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Graphene-Based High-Efficiency Surface-Enhanced Raman Scattering-Active Platform for Sensitive and Multiplex DNA Detection

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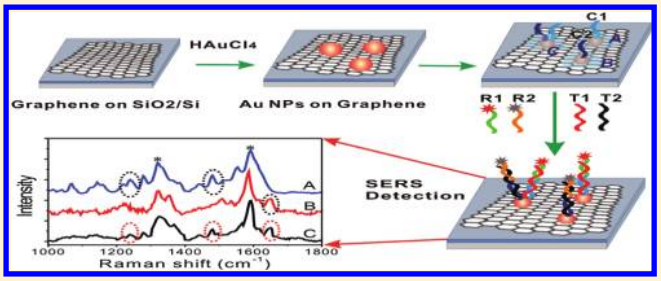
Supporting Information

ABSTRACT: We have developed a surface-enhanced Raman scattering (SERS)-active substrate based on gold nanoparticle-decorated chemical vapor deposition (CVD)-growth graphene and used it for multiplexing detection of DNA. Due to the combination of gold nanoparticles and graphene, the Raman signals of dye were dramatically enhanced by this novel substrate. With the gold nanoparticles, DNA capture probes could be easily assembled on the surface of graphene films which have a drawback to directly immobilize DNA. This platform exhibits extraordinarily high sensitivity and excellent specificity for DNA detection. A detection limit as low as 10 pM is obtained. Importantly, two different DNA targets could be detected simultaneously on the same substrate just using one light source.



Graphene has attracted great interest in a wide range of areas because of its unique electronic, optical, thermal, and mechanical properties[1−6] and potential applications in the fields of nanoelectronics, photonics, and nanomechanical systems.[3,7,8] It is of our particular interest that graphene can effectively enhance Raman signals of absorbed organic molecules that makes it a type of useful surface-enhanced Raman scattering (SERS) substrate.[9−11] Due to the charge transfer between graphene and adsorbed molecules, it was reported that graphene leads to an enhancement factor (EF) of 2−17.[9] Nevertheless, there are several factors that hinder graphene from being an efficient SERS substrate for biomolecular detection. First, chemical synthesized graphene oxide (GO) sheets are usually obtained in small sizes and with many chemical and structural defects.[12] Second, graphene itself only has limited SERS enhancement factors that can not meet the requirements for high-sensitivity detection. Third, it is difficult to directly immobilize biomolecules on graphene due to the lack of convenient and reliable surface chemistry. Herein, we report the preparation of a uniform, high-efficiency SERS-active graphene-based substrate using chemical vapor deposition (CVD)-grown large-sized graphene with in situ grown gold nanoparticles (AuNPs) and demonstrate its application for multiplex DNA sensing.

Sensitive and multiplex DNA detection is vitally important in numerous areas of molecular biology and clinical medicine because it can provide useful diagnostic information for identification of disease and pharmacogenomics.[13−15] Among the developed methods for DNA detection,[16−20] a fluorescence-based method is mostly used.[21−23] However, the harvesting of fluorescent signals are often limited by spectral overlap of different fluorophores, which makes only about three labels generally being detected simultaneously and usually needing excitation light with different wavelength.[22] SERS is an attractive substitute method for multiplex DNA detection since it produces molecular specific vibrational spectra that can easily differentiate between mixtures of fluorescent labels. Thus, SERS had been used to simultaneously detect multiple DNA labels with high sensitivity.[24−29] However, fabrication of uniform and efficient SERS substrates remains to be challenging due to the complex processes, high cost, and poor compatibility with bioassays.[30−32] On the basis of previous findings that carbon nanomaterials can directly reduce metal ions, leading to spontaneous growth of metal nanoparticles on them in the absence of external reducing agents or catalysts,[33,34] we developed a convenient and highly active gold-on-graphene nanostructured SERS substrate for DNA detection. AuNPs not only serve as anchor spots for the assembly of thiolated DNA probe but also provide additional enhancement of Raman signals. We have demonstrated that the as-fabricated SERS

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substrate result in large Raman enhancement and provide a unique platform for simultaneous multiplex DNA analysis with a single excitation light source.

