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Detection of DNA Mutations Using Novel SERS (Surface-Enhanced Raman Spectroscopy) Diagnostic Platform

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Abstract: This article describes the detection of DNA mutations using novel Au-Ag coated GaN substrate as SERS (surface-enhanced Raman spectroscopy) diagnostic platform. Oligonucleotide sequences corresponding to the BCR-ABL (breakpoint cluster region-Abelson) gene responsible for development of chronic myelogenous leukemia were used as a model system to demonstrate the discrimination between the wild type and Met244Val mutations. The thiolated ssDNA (single-strand DNA) was immobilized on the SERS-active surface and then hybridized to a labeled target sequence from solution. An intense SERS signal of the reporter molecule MGITC was detected from the complementary target due to formation of double helix. The SERS signal was either not observed, or decreased dramatically for a negative control sample consisting of labeled DNA that was not complementary to the DNA probe. The results indicate that our SERS substrate offers an opportunity for the development of novel diagnostic assays.

Key words: SERS, SERS-active surface, DNA detection, GaN, chronic myelogenous leukemia.

1. Introduction

Development of simple, reliable and high-throughput methods for DNA mutation detection is of paramount importance for both research and medical diagnostics of genetic diseases [1]. Detection of particular mutations in patients greatly affects the prediction of cancer risk and helps to refine therapeutics aims. Met244Val substitution results from mismatch mutation within the region corresponding to the catalytic domain of BCR-ABL (breakpoint cluster region-Abelson) gene and leads to development of resistance to imatinib. This and other mutations within the BCR-ABL kinase domain are the most commonly identified mechanism associated with relapse of chronic myelogenous leukemia, and rapid detection assays allowing for their identification are of great prognostic importance in oncology.

A variety of methods for identifying mutations have been published in Refs. [2-7]. However, Raman spectroscopy appears as a modern, attractive alternative in genetic analysis and offers an opportunity for the development of novel biosensors. Raman spectroscopy provides unique vibrational "fingerprints", capable of distinguishing structurally similar molecules. In spite of many advantages, the application of this method is limited by very low sensitivity. However, much more sensitive Raman techniques have been developed such as SERS (surface-enhanced Raman spectroscopy) and SERRS (surface-enhanced resonance Raman spectroscopy) [8]. These detection techniques have significant

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advantages over conventional fluorescence [9]. These include: (1) Raman peaks have 10-100 times narrower spectral widths than fluorescence labels, increasing ability to multiplex [10]; (2) Flexibility in the choice of labels [11]; (3) Excellent selectivity and (4) SERS labels are not susceptible to photobleaching [12]. In addition, in comparison to SPR (surface-plasmon resonance) technique [13] or electrochemical detection methods [14], SERRS is molecule-specific and much more sensitive.

SERRS has been extensively used for enzyme activity measurements using lipases [15], alkaline phosphatase [16], proteases [17] and peroxidase [18]. In addition, numerous methods of employing SERRS in immunoassays have been described, e.g., using reporter-labeled nanoparticles conjugated to antibodies [19, 20], glass-coated dye-labeled nanoparticles coated to antibodies [21], dye- [22] or fluorescently-labeled antibodies [23], and even label-free method [24]. However, to date only few studies have been published which demonstrate a successful SERS-based detection of DNA. Fabris et al. [25] and Fang et al. [26] have developed variant assays based on the directly captured oligonucleotide probes tethered to glass slides and Au-Ag coated Si wafers, respectively. Wabuyele and Vo-Dinh [27] and coworkers demonstrated the strategy based on quenching of SERS signal by hybridization of a complementary target sequence to a hairpin ssDNA (single-stranded oligonucleotide) sequence, in the absence of capture, placing the Raman label within the proximity to the metal nanoparticles. More recently, significant progress has been made in the development of alternative nonfluorescent DNA assays [28]. Barhoumi and Halas [29] have developed label-free SERS-based detection of DNA hybridization when the probe of DNA sequence is adenine-free. However, two main impediments prevented the development of label-free methods. First, SERS spectral reproducibility of DNA has been difficult to achieve. Second, SERS spectra of both target and probe DNA are dominated by adenine signature. Therefore, distinguishing a specific SERS signal upon hybridization is extremely difficult.

