS assay was first employed to measure the level of the DNA methylation in a panel of breast cancer cell lines. As indicated in Figure 4A, typical SERS spectra obtained from the no-template control were of only low background signals, while the signals from the three cell lines were of the distinct SERS nanotags signals profiles depending on their methylation states. The intensity ratio between the peak at 1334 cm^{-1} (C-reaction; methylated) and 1080 cm^{-1} (T-reaction; unmethylated) was used to estimate the degree of DNA methylation in the three cell lines (Figure 4B, blue bars). The estimated DNA methylation level were 76% in MDA-MB 231 cell lines, 0.4% in MDA-MB 468 cell lines and 47% with HCC 1937 cell lines. These methylation estimates were then validated with Next Generation Sequencing (Figure 4B; black bars) and were also consistent with the literature[19, 38] thus indicating that LCR/SERS had potential as an accurate DNA methylation analysis platform with single CpG resolution. To further demonstrate clinical viability, a clinically normal serum DNA sample was also successfully tested (Figure S4). DNA agarose gel electrophoresis indicated the presence of the target while SERS measurments estimated methylation to be approximately 58.74%.

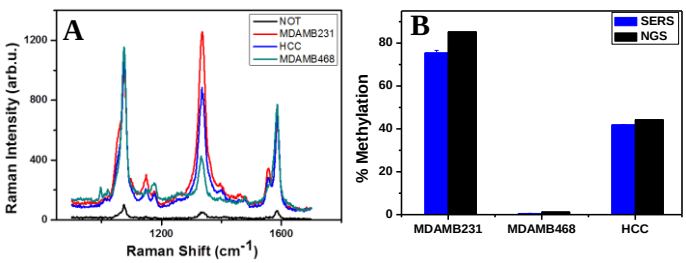


Fig.4. Typical SERS spectra for a panel of cell lines detection (A) and comparison of estimated methylation in three breast cancer cell lines with SERS-LCR assay (B). Error bars represent ±SD, n = 5.

In summary, a simple platform for rapid multiplexed detection of single base changes was developed with SERS via LCR, which could significantly increase detection sensitivity (0.5 pM) while maintaining high specificity. More importantly, the developed assay could be used for DNA methylation analysis by simultaneously detecting the relative Raman intensities between the C and T (methylated and unmethylated respectively) DNA base changes amplified by LCR. As low as 10% differences in methylation could be detected, demonstrating the potential of SERS for sensitive multiplexed (epi)genetic monitoring. The LCR/SERS assay was also successfully applied to breast cancer cell lines and a serum-derived DNA sample to demonstrate its feasibility on complex biological samples. Lastly, assay results validated with Next Generation Sequencing, underscoring its potential for accurate genetic biomarker detection.

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