EXPERIMENTAL SECTION

Materials. DNA oligonucleotides were synthesized and purified by TAKARA Biotechnology (Dalian, China). The sequences of these oligonucleotides are shown in Table 1.

Table 1. Sequence of Oligonucleotides Employed in This Work

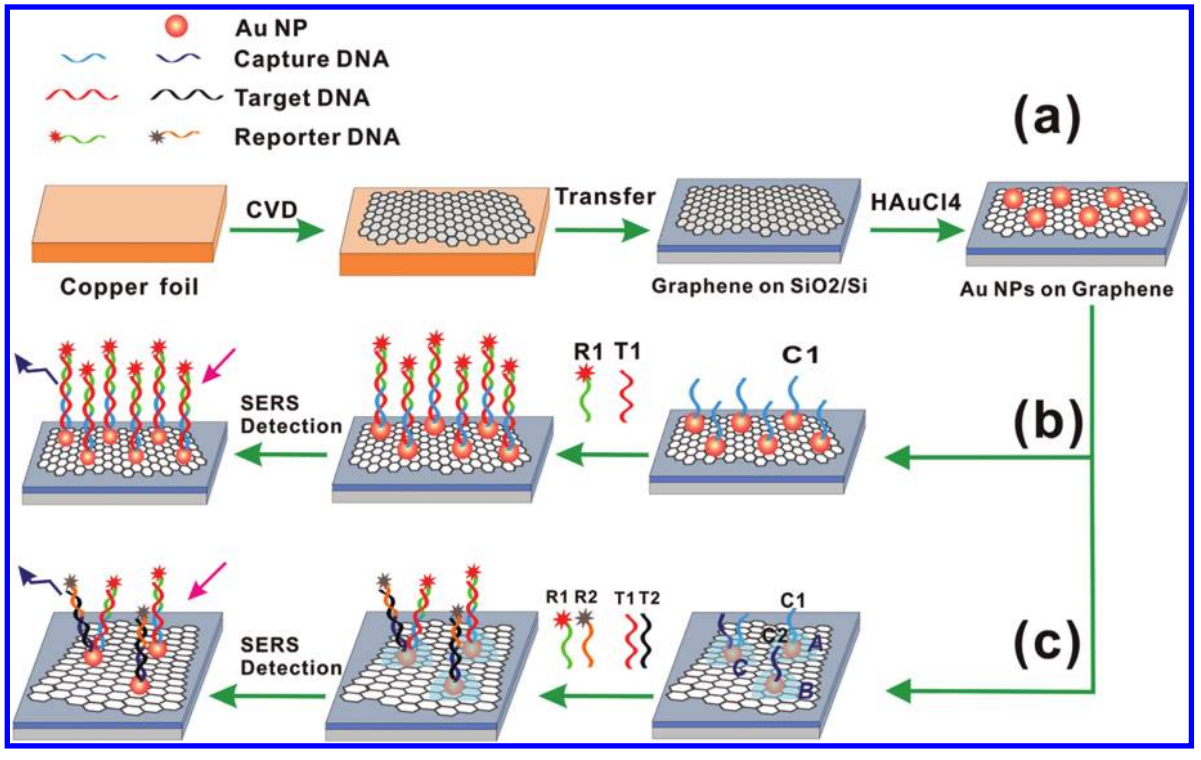
DNA name	sequence (5′→3′)
C1 (Capture DNA No. 1)	TCCATGCAACTCTAAAAAAAA-SH
C2 (Capture DNA No. 2)	ATAACTGAAAGCCAAAAAAAA-SH
T1 (Target DNA No. 1)	TTAGAGTTGCATGGATTAACTCCTCTTTCT
T2 (Target DNA No. 2)	TTGGCTTTCAGTTATATGGATGATGTGGTA
R1 (Reporter DNA No. 1)	**Cy3**-AGAAAGAGGAGTTAA
R2 (Reporter DNA No. 2)	**TAMRA**-TACCACATCATCCAT
NoncDNA	GAGGGATTATTGTTAAATATTGTAAAGGAT

