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REVIEW

Plasmonic SERS biosensing nanochips for DNA detection

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Abstract The development of rapid, cost-effective DNA detection methods for molecular diagnostics at the point-of-care (POC) has been receiving increasing interest. This article reviews several DNA detection techniques based on plasmonic-active nanochip platforms developed in our laboratory over the last 5 years, including the molecular sentinel-on-chip (MSC), the multiplex MSC, and the inverse molecular sentinel-on-chip (iMS-on-Chip). DNA probes were used as the recognition elements, and surface-enhanced Raman scattering (SERS) was used as the signal detection method. Sensing mechanisms were based on hybridization of target sequences and DNA probes, resulting in a distance change between SERS reporters and the nanochip's plasmonic-active surface. As the field intensity of the surface plasmon decays exponentially as a function of distance, the distance change in turn affects SERS signal intensity, thus indicating the presence and capture of the target sequences. Our techniques were single-step DNA detection techniques. Target sequences were detected by simple delivery of sample solutions onto DNA probe-functionalized nanochips and measuring the SERS signal after appropriate incubation times. Target sequence labeling or washing to remove unreacted components was not required, making the techniques simple, easy-to-use, and cost-effective. The usefulness of the nanochip platform-based techniques for medical diagnostics was illustrated by the detection of host genetic biomarkers for respiratory viral infection and of the dengue virus gene.

Keywords DNA detection · Multiplex DNA detection · DNA biosensor · Surface-enhanced Raman scattering · Molecular sentinel-on-chip

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Introduction

Technologies to detect target DNA sequences are important to a wide variety of fields, ranging from medicine to bioanalytical chemistry and forensic science, with many applications such as disease diagnosis, viral load monitoring, gene mutation detection, bacterial and viral strain identification, DNA typing, and gene-expression profiling. The current gold standard of DNA detection involves polymerase chain reaction (PCR), which is highly sensitive with a single DNA copy detection limit [1]. However, the PCR analytical process is quite labor-intensive, time-consuming, and requires relatively bulky and expensive equipment, as well as skilled workers, precluding their routine bedside or field-use [2, 3]. The development of rapid, easy-to-use, cost-effective, high accuracy DNA detection technologies and devices for applications at the POC (e.g., doctors' offices, in the field, first responders' sites) and in resource-limited settings remains a challenge.

Many efforts have been made to address the above challenge. Since the copy number of target DNA sequences is usually low, most of the methods have to employ target amplification or/and signal amplification to achieve sufficient

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sensitivity. In methods employing target amplification, target sequences are amplified using enzymatic reactions, where the sample temperature is modulated using a thermal cycler (e.g., as in PCR) or kept constant (as in isothermal amplification techniques such as loop-mediated isothermal amplification, strand displacement amplification). Since the copy number of target sequences is amplified millions of times or more, enzymatic amplification-based methods are highly sensitive, with detection limits from a single to a few copies. These advantages are offset by the need to use complex reaction mixtures. Enzymatic amplification of highly degraded DNA, which is not unusual in ancient samples, is difficult [4]. As a result of the high level of amplification, trace amounts of DNA contaminants could serve as templates, resulting in amplification of the wrong template (false positives) [5]. Because the potential presence of inhibitors is also a threat for enzymatic amplification, purification is often required [6]. Lab-on-a-chip systems that integrate all steps of the detection process including nucleic acid extraction, purification, amplification, and signal detection can prevent contamination and automate the entire process. Owing to miniaturization and simplicity, such systems are suitable for POC applications. Lab-on-a-chip systems integrating PCR or other DNA detection techniques are continuously being developed and have attracted great interest [3, 7–11].

Alternatively, original target sequences can be directly detected without prior enzymatic amplification. In many cases, such direct detection requires strong signal amplification because of the low copy number of target sequences. Compared to target amplification methods, signal amplification methods can be more cost-effective, simple, and rapid because the nucleic acid purification and enzymatic amplification steps are not required. Target loss and biases associated with the two purification–amplification steps, which may adversely affect accuracy in quantitative analysis, can also be avoided. Therefore, extensive efforts have been devoted to develop highly sensitive DNA techniques using signal amplification.

