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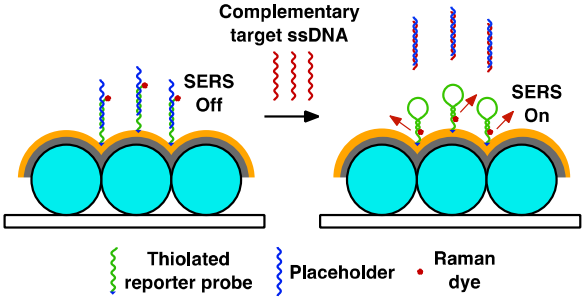

A signal-on DNA bioassay-on-chip using SERS detection and a single incubation step without any washing was developed for dengue diagnosis.

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DNA Bioassay-on-Chip using SERS Detection for Dengue Diagnosis

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A novel DNA bioassay-on-chip using surface-enhanced Raman scattering (SERS) on a bimetallic Nanowave chip is presented. In this bioassay, SERS signals were measured after a single reaction on the chip's surface without any washing step, making it simple-to-use and reducing reagent cost. Using the technique, specific oligonucleotide sequences of the dengue virus 4 were detected.

Dengue fever results from infection with any one of four different serotypes of the dengue virus (DENV), which are transmitted by mosquitoes. It is estimated that over 3.6 billion people worldwide are at risk of dengue infection, and about 230 to 390 million infections occur each year.[1, 2] Current methods for dengue diagnosis include: virus isolation, antigen detection, viral genome detection, and serological tests.[3] Which method is most appropriate depends on the phase of the illness. For the acute phase, the first three methods can be used. While the virus isolation method offers high specificity, its prolonged turnaround time (more than a week) precludes its utility for early detection. Antigen detection based on the detection of non-structural protein 1 is specific but its sensitivity ranges widely in published reports and is significantly lower in secondary compared with primary dengue infections.[4-6]

Recent developments in dengue genome detection using real time RT-PCR show high level of sensitivity and specificity, making this approach suitable for early dengue diagnosis.[4, 7] These assays, however, require skilled labor and expensive laboratory equipment. For low-resource settings and point-of-care applications, the development of inexpensive, sensitive, specific, easy-to-use tests for early detection of dengue is urgently needed. Based on dengue genome detection, several methods have been proposed. Zaytseva et al. developed a microfluidic biosensor using a magnetic-based sandwich hybridization system and liposome amplification for detection of isothermal amplified DENV RNA.[8] Also using sandwich hybridization, Chen et al. created layers of gold nanoparticles on quart crystal microbalance for detecting RT-PCR product of DENV 2.[9] In another work, RT-PCR product of DENV 2 was detected by silicon nanowire-based biosensor developed by Zhang et al.[10] Toh et al. utilized nanoporous alumina membrane and electrochemical detection to detect cDNA PCR sample of DENV 1.[11, 12] Although sensitive, these methods require multiple incubation and/or washing steps, thus increasing the assay cost and complexity. There is a need for simpler methods, ideally homogeneous with sample-to-answer capability.