Gold(III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$) and R6G were purchased from Sigma-Aldrich. Cy3 dye was purchased from Fanbo Biochemicals (Beijing, China). ROX was purchased from Keqing bio. (Suzhou, China). Other chemicals were of analytical grade, and water was purified using a Millipore filtration system (Milli-Q water).

CVD Growth of Graphene. For the CVD growth of graphene on Cu foil, high purity copper foil (25 μm in thickness, purity of 99.8%) was put on a quartz plate and then loaded into the center of a tubular furnace (TF555500030, Lindberg/Blue/M). The chamber was evacuated to ~5 mTorr, and the temperature was increased from room temperature to 1030 °C. Prior to growth, a pretreatment step was performed under a H_2 atmosphere with 400 sccm flow at 500 Torr for 50 min. During the growth step, a gas mixture of methane and hydrogen (CH_4/H_2= 13 sccm/3 sccm at 300 mTorr) was introduced for 20 min. The system was then cooled down to room temperature to complete the growth.

Transfer Process of CVD-Grown Graphene. To transfer the as-grown graphene onto the SiO_2/Si (300 nm thick SiO_2) substrate, the Cu foil after CVD growth was coated with a layer of poly(methyl methacrylate) (PMMA) by the spinning-coating method (step 1: 900 rpm for 10 s; step 2: 2900 rpm for 30 s), followed by baking at 90 °C for 1 min. After that, the PMMA-caped Cu foil was then flipped on the $Fe(NO_3)_3 \cdot 9H_2O$ (4.76 wt %) overnight to etch away the Cu substrate. The PMMA-caped graphene (PMMA/graphene) film was transferred to a Milli-Q water to dilute and remove the etchant and residues. The PMMA/graphene was then transferred to the receiving substrate and dried at hot plate (90 °C for 10 min). The PMMA was removed by warm acetone (80 °C), and then the sample was rinsed with isopropyl alcohol (IPA) and Milli-Q water.

Scheme 1. Schematic Illustration of the Preparation of SERS-Active Substrate and Its Use for DNA Detection[a]



[a](a) The large-sized graphene films were grown by CVD on copper foils and then transferred onto SiO_2/Si substrate. Then graphene film was decorated with AuNPs by immersing it into $HAuCl_4$ solution. (b) The detection of target DNA by Au-G-SiO_2/Si substrate-based SERS sensor. (c) The multiplexing detection of two different target DNAs by Au-G-SiO_2/Si substrate-based SERS sensor; A, B, and C display different locations for multiplex DNA detection on the substrate. To simplify the model, only six (or three) AuNPs are shown in the figure and each AuNP was modified with one (or two) DNA.

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Preparation of SERS Substrate. After transferring, the CVD-grown graphene on SiO_2/Si substrate (G-SiO_2/Si) was rinsed using ethanol and Milli-Q water. Then the G-SiO_2/Si was immersed in $HAuCl_4$ solution which was prepared at 10 mM concentration in Milli-Q water and an equal volume of ethanol for better wetting of the graphene film for about 60 min. The resultant SERS substrates (Au-G-SiO_2/Si) were rinsed with ethanol and Milli-Q water, dried with a gentle flow of nitrogen.