Surface-enhanced Raman scattering (SERS), a phenomenon in which Raman scattering of molecules adsorbed on metallic nanostructures is enhanced millions times or more, is a sensitive analytical technique [12]. The sensitivity of SERS is demonstrated through its ability to detect single molecules [13–16]. Compared to fluorescence, SERS offers several advantages. First, SERS exhibits emission peaks two orders of magnitude narrower than those of fluorescence, making SERS more suitable for multiplex detection [17]. Second, SERS is less sensitive to photobleaching than fluorescence is [17, 18]. Third, resonant SERS (SERRS) has a lower limit of detection (LOD) than that of fluorescence, e.g., SERRS LOD was three orders of magnitude lower than that of fluorescence with the same commercially available fluorophore [19]. Finally, SERS can be excited in the near-IR region where absorption and fluorescence background of biological matrices are low [17, 20]. As a result, optical output is less affected by biological matrices, thus reducing the cost and labor associated with sample preparation and simplifying the assay procedures [21].

Our laboratory has developed different chemical and biological sensing methods based on SERS for environmental monitoring and medical diagnostics [22–25]. Three decades ago, we introduced a unique type of plasmonic-active substrate, a “metal film over nanospheres” (MFON), as an efficient and reproducible SERS-active substrate [26]. We refer to the substrate as a “nanowave” because of the resemblance between its shape and a 2D periodic waveform [27, 28]. Since its introduction in 1984, the nanowave has been extensively used for chemical sensing, bioanalysis and biosensing, and trace organic analysis [28–35]. Using nanowave substrates, we have demonstrated the analytical potential of the SERS effect and its practical use for trace analysis [26]. The nanowave substrate offers several advantages as a SERS detection platform, such as its relatively simple fabrication, stability, and high enhancement factor.

SERS-based DNA detection has attracted a lot of interest. A common strategy is to form a sandwich structure of substrate–target sequence–reporter [36–42]. Mirkin and coworkers developed chips in microarray formats that are functionalized with capture probes to capture target sequences [36]. Dye-labeled gold nanoparticles (AuNP), which are functionalized with reporter probes, were then used to report the presence of the captured target sequences via sandwich hybridization involving capture probe, target sequence, and reporter probe. The SERS signals of the dye-labeled AuNP were further enhanced by silver coating. With this method, a 20-fM detection limit was achieved. Sandwich hybridization has also been used to form sandwich structures of magnetic bead–target sequence–reporter for DNA detection [4, 21, 43–46]. Zhang and coworkers utilized magnetic nanoparticles functionalized with capture probes and dye-labeled AuNP functionalized with reporter probes to form sandwiches of magnetic nanoparticle–target sequence–gold nanoparticle [43]. An external magnet source was used to concentrate the sandwiches onto a small spot for SERS measurements. This method achieved a 10-pM detection limit. Some other SERS-based DNA detection strategies include forming SERS “hot spots” upon particle aggregation [47], utilizing the difference in adsorption properties of single-stranded DNA (ssDNA) and double stranded DNA on metal surfaces [48–50], and labeling target sequences with dye molecules [51].

Using SERS, our group has developed various DNA detection techniques for HIV, breast cancer, viral infection, etc. [28, 52–58]. The sensing mechanisms were based on secondary structure change of DNA probes upon hybridization with target sequences, which in turn results in SERS signal change. In this article, unique DNA detection techniques using DNA probes immobilized on SERS-active nanowave chip platforms

developed in our laboratory over the last 5 years will be reviewed. They include the molecular sentinel-on-chip (MSC), multiplex MSC, and inverse molecular sentinel-on-chip (iMS-on-Chip). Since these systems involve single-step DNA detection, the techniques are simple, easy-to-use, and cost-effective. Details of each technique are discussed in following sections.