Surface-enhanced Raman scattering (SERS), which yields very narrow vibrational peaks of the investigated sample, has long been considered to be a powerful tool for chemical identification. Based on SERS, our laboratory has developed different chemical and biological sensing methods for environmental monitoring and medical diagnostics.[13, 14] Many SERS-based DNA detection techniques have been reported.[15-29] Graham et al. developed a separation free DNA detection based on the higher affinity of single-stranded DNA (ssDNA) for metal surface than double stranded DNA.[30] Johnson et al. utilized sandwich DNA hybridization and magnetic nanoparticles to capture Raman-active gold nanoparticles.[31] We demonstrated a SERS-based homogeneous DNA detection strategy in solution referred to as the 'molecular sentinel'.[32, 33] Recently, we showed that this strategy can be applied to SERS-active chips, referred to as 'molecular sentinel-on-chip' (MSC) technique.[34-36] The SERS chips used in these studies were the triangular-shaped nanowire and more recently the Nanowave, also known as 'metal film over nanospheres' (MFON). The MFON concept was first introduced by our group in 1984[37] and has been used by other groups due to its particularly high SERS enhancement factor (EF), 10^6-10^8, and facile fabrication.[38-43] Using bimetallic film (gold and silver), Nanowave's SERS enhancement can be further improved compared to single gold film while still maintaining the stability.[36, 44] Compared to fluorescence, SERS yields much narrower peaks. SERS, therefore, is highly specific and has great potential for multiplex detection of DENV. Multiplex detection capability is desirable for DENV diagnosis given that any one of four different DENV serotypes can exist in the sample. There have been few reports of using SERS for DENV diagnosis so far. Huh et al. described a microfluidic SERS detection chip composed of electrokinetically active microwells for detection of nucleic acid sequences of DENV 2.[45] Chung et al. detected the same targets using a flexible SERS-active substrate.[46] Also in these works, the target sequences needed to be labelled with fluorescent dyes.

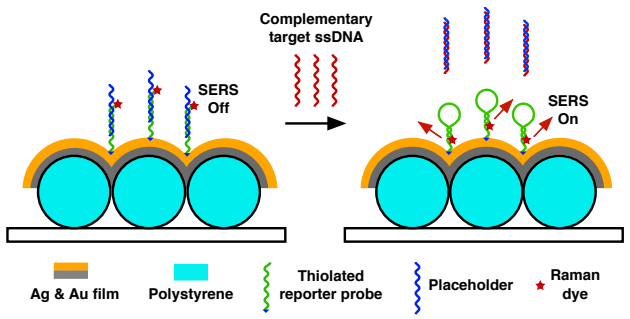

Fig. 1 DNA bioassay-on-chip detection scheme

Herein we report a unique homogeneous DNA bioassay-on-chip using SERS detection on a bimetallic Nanowave chip. The detection strategy (Fig. 1) is developed based on the plasmonic modulation concept which has been previously employed on nanoparticle for solution-based nucleic acid detection.[47] In the absence of complementary target ssDNA, the reporter probes and placeholders maintain partial duplex structures, keeping Raman dyes tagged at 3' ends of the reporter probes away from the Nanowave chip's metal surface. At such Raman dye-chip's surface distance, the SERS signal is low due to the fact that SERS enhancement exponentially decreases with increase in Raman dye-metal surface distance ('Off state'). When complementary target ssDNA targets are introduced, they hybridize with the placeholders, and the reporter probes are free to form hairpin structures due to their design. With hairpin structures, Raman dyes are brought into close proximity of Nanowave chip's metal surface, inducing strong SERS signals ('On state').

The bioassay has "homogeneous" format, i.e., SERS signals are measured after a single incubation step without any washing to remove unreacted components, making it simple-to-use and reducing reagent cost. Note that single-step DNA bioassay has been reported by Plaxco et al. before.[48] However, while the Plaxco group's assay is based electrochemical detection, our bioassay is different and based on SERS detection, which is particularly suitable for multiplex detection in a single reaction. Compared to the MSC technique where SERS intensity decreases in the presence of complementary target ssDNA (signal-off), the new bioassay has signal-on with SERS intensity increasing in the presence of target. Hence, it is less susceptible to false-positive responses. The target ssDNA do not have to be labelled for detection, which is highly desired in many applications. We have applied the new technique for detecting specific sequence of DENV 4. As low as ~6 attomoles of the target ssDNA inside the probed area could be detected. To the best of our knowledge, this is the first time the presented DNA bioassay-on-chip is reported.