Instrumentation. The Raman spectroscopy of CVD-grown graphene film was obtained with a NT-MDT confocal Raman microscopic system (laser wavelength of 473 nm and laser spot size is 0.4 μm). The Si peak at 520 cm^{-1} was used as reference for wavenumber calibration. Scanning electron microscopy (SEM, Oxford Instruments) was carried out at 10 KV to confirm the formation of gold nanoparticles on graphene film. The SERS analysis was performed using a HR800 Raman microscope instrument (HORIBA, Jobin Yvon, France) using the standard 633 nm HeNe 20 mW laser with a laser spot of 1 μm. All these SERS spectra were obtained at the same parameters (e.g., objective: 50× NA 0.7, acquisition time: ~3 s, hole: ~400, slit: ~100, grating: ~600 g/mm). LabSpec 5 software was used for Raman data acquisition and data analysis.

DNA Detection Using SERS. The schematic for the typical detection of target DNA was shown in Scheme 1b. First, 20 μL of thiolated capture DNA (C1, 1 μM) was added onto the Au-G-SiO_2/Si substrate which was kept in a sealed humidity box at room temperature for 16 h, followed by rinsing with phosphate buffer saline (PBS, containing 0.3 M NaCl and 10 mM phosphate, pH 7.4) to remove the unbounded capture DNA and drying with a gentle flow of nitrogen. Second, the functionalized Au-G-SiO_2/Si substrate was hybridized with the different concentration of target DNA (T1, 20 μL) and reporter DNA (R1, 100 nM, 20 μL) in the sealed humidity box at room temperature for 4 h, respectively. The resultant substrate was rinsed with Milli-Q water and dried with nitrogen and then used for SERS detection. The schematic for the typical multiplex detection of two types of target DNA was shown in Scheme 1c. First, 15 μL of thiolated capture DNA (C1, C2, and C1&C2, 1 μM) was dropped onto the different locations of Au-G-SiO_2/Si substrate (A, B, and C, respectively) which was kept in a sealed humidity box at room temperature for 16 h, followed by rinsing with PBS to remove the unbounded capture DNA and then drying with a gentle flow of nitrogen. Second, the functionalized Au-G-SiO_2/Si substrate was hybridized with the mixture solution of two types of target DNA (T1&T2, 15 μL, 1 nM) and a mixture solution of two reporter DNAs (R1&R2, 15 μL, 100 nM) on the selected locations (A, B, and C, respectively) in the sealed humidity box at room temperature for 4 h, respectively. The resultant substrate was rinsed with Milli-Q water and dried with nitrogen and then used for SERS detection. All these DNAs were dissolved in PBS.

RESULTS AND DISCUSSION

The preparation of SERS-active substrate is illustrated in Scheme 1a. The large-sized graphene films were grown by CVD on copper foils and then transferred onto SiO_2/Si substrate (300 nm SiO_2). The thickness and uniformity of the graphene films were evaluated via color contrast under optical microscope and Raman spectroscopy. The optical image of a typical CVD-grown graphene is shown in Figure 1a. It is evident that even for a relatively large area of 120 μm × 120 μm, continuous and uniform graphene film was obtained. Figure 1b shows the typical Raman spectra of transferred graphene films measured from the spots marked with red circles in Figure 1a. The excitation wavelength is 473 nm. Regions indicated by a, b, and c in Figure 1a are identified as single-, double-, and multilayered graphene by their characteristic intensity ratio of 2D (~2700 cm^{-1}) peak to G (~1585 cm^{-1}) peak (I_{2D}/I_G) (~2.0, ~1.0, ~0.67). The Raman maps and atomic force microscopy (AFM) image of the graphene film were also used to evaluate the number of layers of the graphene film (Figures S-1a and S-1b, Supporting Information), suggesting the majority of the film is 1−2 layers of graphene. The resulting graphene film on SiO_2/Si substrate was referred to as G-SiO_2/Si. To decorate graphene with AuNPs, graphene film on SiO_2/Si substrate was simply immersed into 10 mM $HAuCl_4$ solution for about 60 min. Gold nanoparticles will form spontaneously on graphene film due to direct redox reaction between metal ions and graphene.[33,34] The SEM images of the graphene film before and after gold decoration are shown in Figure 1c,d. The AuNPs are obviously seen on the graphene film (Figure 1d). The size of AuNPs ranges from about 20 to 50 nm (the inset of Figure 1d). The variation in size might result from the PMMA residuals on the graphene film and the differential thickness at the different location of graphene film.[34] The as-fabricated AuNP-decorated graphene film on SiO_2/Si was referred to as Au-G-SiO_2/Si.