Molecular sentinel-on-chip

Our laboratory first developed the molecular sentinel (MS) method, which involves Raman dye-labeled hairpin DNA probes on plasmonic substrates utilizing SERS as the signal readout [52]. It has the same stem-and-loop structure as that of molecular beacons, which were first introduced by Tyagi and Kramer [59]. However, instead of using fluorescence detection as in molecular beacons, molecular sentinels utilize SERS. In the absence of target sequence, the molecular sentinel forms a stem-and-loop structure as a result of its two complementary arm sequences that are on either side of the probe. A Raman reporter attached to the end of one arm is in close proximity to a SERS-active nanostructure, which is attached to the end of the other arm. In this configuration, a strong SERS signal is induced. In the presence of a target sequence, the target sequence hybridizes with the loop part of molecular sentinel, forcing the two arm sequences apart. The Raman reporter moves away from the SERS-active nanostructure, resulting in a SERS signal decrease.

We have developed DNA biosensors based on molecular sentinels for HIV 1 [52], breast cancer [53, 54], and respiratory viral infection [28, 55, 58]. Following our development of the MS methodology [52], several other groups have used the MS sensing mechanism for various applications [60–63]. Wei et al used similar MS stem-loop oligonucleotides immobilized on a gold nanoparticle-decorated silicon nanowire array for identification of single-base mismatches and detection of multiplexed DNA [46]. Pang et al. utilized molecular sentinel probes immobilized on MFON for the detection of an RNA genetic marker associated with highly pathogenic avian influenza (HPAI) virus [61]. Silver nanoparticle-decorated silicon wafers which were functionalized with molecular sentinel probes have been used by Wang et al. for deafness mutation discrimination [62]. Qi et al. described the use of a label-free MS method for in situ monitoring of individual DNA hybridization in microfluidics. By immobilizing MS probes on nanoporous gold disks, sensitivity approaching the single-molecule limit via SERS detection was reported [49].

In our works, SERS-active nanostructures were nanoparticles or nanochips. One of the nanochips was a nanowave SERS substrate (Fig. 1a, b). Nanowave substrate was chosen owing to its strong SERS enhancement, reproducibility,

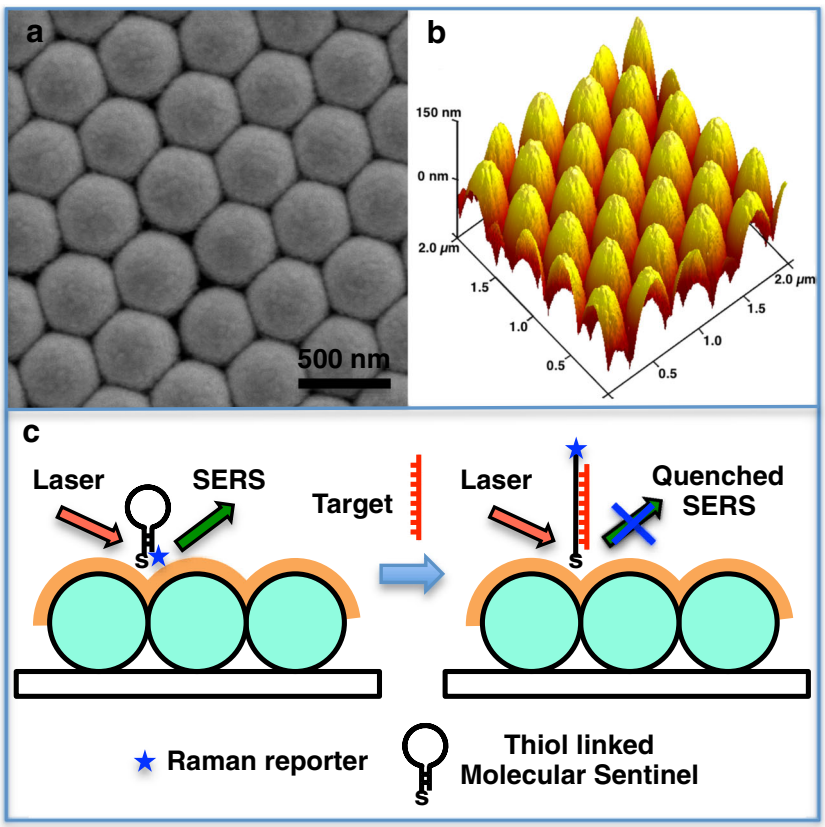

Fig. 1 **a** SEM and **b** AFM images of nanowave chip. **c** Molecular sentinel-on-chip (MSC) technique detection scheme (adapted from refs. [28] and [57])

