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DNA detection using nanostructured SERS substrates with Rhodamine B as Raman label

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ABSTRACT

A technique is demonstrated to detect DNA hybridization at low concentrations, based on Surface-Enhanced Raman Scattering (SERS) using silicon nanostructures coated with gold–silver as substrate. Standard silicon process technologies were employed to fabricate the SERS substrates featuring nanogaps with a characteristic distance of 15 ± 10 nm. Target DNA was hybridized with cysteine-modified Peptide Nucleic Acids (PNA), which was previously fixed into the nanogaps as the capture sites. After hybridization, the introduced phosphate groups from the backbone of the target DNA showed strong affinity to an inorganic linker, Zr^{4+}, so that resulting in the assembly substrate–PNA–DNA–Zr. Since PNA does not possess phosphate groups, the linker is avoided when there is no hybridization from the complimentary DNA. Subsequently, the assembly of substrate–PNA–DNA–Zr was incubated with a Raman label, Rhodamine B (RB). The carboxylic acid group in RB reacted with the linker Zr^{4+} allowing this Raman Label to be attached to the assembly substrate–PNA–DNA–Zr. The Raman peaks corresponding to RB were selected to detect the target DNA, with a detection limit of 1×10^{-12} M.

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1. Introduction

Since its discovery in 1977, SERS (Surface-Enhanced Raman Scattering) has received more and more attention from researchers around the world (Kneipp et al., 1999; Haynes et al., 2005a), not only because of its high sensitivity and the small volume of sample needed (Etchegoin et al., 2003), but also due to the possible wide applicability (Vandenabeele et al., 2007; Seydack, 2005). The extremely high sensitivity, which comes from the enhancement on substrate surface, makes it possible to become a new tool for single molecule detection, similar to some other methods, such as laser-induced fluorescence, frequency-modulated optical absorption at low temperature, and electrochemical detection of redox-active species as well (Nie and Emory, 1997; SaSic et al., 2005). Some commonly used substrates for SERS include electrochemically roughened metal surface (Liu et al., 2006), metallic nanoparticles array (Haynes et al., 2005b), nanofabricated substrates (Dootz et al., 2006), etc. With regard to roughened metal surfaces or nanoparticles arrays, although high enhancement efficiency has been achieved, the distribution of so-called hot spots is random, resulting from imprecise control of such nano-scale structures. Controllable nanostructures, however, are crucial for the SERS effect because the strong electromagnetic field on a metal surface, which is the main contributor for enhancement beyond the chemical enhancement, exponentially degrades with a characteristic length-scale of ~2 nm (Haynes et al., 2005a). The surface electromagnetic field is stronger at the sharp tip than on the flat surface. If two sharp tips are arranged closely, the electromagnetic field can be overlapped and a further enhancement for SERS is then expected. Based on this understanding, another version of SERS, Tip-Enhanced Raman Scattering was developed to further enhance the Raman response by controlling the distance between the tip and the substrate (Rasmussen and Deckert, 2006; Domke et al., 2007). The complicated instrument is however limited in widespread application, especially when one would like to have a portable system for field use. Nanofabricated substrates have been then produced (Haynes et al., 2005a; Gunnarsson et al., 2001). Lots of technologies have been developed to control the substrate structure on the nano-scale so that a uniform distribution of nanostructures is possible. Further work in this area has been performed and potential applications have been identified (Tan et al., 2007).

The mechanism of SERS remains an active research topic since the theoretical understanding is not clear so far, but the application

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has been developed over the past decades. The SERS detection of various bio-molecules or species has been reported, such as proteins (Driskell et al., 2007), enzymes (Ruan et al., 2006), viruses (Wabuyele and Vo-Dinh, 2005), bacteria (Jarvis et al., 2006), cancer marker (Gong et al., 2007), and nucleotides (Nie and Emory, 1997; Kneipp et al., 1998, 1999; Sackmann and Materny, 2006). Among those kinds of application, the detection of nucleotides or DNA is very interesting because the nucleotide bases show well-distinguished SERS peaks while the sugar or phosphate groups on the backbone have little interference (Green et al., 2006). The direct detection of DNA sequence at low concentration with SERS, however, is still elusive (Wu et al., 2006). The resulting spectrum strongly depends on the sequence of the bases (Deng et al., 1999), and also on the binding modes of DNA strands (Gearheart et al., 2001), which results in the difficulty to identify the DNA sequence. Additionally, the methodologies used for DNA recognition are usually based on the hybridization of the target nucleic acid with its complementary bases. Difficulties arise since there is little difference between the Raman spectra of the target nucleic acid and its complementary bases. Consequently, there is always a need to label the DNA with a Raman scatterer, such as cresyl fast violet (Vo-Dinh et al., 2005), Rhodamine B (Culha et al., 2003), Rhodamine 6G and others (Cao et al., 2002). Some metal nanoparticles have also been conjugated with DNA to help the detection (Wabuyele and Vo-Dinh, 2005; Braun et al., 2007).