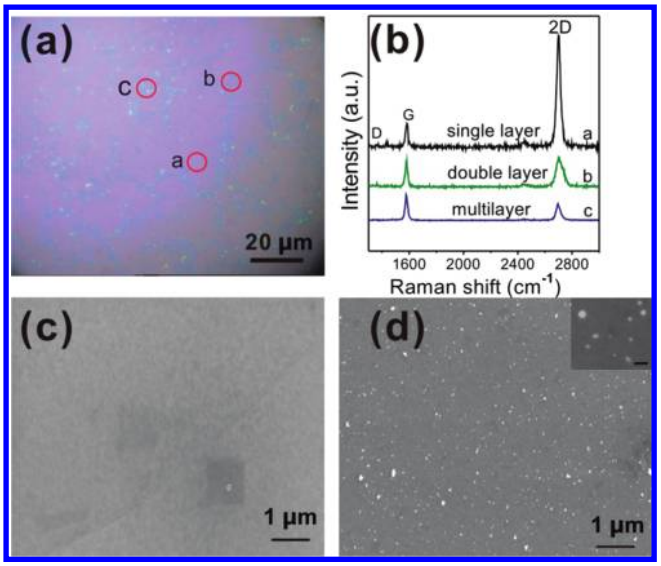

Figure 1. (a) Optical micrograph of the graphene grown on a Cu foil and transferred onto a SiO_2/Si (300 nm thick oxide layer) substrate. (b) Representative Raman spectra from the spots marked with red circles in (a). The number of graphene layers can be determined by the peak intensity ratio of 2D and G bands in the Raman spectrum. SEM images of the surface of bare graphene film (c) and same film after 60 min of immersing in 10 mM $HAuCl_4$ solution (d). The inset is a fractional SEM of panel d, and the scale bar is 100 nm.

To investigate whether the Au-G-SiO_2/Si substrate can be used as an effective SERS substrate, a droplet of ethanol solution of Cy3 dye (10 μM) was added onto the surface of the substrate. SiO_2/Si substrate (SiO_2/Si) and bare graphene film on SiO_2/Si substrate (G-SiO_2/Si) were used as controls. Their Raman spectra were measured after the evaporation of ethanol. As shown in Figure 2a, no visible Raman signal of Cy3 is observed on the SiO_2/Si substrate, while obvious SERS peaks of Cy3 are observed on the G-SiO_2/Si and the Au-G-SiO_2/Si substrate. The assignments of the peaks are consistent with

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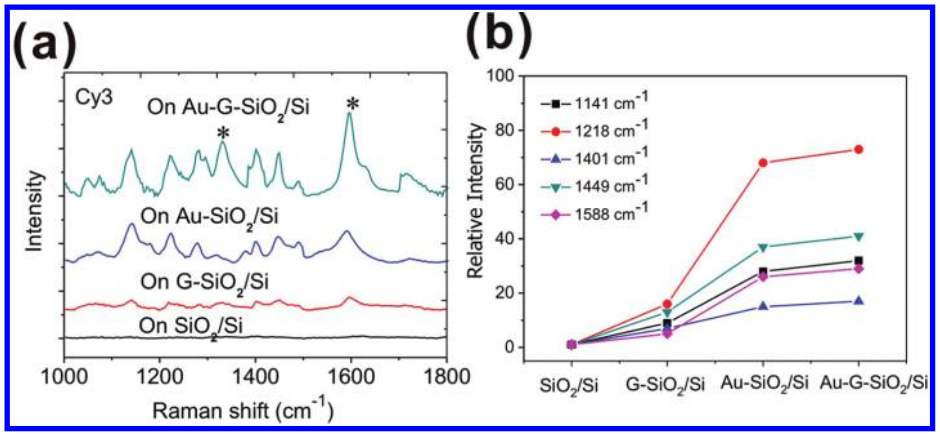