stability, and relatively simple fabrication. We have previously demonstrated that bimetallic nanowave's SERS enhancement was approximately two orders of magnitude higher than that of Klarite, a commercially available SERS substrate [57]. Nanowave substrate's stability over large temperature and potential ranges has also been demonstrated by Van Duyne and coworkers [64]. Large areas of nanowave substrates can be produced using a self-assembly on water–air interface method, making the fabrication relatively simple, low-cost, and highly reproducible [28].

With molecular sentinel probes immobilized on the nanowave chip, target DNA sequences can be detected using a homogeneous assay format (Fig. 1c). The technique is referred to as the molecular sentinel-on-chip (MSC) technique. In the absence of target sequence, the molecular sentinel probes form stem-and-loop structures, keeping the Raman reporters in close proximity to the nanowave chip's gold surface (less than 1 nm), inducing a strong SERS signal. When target DNA sequences encounter the molecular sentinel probes, they hybridize to the probes, forcing the loops to open. The Raman reporters are displaced away from the nanowave chip's gold surface (ca. 10 nm), leading to a decrease in SERS signal. MSC is a single-step DNA detection technique. Target DNA sequences are detected simply by delivering sample solutions onto molecular sentinel-functionalized nanowave chips followed by signal measurements without any additional steps. Because the target sequence does not need to be labeled, and secondary hybridization or post-hybridization washing is not required, the MSC technique is simple to use and cost-effective. In addition, since MSC is based on a nanochip platform, it is suitable for being extended into DNA microarrays, which can provide high-throughput analysis.

We have illustrated the application of the MSC technique for medical diagnosis, using the *RSAD2* (Viperin) gene, a host genetic biomarker for respiratory viral infection, as a test model [28]. Figure 2 shows the results of *RSAD2* gene detection using the MSC technique. SERS spectra were taken 2 h after samples containing buffer (blank), non-complementary DNA sequences, or complementary target DNA sequences (*RSAD2*) were delivered on molecular sentinel-functionalized nanowave chips. The SERS intensity of complementary target DNA sample was clearly lower than that of blank and non-complementary samples, indicating hybridization between the molecular sentinels and complementary target sequences. The molecular sentinel loops were opened, bringing the Cy3 dyes away from the nanowave chip's surface, resulting in lower SERS intensity. Meanwhile, SERS intensities of blank and non-complementary DNA samples are similar, suggesting that non-complementary DNA sequences did not disturb molecular sentinels' stem-and-loop structures. The results demonstrated that the MSC technique could detect a specific DNA sequence, herein a genetic biomarker for respiratory viral infection.

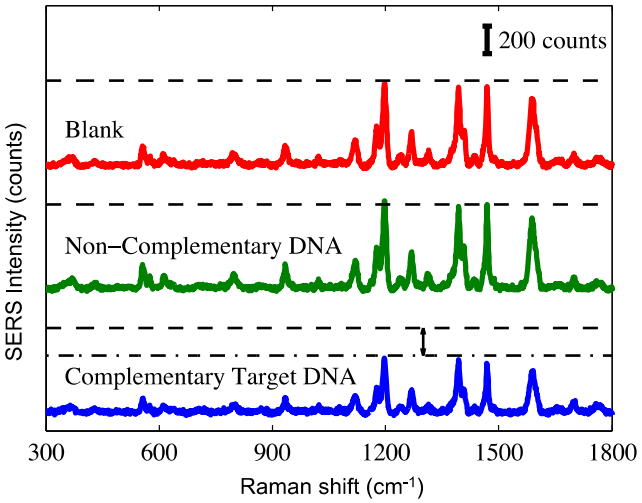

Fig. 2 SERS spectra of molecular sentinel probes on nanowave chip in the presence of blank, non-complementary DNA, or complementary target DNA sample. The *dashed lines* mark the blank sample's SERS intensity. The *dashed–dotted line* marks the complementary target DNA sample's SERS intensity (adapted from ref. [28])

Multiplex molecular sentinel-on-chip

Multiplexed DNA detection (i.e., simultaneous detection of distinct DNA targets) is highly desired. It can be used to simultaneously detect multiple genetic biomarkers that are usually involved in disease onset and progression. Also it can be used to detect both target sequences of interest and control sequences, which is usually required for proper data analysis. Since multiple distinct DNA sequences are detected simultaneously, multiplex DNA detection could reduce running time, cost, and sample volume needed.