Development of a SERS-based method to detect DNA and RNA qualitatively and quantitatively still remains a challenge. A standard application of SERS in biological and biomedical tests does not exist despite almost 40 years of studies. One major obstacle in transferring this technique into industry has been the challenge of fabricating low cost, stable, reproducible and sensitive SERS substrates. Especially reliable reproducibility and good temporal stability of SERS substrates are highly desired properties for designing a DNA hybridization assay. Typical assay steps include culturing of the sample, modification of the SERS surface, immobilization of ssDNA probe on the modified surface, performing hybridization, washing, and reading the results. The processing time can be lengthy, up to 32 h. The hybridization time, up to several hours, is the most time-consuming step. Therefore, the crucial aspect of the SERS-based method of DNA detection is reproducibility and temporal stability of SERS signals from a SERS-active surface. Although numerous SERS studies of DNA have been performed to date [27], only few papers discussed the stability of SERS signals over time [30]. This is particularly important in the studies of large complex molecules such as DNA, because the SERS signal in these molecules depends on molecular conformation, orientation and physical and compositional stability of substrate surface for an extended period of time.

The authors have developed a novel SERS active substrates based on Au or Au-Ag coated GaN with bunched nano-pillars [31, 32]. The resultant SERS platforms exhibit very strong surface-enhancement factor (up to 1×10^7), high stability (up to three months under ambient conditions) and high reproducibility, which could be used in the design of efficient SERS-active platforms for analytical applications. In this work, we report the results of

using this platform for the detection of mutations within BCR-ABL fusion gene translocation associated with development of imatinib-resistance in chronic myelogenous leukemia [33].

2. Experiments

2.1 Chemicals

The chemicals used for the immobilization reaction were purchased from the following sources:

Thiolated single DNA probe sequences were purchased from Oligo Company.

Sets of 3' MGITC (green malachite isothiocyanate)-labeled single-strand DNA oligonucleotide targets (22 nucleotide long) containing known BCR-ABL mutations were obtained from Genomed Company. Their base sequences were as follows:

Probe DNA: 5'SH-AAC ACG AAG TGC CAC TAC AGG C-3';

Complementary target DNA: -5'-GCC TGT AGT GGC ACT TCG TGT T-3'-MGITC;

Single-base-mismatched DNA; Mutation: Met244Val 730A>G: 5'-GCC TGT AGT GGT ACT TCG TGT T-3'-MGITC.

Stock solutions of the oligomers (100 μg/L) of all oligonucleotides were prepared using TE buffer (10 mM Tris-HCl, 1 mM EDTA (ethylenediaminetetraacetic acid), pH 8.0). Less concentrated solutions were obtained by diluting the stock solution with the same buffer. All chemicals were of analytical grade and used without further purification. All solutions were prepared with deionized water. Mercaptohexanol (Sigma-Aldrich), potassium peroxodisulfate (Merck; analytical grade), potassium hydroxide, as well as nitric acid (Sigma-Aldrich, puriss grade) were used as supplied. Gold-silver metallic alloy used as a target (99.99% pure for both elements) was bought from Mint of Poland.

2.2 Preparation of the SERS Substrate

The SERS substrate was synthesized according to previously published procedures [32]. GaN epitaxial layers grown on sapphire by MOCVD (metal organic chemical vapor deposition) method were subjected to photo-etching in KSO-D solution (0.02 M KOH + 0.02 M $K_2S_2O_8$) [34]. The photo-etching in alkaline solutions (both in KOH and in KOH/$K_2S_2O_8$) is normally used for revealing and analyzing dislocations and electrically active defects in GaN and in SiC [35]. Due to effective recombination of photo-generated carriers (electron-hole pairs) on dislocations, the protruding pillars are formed around these linear defects and after long etching form "sheafs" (Fig. 1(a)).

A metallic layer was deposited on photo-etched GaN samples by sputtering technique using Quorum Q150R S sputter coater. Au alloy of gold with silver (30%-70% by weight) was used as target. The thickness of the deposited layer measured on a flat surface was 90 nm. The coated samples were immersed in 65% nitric acid (Sigma-Aldrich) for 24 h in order to perform dealloying and to obtain porous structure [36]. Subsequently, the substrates were washed in deionized water and dried in a stream of nitrogen.