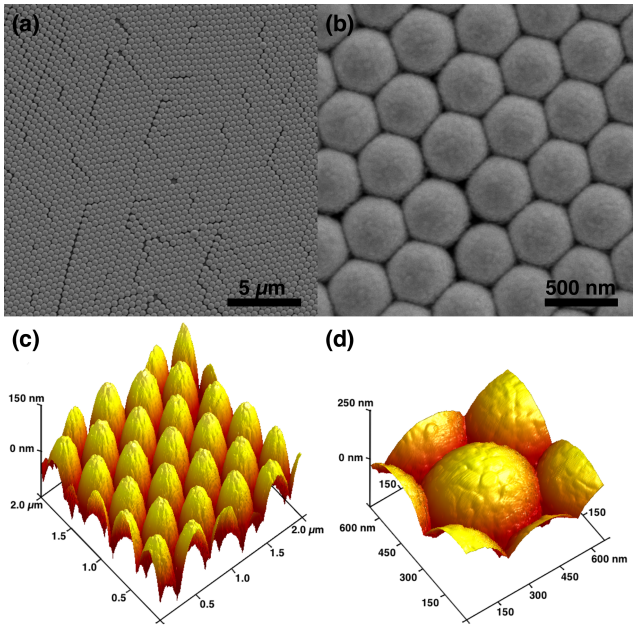

Fig. 2 SEM images (a,b) and AFM images (c,d) of bimetallic Nanowave chip

Bimetallic SERS Nanowave chips were fabricated as previously described.[36] Briefly, a monolayer of closely packed polystyrene beads (PS, 430 nm diameter) was prepared on microscope glass slides using the self-assembly at water-air interface method. The as-prepared samples were then annealed at 80 °C for 1 hour followed by metal coating (5nm Ti, 100 nm of Ag, and 100 nm of Au sequentially). With this method, a large-area of bimetallic Nanowave chip can be fabricated with high reproducibility (Fig. 2a and Fig. S1). Furthermore, by replacing the microscope glass slide with silicon wafer, we have been able to fabricate bimetallic Nanowave chips at wafer scale, opening the possibility for mass production (Fig. S2). The higher magnification SEM image shows the periodic hexagonal pattern of the chip and crevices between metal-coated PS (Fig. 2b). The image also indicates considerable surface roughness on the chip. To confirm this, we conducted AFM measurements, and results are shown in Fig. 2c and 2d. From these images and cross-section profiles (Fig. S3), deep nanosize crevices between metal-coated PS and substantial surface roughness can be clearly observed. In addition, reflectance measurements show a good match between the localized surface plasmon resonance of our chip and wavelength of the excitation laser.[36] All these characteristics are believed to contribute to the high SERS enhancement of the bimetallic SERS Nanowave chip. Compared to single-metal (gold) Nanowave chip, the bimetallic Nanowave chip has a ×3.6 higher SERS intensity.[36] Meanwhile, compared to a commercially available SERS substrate (Klarite), the bimetallic Nanowave chip has approximately ×100 higher SERS intensity (Fig. 3).

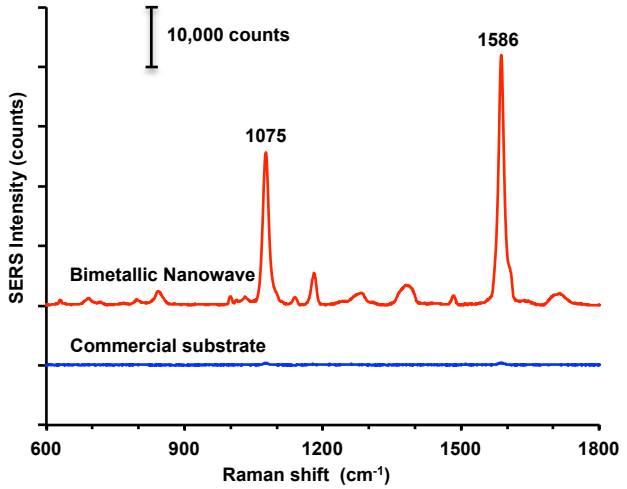

Fig. 3 SERS spectra of p-mercaptobenzoic acid on bimetallic Nanowave chip and commercial substrate