The multiplex detection scheme using MSC technology is shown in Fig. 3 [56]. Two different molecular sentinel probes tagged with two different Raman reporters were immobilized on a bimetallic nanowave chip via gold–thiol bonds. Each molecular sentinel probe was designed to be complementary to a specific target DNA sequence. The detection mechanism was similar to the one described in the previous section. Two distinct target DNA sequences were simultaneously detected on the basis of SERS intensities from the two probes.

The illustration of multiplex detection for two distinct target DNA sequences, viz. interferon alpha-inducible protein 27 (*IFI27*) gene and interferon-induced protein 44-like (*IFI44L*) gene, which are host genetic biomarkers for respiratory viral infection, is depicted in Fig. 4 [65]. The SERS peaks marked with black numbers are from molecular sentinel probes for the *IFI27* gene. The SERS peaks marked with red numbers and an asterisk are from probes for the *IFI44L* gene. For blank samples (i.e., buffer only), the SERS intensities from the two probes were high (Fig. 4a). For samples containing only *IFI27* target sequences, the SERS intensities from *IFI27*

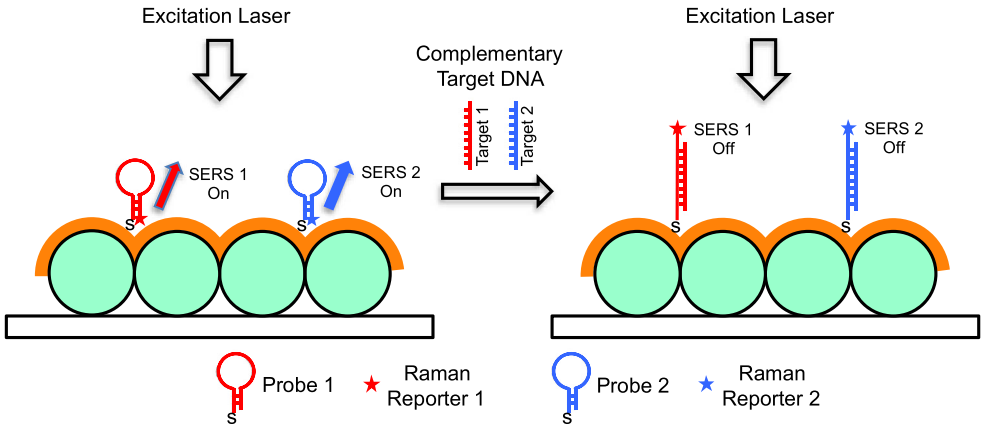

Fig. 3 Multiplex molecular sentinel-on-chip technique detection scheme (adapted from ref. [56])

probes were decreased (Fig. 4b). Similarly, for the samples containing only *IFI44L* target sequences, the SERS intensities from *IFI44L* probes were decreased (Fig. 4c). On the other hand, for samples containing both *IFI27* and *IFI44L* target sequences, the SERS intensities from both probes were both decreased.

Although the MSC technique was effective, its SERS signal decrease in the presence of target sequences was relatively counterintuitive to normal users. To present the detection results in a more intuitive way, a parameter called relative diagnostic index (RDI), defined as $RDI_{sample}=(I_{SERSblank} - I_{SERSsample})/I_{SERSblank}$, was introduced [28]. On the basis of this definition, the SERS intensities in Fig. 4 were converted to the RDI and plotted on Fig. 5. Using RDI, one could easily determine the existence of IFI27 targets and IFI44L targets in the samples of interest. For blank samples, the RDI values of both IFI27 probe and IFI44L probe were zero. For samples containing only IFI27 target sequences, the RDI of IFI27 probe substantially increased while the RDI of IFI44L probe was statistically unchanged. For samples containing only IFI44L target sequences, the RDI of IFI44L probe increased while the RDI of IFI27 probe was zero. Finally, for samples containing both IFI27 and IFI44L target sequences, the RDI values of IFI27 and IFI44L probes both increased. The use of the RDI to represent detection results is simple and more intuitive.