Here, we demonstrate the SERS detection of DNA using Rhodamine B (RB) as a Raman label. Standard silicon process technologies were used to fabricate a SERS substrate with high reproducibility and massive productivity. The fabrication process includes conventional deep UV photolithography, reactive ion etching, thermal oxidation and physical vapour deposition of silver and gold as well. Further details can be found in previous reports (Tan et al., 2007). There is a high electromagnetic field in the fabricated nanogaps on the SERS substrate (Haynes et al., 2005a). When the "to-be-detected" species are located in the nanogaps, the extremely high electromagnetic enhancement of Raman scattering is expected. PNA with a specific sequence was introduced into the nanogaps and was hybridized with the complementary target DNA with high selectivity. Subsequently, the phosphate groups in the target DNA backbone (in the substrate–PNA–DNA assembly) then reacted with Zr^{4+}. Zr^{4+} has been previously used as a linker in nucleotide studies (Bakiamoh and Blanchard, 1999, 2001; Fan et al., 2007b). The resulting substrate–PNA–DNA–Zr was exposed to RB that was employed as a Raman label. The Raman signal from RB promoted detection of the target DNA at low concentration. Although the intrinsic Raman scattering of DNA bases is also measurable, its intensity depends on lots of factors, not just on the quantity of target DNA alone (Deng et al., 1999; Gearheart et al., 2001). Particularly, when the concentration of complimentary DNA is low, the Raman signal from the target DNA is far lower than that from capture PNA. The difference between the spectra before and after the hybridization is negligible. Therefore, the introduction of Raman label is necessary. A Raman label, rather than a fluorescent label, is selected here because the high SERS enhancement from the nanostructured substrate is expected due to the high electromagnetic field in the nanogap. Furthermore, the specific Raman scattering can provide high information content about molecular vibrations and the potential for multiplexed labeling (Doering et al., 2007).

2. Experimental

2.1. Materials

All reagents were obtained from Sigma–Aldrich and used without further purification. The sequences of PNA and DNAs purchased from Biolabs. are as follows:

(i). Cysteine (cys)-modified PNA, 3′-ACT CCA TCA TCC AAC ATA CCA A-cys-5′,
(ii). DNA, 5′-TGA GGT AGT AGG TTG TAT GGT T-3′,
(iii). DNA, 5′-TGA GGT AGT AGG TTG TGT GGT T-3′.

In addition, a long strand DNA (λ-DNA from Biolabs. too) (duplex DNA is isolated from bacteriophage lambda and is ca 48,502 base pairs in length) was also used in control experiments.

The PNA was derivatized with cysteine, the resulting thiol groups of which anchored the PNA onto the gold or silver surface. DNA (ii) was used as target DNA, whereas the DNA (iii) a mismatch for a negative control. The hybridization was conducted in a TE buffer solution (10 mM Tris–HCl + 1.0 mM EDTA + 0.15 M sodium chloride, pH ∼8.0) at room temperature.

2.2. Fabrication of SERS substrate

The highly ordered arrays of silicon nanostructures coated with silver and gold were fabricated on an 8-in. diameter, single crystal, and *p*-type silicon wafers. The process steps included deep UV photolithography to pattern the needed nanostructures, reactive ionic etching to realize the nanostructure pattern arrays, and so on. The silicon etching was performed with SF_6 in C_4F_8, and the depth of the nanostructures was about 150 nm. Dry oxidation was done at 900 °C in order to control the nanostructures' spacing. Physical vapour deposition of the bottom silver layer with thickness of 30 nm and the top gold layer with thickness of 15 nm was done using an E-beam evaporation system. Scanning electron microscopy (SEM, JSM-6700F, Japan) and transmission electron microscope (TEM, Philip, with PW6061/25 EDX) were used for physical characterization of the substrates. The resulting nanostructured SERS substrate and the schematic drawing of a nanogap with the dimensions of the various layers are shown in Fig. 1.

2.3. Modification of the substrate

The SERS substrate was washed with chloroform, acetone, 0.1 M $NH_3{\cdot}H_2O$, 0.1 M HCl and deionized water, respectively. Then it was dried under nitrogen flow to get the clean substrates for each analysis.

Firstly, a clean substrate was immediately immersed into a solution of 1×10^{-6} M PNA (0.1 × PBS) over weekend, then thoroughly washed with deionized water. The immobilized PNA was then hybridized with different concentrations of target DNA in TE buffer solution for 1–2 h with slight shaking. A little bit longer period of time is necessary since the hybridization efficiency in nanogap is low due to the steric inhibition. That is, the transportation kinetics of target DNA into the nanogap is a slow process. After that, the substrate was washed with solution # 1 (1 × SSC + 0.1% SDS) (SSC, solution of 0.3 M sodium citrate +3 M NaCl, pH ∼7.0 in water; SDS, aqueous solution of sodium dodecyl sulfate, pH ∼7.0), solution # 2 (0.1 × SSC + 0.1% SDS) and 6 mM NaCl, respectively. Then it was dipped into a fresh solution of 5 mM zirconyl chloride in 60% ethanol solution for 20 min in order to attach Zr^{4+} onto the phosphate groups of the target DNA backbone. After being washed with a 60% ethanol solution, the substrate was immersed into a solution of 1×10^{-6} M RB + 1 mM NaCl for different period of time. After this incubation period, the substrate–PNA–DNA–Zr–RB assembly was washed with 6 mM NaCl and dried with nitrogen blow. The modification procedure and the substrate–PNA–DNA–Zr–RB assembly are represented in Fig. 2. The molecular structure of RB is also listed.

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