Figure 2. (a) Raman spectra of 10 μM Cy3 on SiO_2/Si, G-SiO_2/Si, Au-SiO_2/Si, and Au-G-SiO_2/Si substrate. (b) The relative Raman intensity of Cy3 on different substrates at the selected 5 Raman peaks. The signals on the SiO_2/Si substrate are set to "1". The peaks marked by the star (*) are the D-band (~1330 cm^{-1}) and G-band (~1595 cm^{-1}) of graphene.

previous reports.[35,36] Importantly, we find that the Raman signal of Cy3 on the Au-G-SiO_2/Si substrate is much stronger than that on the G-SiO_2/Si substrate, indicating that the former is a high-efficiency SERS substrate. It has much better performance of SERS than G-SiO_2/Si substrate. To investigate the contribution of enhancement from graphene, we prepared the Au-SiO_2/Si substrate (Supporting Information experimental section and Figure S2) and a droplet of ethanol solution of Cy3 dye (10 μM) was added onto the surface of Au-SiO_2/Si substrate; the signals of Cy3 on this substrate were obtained and compared with that on other substrates (Figure 2). In order to calculate the Raman enhancement magnitude, we chose the SERS intensity of Cy3 on the SiO_2/Si substrate at the selected 5 obviously Raman peaks (1141, 1218, 1401, 1449, 1588 cm^{-1}) as the normalization reference (set to "1", Figure 2b). As shown in Figure 2b, the relative intensities of these 5 peaks of Cy3 on G-SiO_2/Si substrate are 9, 16, 7, 13, and 5, respectively, which is consistent with previous reports.[9] Significantly, the relative intensities of 5 peaks of Cy3 on Au-SiO_2/Si substrate are 28, 68, 15, 37, and 26, while the relative intensity of 5 peaks of Cy3 on Au-G-SiO_2/Si substrate is 32, 73, 17, 41, and 29, respectively.

SERS enhancement typically comes from two mechanisms: chemical mechanism (CM) and electromagnetic mechanism (EM).[31,32] CM is based on a charge transfer between the molecule and the substrate. It is a short-range effect which usually requires the molecule to be close enough to the substrate.[37] On the other hand, the EM results from the large increase of the local electric field caused by surface plasmon resonances (SPR) of nanosized metals, such as silver (Ag) and gold (Au).[32] These two mechanisms always contribute simultaneously to the overall enhancement, and EM is thought to contribute the main enhancement. SEM images in Figure 1d show that a large amount of AuNPs formed on graphene film and the relative strong SERS signals were observed on this SERS-active substrate (Figure 2). Although the SERS signals on the Au-SiO_2/Si substrate was much stronger than that on the G-SiO_2/Si substrate, the relative Raman intensity of Cy3 is weaker than that on the Au-G-SiO_2/Si substrate (Figure 2). Meanwhile, the Raman intensities of D-band and G-band on the Au-G-SiO_2/Si substrate are much stronger than that on the G-SiO_2/Si substrate, which was attributed to the coupled surface plasmon resonance (SPR) absorption of AuNPs on the graphene film.[38−40] All these results suggested the enhancement was from not only AuNPs but also graphene. For further investigation of the SERS of other common Raman reporter molecules on this substrate, a drop of ethanol solution of ROX or R6G was added onto the surface of the SiO_2/Si, G-SiO_2/Si, and Au-G-SiO_2/Si substrate; the intensity of these selected peaks of the dye on Au-G-SiO_2/Si substrate is a huge enhancement compared with G-SiO_2/Si and SiO_2/Si substrate, which was similar with Cy3 dye (Figure S3 and Table S1, Supporting Information). Therefore, these results demonstrated the ability of Au-G-SiO_2/Si substrate by combination of AuNPs and graphene to enhance the Raman signals of dyes.