Inverse molecular sentinel-on-chip

The MSC technique discussed above involves an "on-to-off" signaling technique, with SERS signal decreasing in the presence of target sequences. This feature could make the MSC technique more susceptible to false positives. We developed another version of the MSC technique with an "off-to-on" signaling process upon target introduction [57]. This

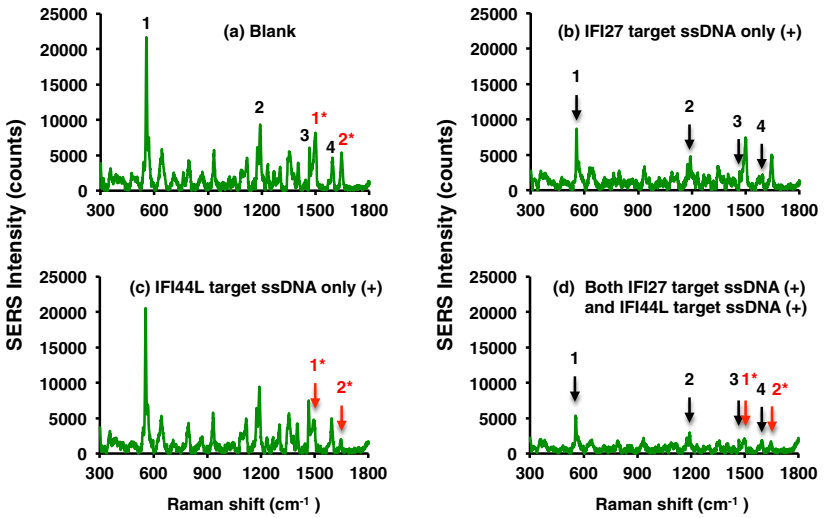

Fig. 4 SERS spectra of a mixture of *IFI27* probes and *IFI44L* probes in the presence of different samples. **a** Blank (i.e., buffer only). **b** Only *IFI27* target sequences. **c** Only *IFI44L* target sequences. **d** Both *IFI27* and *IFI44L* target sequences. Major Raman bands from *IFI27* probes are marked with *black number*, and major Raman bands from *IFI44L* probes are marked with *red number* and *asterisk*. The *arrows* illustrate the decreased SERS intensity of the major Raman bands in the presence of corresponding target sequences (adapted from ref. [56])