The fabricated bimetallic Nanowave chip was then functionalized with DNA probes for dengue virus oligonucleotide detection. First, Nanowave chip were cut into smaller pieces with a diamond tip. Thereafter, pieces of Nanowave chip were soaked in aliquots of 1 μM reporter probe solution in buffer (0.5 M NaCl, 10 mM sodium phosphate buffer in water) for 16 h at room temperature. The chips were then rinsed with buffer followed by soaking in solutions of 1 mM mercaptohexanol (MCH) in buffer for 1 h. The purpose of this step is to displace non-specifically adsorbed probes and to passivate the gold surface.[49] Subsequently, the chips were rinsed with water, followed by soaking in aliquots of 1 μM placeholder solution in buffer for 24 h. Upon hybridization between reporter probes and placeholders, reporter probe–placeholder partial duplexes were formed on the surface of the bimetallic Nanowave chip (Fig. S4). This resulted in decrease in SERS signal (Fig. S5). The decrease is attributed to the opening of reporter probes' hairpin structures, thus displacing Cy5 Raman dyes tagged at 3' ends of the reporter probes away from chip's surface. Finally, excess placeholders were washed with buffer, and the functionalized chips were ready for use.

Sequences of ssDNA used in this study are shown in Table S1. Complementary target ssDNA is a specific DNA sequence of DENV 4 (GeneBank accession number KC963425.1). Based on the target sequence, placeholder and reporter probe were designed such that the whole sequence of placeholder is complementary to the target sequence, whereas only a part of it is complementary to the reporter probe. This created a single-stranded overhang region (know as a toehold) upon formation of reporter probe-placeholder partial duplex. The toehold ensures that the target sequence can successfully displace the reporter probe to hybridize with the placeholder via toehold-mediated strand displacement.[50] The reporter probe was further designed to be able to form hairpin structure after being displaced from the reporter probe-placeholder partial duplex.

The capability of detecting Dengue virus oligonucleotides is demonstrated in Fig. 4. To demonstrate the specificity of the method, the functionalized Nanowave chips were tested against three different samples including: 1 μM synthetic DENV 4 ssDNA in buffer (complementary target), 1 μM non-complementary ssDNA in buffer, and buffer only (blank). First, pieces of functionalized chip were incubated in 30 μL aliquots of sample solutions for 2 h at 37 °C. After incubation, the chips where removed from the sample solutions and transferred to a Renishaw confocal Raman Microscope for SERS detection (using 632.8-nm laser excitation) without any washing steps. Note that the chips were kept wet during SERS measurements to prevent conformational change of DNA structures. Only 1 % of the laser power, that is, ~50 microwatt, was used to prevent potential damaging effects to the DNA sequences. In addition, this laser power also helped to avoid saturating the SERS detection system's CCD camera with high fluorescent background of the Cy5 resonant Raman dye. The acquired SERS spectra were background subtracted and smoothed using a Savitsky-Golay filter (five point window and first-order polynomial).

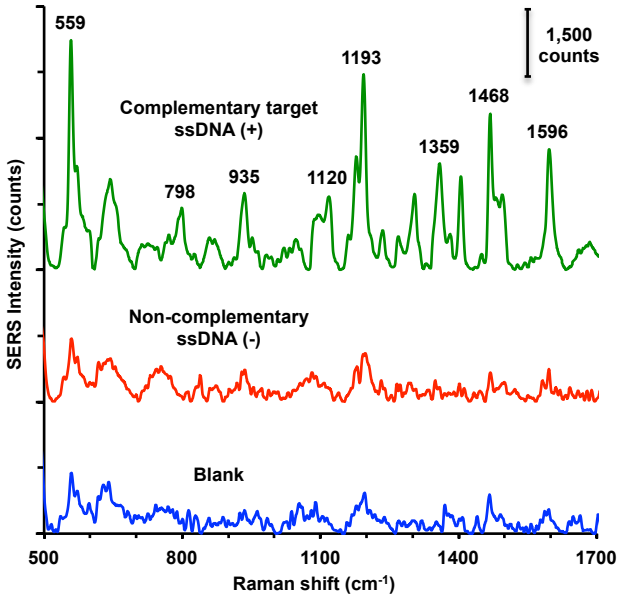

Fig. 4 SERS spectra after incubation of functionalized Nanowave chip in blank, non-complementary ssDNA, and complementary target ssDNA samples