Having established that Au-G-SiO_2/Si is a SERS-active substrate, we employed it for multiplex DNA detection by immobilizing thiolated DNA on AuNPs. Scheme 1b schematically shows the principle for DNA sensing with this novel SERS substrate. The AuNPs on the SERS-active substrate were first modified with the thiolated capture DNA (C1) as the previously reported method.[41] Then the DNA-functionalized Au-G-SiO_2/Si substrate was incubated with a target DNA (T1) followed by reporter DNA labeled with Cy3 (R1). The resulting sandwich complex of capture/target/reporter DNA was used for SERS detection. The equal amounts of phosphate buffer and noncDNA (NC-DNA) at the same concentration were employed as the blank control and the negative control (Scheme 1b).

As shown in Figure 3a, strong SERS signals from Cy3-labeled DNA were observed only when the complementary target DNA was added while there are no obvious signals in the blank control and negative control (NC-DNA) experiments, indicating the high specificity of the SERS-based DNA sensor. It is worthwhile to point out that the length of the DNA sequence is shorter compared with the size of AuNPs and the dye in general is not absorbed efficiently on the AuNPs;[10] most of the Cy3 dye was away from the substrate. The Raman enhancement obtained here is mainly attributed to the long-range effect of electromagnetic enhancement on the AuNP decorated graphene substrate.[31,42] The experimental SERS enhancement factor (EF) of the Au-G-SiO_2/Si substrate with respect to the SiO_2/Si substrate is evaluated to be 1.8×10^4 (see more details in Supporting Information) which is in the order of electromagnetic enhancement.[31] However, some Cy3 dyes were probably close to the surface of the graphene film; the CM of graphene may also contribute to the overall enhancement.[9]

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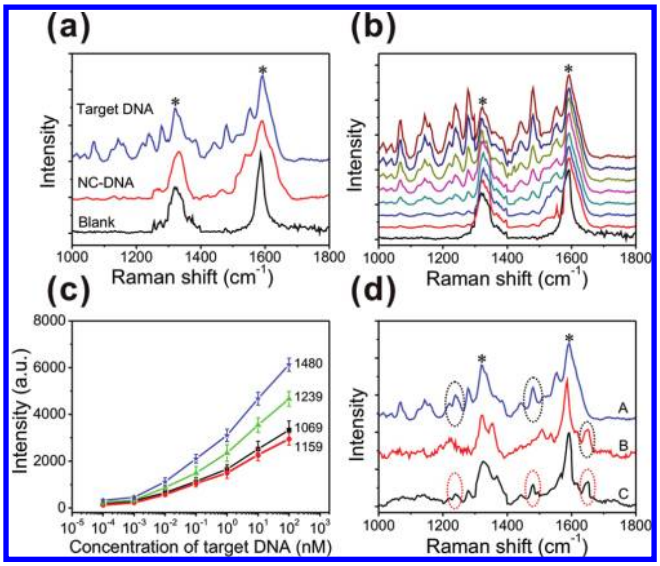

Figure 3. (a) SERS spectra obtained in the absence of target DNA (black) and in the presence of 1 nM noncDNA (NC-DNA, red) and complementary target DNA (blue). (b) SERS spectra obtained in the presence of different concentration of target DNAs (the concentrations from bottom to top are 0, 10^{-4}, 10^{-3}.10^{-2}, 10^{-1}, 1, 10, and 100 nM). (c) The calibration curve for the SERS intensity versus target DNA concentration at different Raman peaks (red for 1159 cm^{-1}, black for 1069 cm^{-1}, green for 1239 cm^{-1}, and blue for 1480 cm^{-1}). The data were measured from at least three different locations of the substrate. (d) SERS spectra obtained from different locations (A, B, and C) which are marked in Scheme 1c. The circled peaks represent the peaks which will be monitored to identify the specific sequence within the multiplex: A (blue line) Cy3, 1239 cm^{-1} and 1480 cm^{-1}. B (red line) TAMRA, 1649 cm^{-1}; all these peaks are marked using a red circle in the mixture spectra of C (black line).The peaks marked by the star (∗) are the D-band and G-band of graphene.