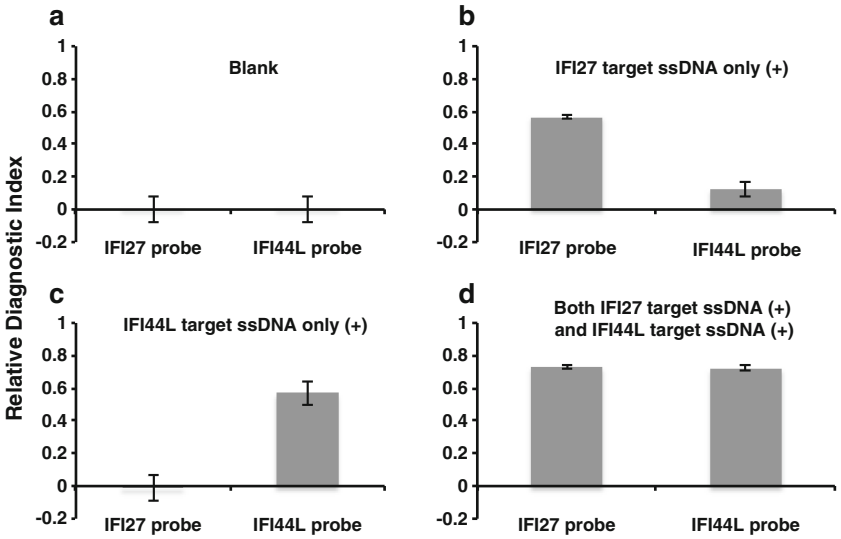

Fig. 5 Relative diagnostic index of a mixture of *IFI27* probes and *IFI44L* probes in the presence of different samples. **a** Blank (i.e., buffer only). **b** Only *IFI27* target sequences. **c** Only *IFI44L* target sequences. **d** Both *IFI27* and *IFI44L* target sequences. *Error bars* represent standard deviation (adapted from ref. [56])

technique is referred to as the inverse molecular sentinel-on-chip (iMS-on-Chip). The detection scheme of iMS-on-Chip is depicted in Fig. 6. In the absence of target sequences, the reporter probes and placeholders form partial duplex structures. Raman reporters tagged at the 3′ ends of reporter probes are kept away from the chip's metallic surface, resulting in weak SERS signals ("off state"). When target sequences are present, they form hybrids with the placeholders. The reporter probes are now free to form stem-and-loop structures owing to their complementary arm sequences. These stem-and-loop structures bring Raman labels in close proximity to the chip's metallic surface, inducing strong SERS signals ("on state"). Several distinct advantages are inherent to the iMS-on-Chip technique: (1) single-step DNA detection without any washing to remove unreacted components; (2) the target sequences do not have to be labeled; and (3) the "off-to-on" signal upon target introduction is more intuitive for diagnostics and less susceptible to false-positive responses. With proper design, the iMS-on-Chip technique is simple to operate and interpret, has low reagent costs, and returns rapid results.

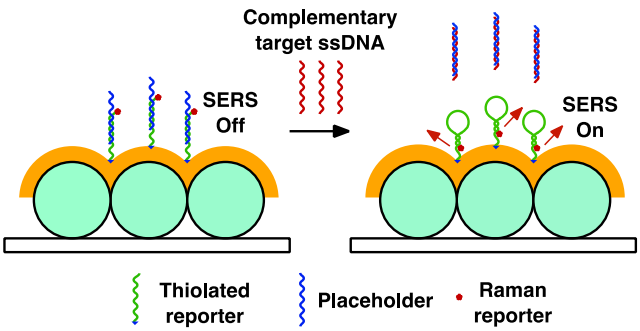

Fig. 6 Inverse molecular sentinel-on-chip technique detection scheme (adapted from ref. [57])

To test the iMS-on-Chip technique's performance, a specific DNA sequence of dengue virus 4 (DENV4) was used as a model target. A non-complementary sequence and buffer only (blank) were used as negative controls. Detection results are shown in Fig. 7. For blank samples and non-complementary samples, the SERS intensities were low and unchanged, indicating that the reporter probe–placeholder partial duplexes were not disturbed by these samples. The distance between Cy5 Raman reporters tagged at the 3' end of the reporter probes and the nanowave chip's metal surface was maintained at ca. 13.5 nm (equivalent to 40 nucleotides). At this distance, SERS enhancement was weak, resulting in low SERS intensities ("off" state). For complementary DENV4 target

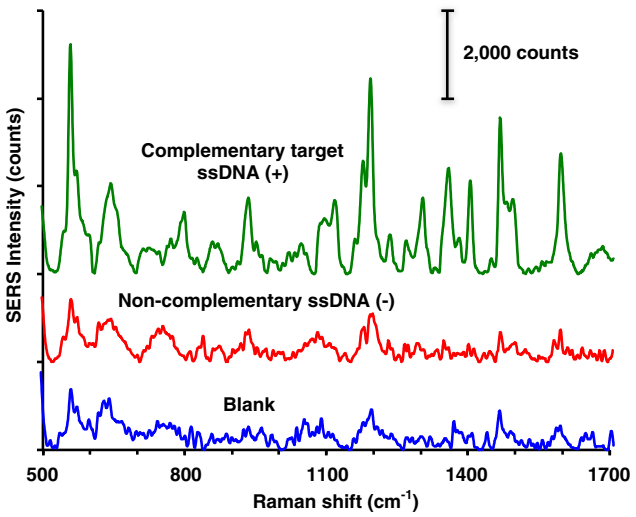

Fig. 7 SERS spectra of inverse molecular sentinel probes on a nanowave chip in the presence of blank (*bottom* spectrum), non-complementary ssDNA (*middle* spectrum), or complementary target ssDNA sample (*top* spectrum) (adapted from ref. [57])