2.3 Immobilization of Oligonucleotide Probes and Hybridization

Single-strand DNA probe sequences were attached to the surface through a thiol group located at the 5' end of the oligonucleotide sequence by soaking GaN/Au-Ag surfaces in a 10 μM solution of the probe at pH 8.0 TE buffer for 40 h at 10 ^{o}C. After immobilization, the excess of ssDNA was removed by rinsing with TE buffer. Following probe attachment, the SERS substrates were incubated in 15 μM mercaptohexanol for 8 h to remove nonspecifically bound DNA. The mercaptohexanol has the effect of reorienting the ssDNA immobilized on a surface into more upright conformation [37]. Due to weak intensity of Raman spectra obtained for DNA alone, a suitable SERS label was required for adequate detection specificity and sensitivity. MGITC (green

malachite isothiocyanate), a molecule which has electronic transition in resonance with the laser excitation (632.8 nm), such that surface enhancement is accompanied by a further resonant enhancement of 1×10^3 to give SER(R)S, has been used as a labeling reporter.

In the next step, a hybridization buffer containing MGITC target oligonucleotides (10 μM) was passed over the surface. The bimolecular recognition reaction between capture- and target DNA was performed by using a thermal management, which allows the adjustment of the optimal hybridization temperature. Hybridization was carried out in 10 μM solution of labeled targets at pH 8.0 for 7 h at 37.7 ^{o}C.

2.4 Raman Experiment

SERS measurements were carried out on dried samples using a Renishaw in Via Raman system equipped with a 100-mW laser emitting a 632.8 nm line used as the excitation source. The light from the laser was passed through a line filter, and focused on a sample mounted on an X-Y-Z translation stage with a 50× microscope objective. The Raman-scattered light was collected by the same objective through a holographic notch filter to block out Rayleigh scattering. A 1,800 groove/mm grating was used to provide a spectral resolution of 5 cm^{-1}. The Raman scattering signal was recorded by a 1,024 × 256 pixel RenCam CCD detector. The beam diameter was approximately 2.5 μm. Typically, for the MGITC-labeled oligonucleotides, the spectra were acquired for 10-30 s, either in a static mode, centering at 1,200 cm^{-1}, or in an extended mode, between 300-3,400 cm^{-1}, with the laser power measured at the sample being 5 mW. The spectra have been normalized by the laser power and the collection times. SEM (scanning electron microscopy) images were taken by a Zeiss Ultra Plus scanning electron microscope.

3. Results and Discussion

3.1 Surface Characterization

The morphologies of the SERS substrates prepared in this work were monitored by SEM and representative images are presented in Fig. 1. The etching process created nanopillars at defects emerging on the GaN surface, with the average distance between the nanopillars fixed by the density of the defects. The dislocation-related nano-pillars are well-developed and stick together forming conical "sheafs" (Fig. 1(a)) veered by the Au-Ag bumps on the side walls of "sheafs" (Fig. 1(b)).

3.2 Stability of the Surface

One of the principal objectives of the current study was to prepare SERS-active substrates that are suitable for DNA detection. Previous reports have shown that the spectral quality and reproducibility of

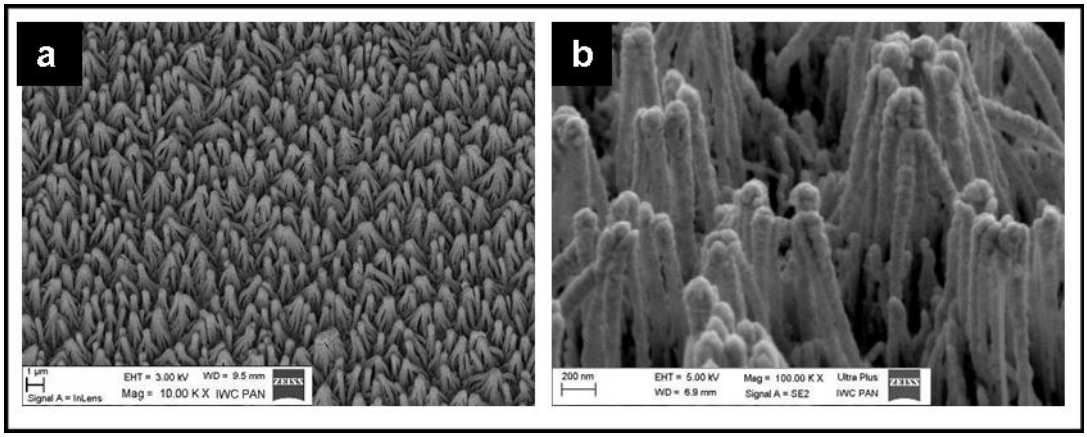


Fig. 1 (a) SEM images of a GaN platform after KSO-D photo-etching and sputtering of 70/30 % Au-Ag alloy; (b) the same surfaces after 24 h dealloying in HNO_3; samples tilted by 45°.