Evaluation of quantitative DNA detection was further performed by measuring the SERS intensity of Cy3-labeled DNA with the variation of target DNA concentration. As shown in Figure 3b, the SERS intensity was decreased with the descent of the concentration of DNA. No obvious intensity could be measured when the concentration of target DNA is reduced to 1 pM. The SERS intensity versus target DNA concentration is plotted as a dose−response curve at selected peaks (1069, 1159, 1239, and 1480 cm^{-1}) (Figure 3c).[35,36,43] It is obvious that the SERS signals could be observed at a target DNA concentration as low as 10 pM, which corresponds to 200 amol of DNA molecules in 20 μL of samples. Thus, the limit of detection of our SERS-based DNA sensor is 10 pM.

To prove our SERS sensor can be served as a platform for multiplex DNA detection, the AuNPs at different locations (labeled as A, B, and C, Scheme 1c) of the same graphene substrate were modified with different capture DNA (C1 for A, C2 for B, and C1&C2 for C, 1 μM). Because the hydrophobic property of the graphene film demonstrated by the contact angle (CA) measurement of water droplet on it (see Supporting Information for experimental details and CA data), the samples dropped onto the different locations were not mixed with each other. This made it possible to assemble different capture DNA at selected locations. Then the DNA-functionalized Au-G-SiO_2/Si substrate was incubated with mixed solution of two target DNAs (T1 and T2, 1 nM). The targets are segments of sequences for the hepatitis A virus Vall7 polyprotein gene (HVA) and hepatitis B virus surface antigen gene (HVB),[44] followed by a mixed solution of two reporter DNAs (R1 and R2, 100 nM) labeled with Cy3 and TAMRA, respectively (Scheme 1c).

As shown in Figure 3d, because of specificity of the DNA sequence, the C1 (C2) only hybridized with T1 (T2) and R1 (R2). No cross hybridization between different target DNA is observed, which is demonstrated in the SERS spectra obtained from locations of A and B (blue and red line, respectively, Figure 3d). When the AuNPs were modified with two capture DNAs (C1&C2, location C), the SERS spectra obtained from the location C was the mixture spectra of these two dye-labeled DNAs which were monitored from peaks at 1239 cm^{-1}, 1480 cm^{-1} (Cy3), and 1649 cm^{-1} (TAMRA).[35] The resultant mixture SERS spectra demonstrated that two types of targets could be detected simultaneously on the same substrate using just only one excitation laser source (line C, Figure 3d). It is very important to detect multiple tumor suppressor genes in order to identify early phase cancers in asymptomatic individuals.[45] Simultaneous detection of multiple targets with our AuNP decorated graphene-based SERS-active platform brings about new opportunities for molecular diagnostics.[46,47]

CONCLUSION

In summary, we have demonstrated the AuNP formation on graphene film. The gold-decorated graphene could serve as a high-efficiency SERS-active substrate for specific, sensitive, and multiplex DNA detection. The graphene-based SERS sensor for DNA detection has several advantages. First, the preparation of the SERS substrate is simple and convenient and has low cost. Second, exploring the electromagnetic enhancement of AuNPs in situ grown on the graphene film, dye labeled DNAs could easily assemble onto the substrate and the SERS intensity on the Au-G-SiO_2/Si substrate could be significantly enhanced. Third, due to the large planar surface of CVD-grown graphene film and its unique properties, it is possible to detect simultaneously many more types of target DNAs on the same substrate with single-laser excitation. Therefore, we expect the AuNP decorated graphene-based SERS platform will become a practical and powerful tool for molecular diagnostics.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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