samples, SERS intensities increased. Strong SERS signal suggested hybridization between the complementary target sequences and the placeholders. The reporter probes were now free to form stem-and-loop structures and consequently bringing the Cy5 Raman reporters closer to the chip's surface (less than 1 nm). The closer distance resulted in strong SERS intensity ("on" state). It is noteworthy that the Cy5 dye was chosen as Raman reporter because its absorption maximum (at 643 nm) is close to wavelength of the excitation laser (633 nm). Under this condition, SERRS was achieved, which provided additional enhancement to SERS [66]. SERRS enhancement was found to be three orders of magnitude higher compared to SERS enhancement [67]. Quantitative analysis showed that as low as ca. 6 amol of DENV4 target sequences inside the probed area (defined by the laser spot size) could be detected.

Conclusion

This report provides an overview of three nanochip-based DNA detection systems developed in our laboratory. The first system is the MSC method utilizing molecular sentinels immobilized on a SERS-active nanowave chip. The second system is the multiplex MSC technique using two distinct molecular sentinel probes on a single nanowave chip. The third system is the iMS-on-Chip technique with "off-to-on" signal response. All these systems use single-step DNA detection techniques with the SERS signals measured after incubation of sample solutions on DNA probe-functionalized nanowave chips without any washing. These techniques allowed host genetic biomarkers for respiratory viral infection and a specific DNA sequence of DENV4 to be detected, demonstrating the potential for POC medical diagnosis applications. Several important features of the presented techniques involve seamless integration of nanotechnology (nanoprobes), physics (plasmonics), molecular biology (nucleic acid target recognition), and molecular spectroscopy (SERS detection). The detection approaches are similar to a "label-free" scheme as the targets do not need to be labeled. The bioassay schemes of the MSC and iMS-on-Chip techniques require no sample processing steps (homogeneous assay), which is a desirable feature for many field applications. Multi-functional bioprobes equipped with nucleic acid-based platforms are capable of sensing and imaging various disease processes by targeting DNA sequences (gene mutation), mRNAs (gene expression), miRNAs (post-transcriptional regulation).

It is noteworthy that research discussed here involves only the detection aspects of diagnostic systems, which, ultimately, must also involve sample collection and treatment. For widespread implementation of chip-based systems it is desirable to produce low-cost devices that can be fabricated using micro/nano-fabrication technology. The chip format is suitable for the assembly of the various system components by integration of multiple elements on a single platform. Such a system could have a wide variety of applications ranging from field sensing of environmental pollutants, monitoring plant genomics for bio-energy research to medical diagnostics. For medical applications, this integration will produce extremely low-cost, disposable biochips that have great potential for use in home medical diagnostics of diseases without the need to send samples to a laboratory for analysis. Whereas there has been rapid progress in probe development, important areas related to system integration, such as microfluidics, biosensing surfaces, and detection technology into a seamless "lab-on-a chip" system, still remains an urgent goal requiring further research efforts. Finally, beyond in vitro diagnostics there is the ultimate goal of biochip applications, a truly implantable in vivo sensor that can provide a reliable and real-time snapshot of in vivo health status over long periods of time. In many ways, this will be the greatest challenge to overcome. Technology advances will be required in various areas to deal with issues of biocompatibility, remote detection wireless telemetry, and miniaturization for subcutaneous (or other sites) implantation. Currently a number of investigators including our group are actively addressing these challenges using surface nano-engineering, biomimicry, novel chemistry, ultrasensitive detection, and system miniaturization in order to minimize the foreign body response, enhance sensitivity and specificity as well as improve the operating shelf life of the biosensing system.

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Compliance with ethical stndards

Conflict of interest The authors declare that they have no conflict of interest.

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