DNA SERS spectra can be severely limited by large variations in molecular conformation and/or physical and compositional stability of the substrate surface for an extended period of time. Murphy et al. [38] reported that the differences in intensity of SERS spectra of DNA oligonucleotides adsorbed on gold nanoparticles which resulted from aggregation of the nanoparticles over time.

The stability of the current platform was tested by measuring the Raman activity with time. We have recorded the SERS spectra, after washing, for adsorbed thiolated ssDNA, with 10^{-6} M concentrations, after 20 min, 60 min, 2 h, 24 h, and 7 days of storage under ambient desk-top conditions (Fig. 2). Fig. 2 contains the results obtained in five similar experiments with different pieces of the same substrate. The SERS spectra of DNA display two major bands. The 731 cm^{-1} band is assigned to the ring-breathing mode of adenine, while the multicomponent band peaking at 1,330 cm^{-1} is assigned to mixed in-plane stretching motions of the six-member ring (ring skeleton vibrations). The SERS study revealed excellent reproducibility and stability of our surface. The intensity of the strongest band (at

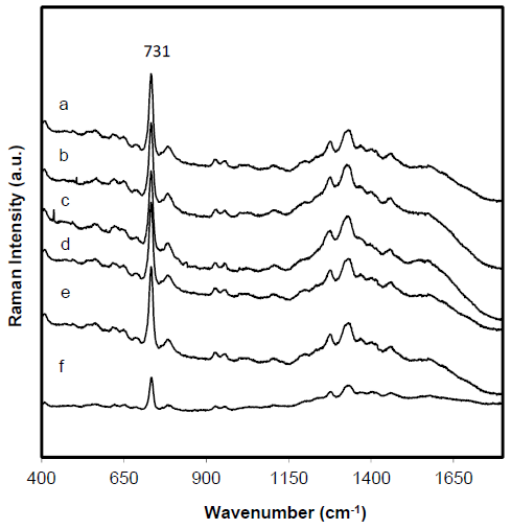


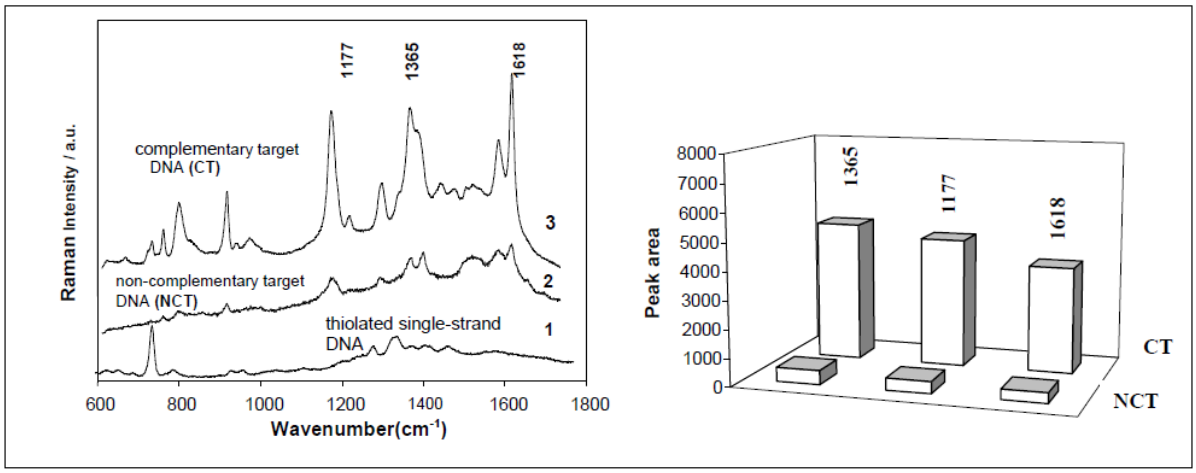
Fig. 2 SERS spectra of ssDNA thiolated (5'SH-AAC TCG AAG TGC CAC TAC TGG C-3') sequence adsorbed from 10^{-6} M concentrations obtained after (a) 20 min, (b) 60 min, (c) 2 h, (d) 6 h, (e) 24 h and (f) 7 days of storage under ambient conditions.