As shown in Fig. 4, for blank samples and non-complementary ssDNA samples, the SERS intensities were low and similar to SERS intensity before these samples had been applied (i.e., signal from reporter probe-placeholder partial duplexes, Fig. S5). It indicated that the reporter probe–placeholder partial duplexes were not disturbed by the blank or the non-complementary ssDNA. Cy5 Raman dyes tagged at 3' end of the reporter probes were therefore kept away from the Nanowave chip's metal surface by 40 nucleotides (approximately 13.5 nm). At such long Raman dye–metal surface distance, SERS enhancement was weak, resulting in low SERS background ('Off' state). Alternatively, there might be small amount of non-specifically bound reporter probes remain lying on the chip's surface after MCH. Raman dyes tagged with these probes stayed close to the metal surface and could contribute to the low SERS background. On the other hand, upon addition of complementary target ssDNA samples, the SERS intensity increased (Fig. 4). This increase is explained by the hybridization between the complementary target ssDNA and the placeholders, leaving the reporter probes free to form hairpin structures. As a result, the Cy5 Raman dyes were

brought closer to the Nanowave chip's surface, resulting in increased SERS intensity ('On' state).

The possibility for quantitative analysis is shown in Fig. 5. Sample solutions of complementary target ssDNA in buffer at different known concentrations (0.0 μM, 0.1 μM, 0.4 μM, and 1.0 μM) were tested using the above procedure. The calibration curve was plotted based on SERS intensity of 559 cm^{-1} peak. Since 30 μL aliquots of sample solutions were used for detection, the absolute amounts of target ssDNA in sample solutions were 0, 3, 12, and 30 picomoles, respectively. We assumed that these amounts of target ssDNA evenly distributed across surface areas of the chips (equivalent of ~3 mm diameter each). Within the probed areas (as defined by the laser spot size, ~3 μm diameter), the amounts of target ssDNA samples that were effectively excited by the laser beam were even smaller, approximately 0, 3, 12, and 30 attomoles respectively (i.e., 10^{-6} fraction of the amounts applied on the chip). A linear trend line was fitted to the data points using linear regression. Based on slope (s = 128.56) and residual standard deviation (σ = 255.02) of the regression line, as low as ~6 attomoles of the target ssDNA (DENV 4) inside the probed area could be detected.

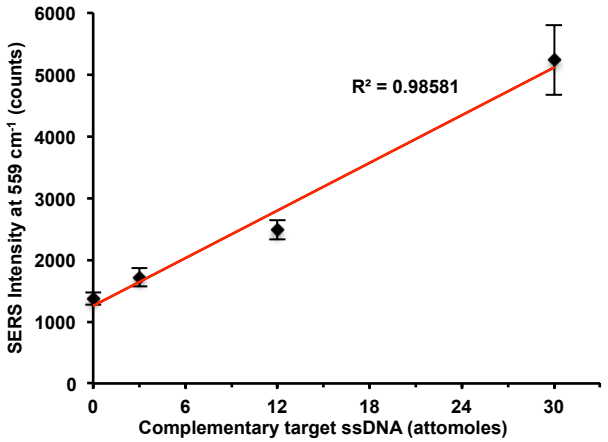

Fig. 5 SERS intensity in the existence of different amounts of complementary target ssDNA inside probed area

Conclusion

In conclusion, we have first demonstrated the application of a bioassay-on-chip platform using SERS detection. The bioassay offers a homogeneous format, making it simple-to-use and reducing reagent cost. The usefulness for clinical diagnosis is demonstrated by the detection of nucleic acid sequence of dengue (DENV 4), which is used as the reference model. As low as ~6 attomoles of the target ssDNA inside the probed area could be detected. For future work, the bioassay can be combined with sample preparation techniques to create new technologies for point-of-care diagnostics and global health applications.

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Notes and references

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