731 cm^{-1}) was reduced approximately by only 20% after 24 h and by 60% after 7 days of keeping the surface on the shelf. We did not detect any peak shift or appreciable change of intensity of other DNA bands. This stability contrasts sharply with other common SERS substrates [39]. Similar experiments were repeated using Klarite, a commercially available substrate. The intensity of ssDNA SERS signals decreased dramatically with time, already after 1 h. These results demonstrate outstanding stability characteristics enabling clear and unambiguous detection of DNA.

3.3 DNA Detection

The 22-mer probes for detecting the wild type and mutated sequences in the BCR/ABL translocation were attached to the GaN/Au-Ag surface. The integration of a Raman label into the DNA system was necessary in this experiment because the SERS spectra of both target and probe DNA are dominated by adenine signature. Other nucleobases have lower signature intensity, so that distinguishing a specific SERS signal upon hybridization should be extremely difficult. Therefore, Green Malachite isothiocyanate has been used as a labeling reporter, giving rise to the characteristic SERS signature which is necessary for identification of hybridization.

Fig. 3 shows the results for sequence-specific DNA detection. Three different samples were compared: a surface with the thiolated ssDNA (1), a surface with ssDNA after hybridization with non-complementary DNA (Met244Val mutation) (2), and a surface with ssDNA after hybridization with complementary DNA (3). Due to the highly localized DNA spots on GaN/Au surface, it was possible to detect SERS spectra of the dye-labeled complementary DNA. Typical Green Malachite spectra with the specific fingerprint (Fig. 3(a)) were observable, proving a successful hybridization using SERS as a detection method. The two prominent bands at 1,618 cm^{-1} and



(a) (b)

Fig. 3 (a) SERS spectra of a surface with the thiolated ssDNA (1), a surface with ssDNA after hybridization with non-complementary DNA (Met244Val mutation) (2), and a surface with ssDNA after hybridization with complementary DNA (λ_{exc} = 632.8 nm) (3); (b) Raman intensity of three different Raman bands at 1,177, 1,365 and 1,618 cm^{-1} (marked in Fig. 3(a)) detected for the CT and NCT target DNA sequences, respectively.

1,365 cm^{-1} (with a shoulder at 1,393 cm^{-1}) have been assigned to stretching of phenyl-N and aromatic ring according to Lueck et al. [40]. A strong intensity band at 1,177 cm^{-1} was assigned to the benzene ν_9 mode, while a weak mode at about 1,184 cm^{-1} has been assigned mainly to a vibration (methyl bend and C-N stretch) localized on the dimethylamino group. An intensive band observed at 806 cm^{-1} corresponds to out-of-plane C-H bending from benzene ring. The band at 762 cm^{-1} has been assigned to the ν_{17} benzene-like vibration. The Raman signal intensity of the three most prominent bands at 1,177, 1,365 and 1,618 cm^{-1} in the SERS spectrum of MIGTC was determined by their peak areas. The mean values of the signal intensities were plotted for both complementary and non-complementary target DNA sequences (Fig. 3(b)). The largest signal intensities were found for the complementary case (Fig. 3(a)) due to the most specific interaction between the probe and target molecule. The Raman signal intensities for the mismatch (Met244Val) case were about an order of magnitude lower than for the complementary case or not observed at all, thus demonstrating the mismatch specificity of the used method.

4. Conclusions

Surface-enhanced Raman spectroscopy is a modern technique in genetic analysis which offers an opportunity for the development of novel biosensors. In this work, it is showed that the GaN/Au-Ag substrates can be used to obtain stable, reproducible SERS signals for DNA detection. Furthermore, we have used this platform for the detection of point mutation (corresponding to Met244Val substitution) in BCR-ABL fusion gene associated with development of resistance to imatinib treatment in patients with chronic myelogenous leukemia. Probe recognition is based on the molecular hybridization process, which involves combining a strand of nucleic acid with a complementary sequence. Due to the weak Raman spectra obtained for DNA alone, a Green Malachite isothiocyanate label was used for adequate detection specificity and sensitivity.

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