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## DNA methylation detection with end-to-end nanorod assembly-enhanced surface plasmon resonance†

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**The Au nanorod (AuNR) assembly-enhanced surface plasmon resonance (SPR) system coupling with polymerization and nicking reactions was developed for amplified detection of DNA methylation and adenine methylation (Dam) methyltransferase (MTase) activity assay. The biosensor showed a good linear relationship between the SPR angle shift and the Dam MTase concentration over a range from 0.5 to 120 U mL<sup>-1</sup>, with a detection limit of 0.2 U mL<sup>-1</sup>. This study provided a sensitive platform to screen inhibitors for Dam MTase with great potential to be further applied in early clinical diagnosis.**

Surface plasmon resonance (SPR) sensors are powerful tools for label-free, real-time analysis of interfacial behaviors of various biological analytes and their interactions.<sup>1</sup> Their unique abilities for characterizing and quantifying low molecular weight molecules are routinely used in a wide variety of research fields, such as theranostics, clinical diagnosis, food safety and environmental monitoring.<sup>2</sup> With the growing availability of SPR instruments, it is also gaining popularity for sensing applications and has been employed for the detection of cells,<sup>3</sup> proteins,<sup>4</sup> and nucleic acids.<sup>5</sup> However, the use of the SPR method is impeded by the fact that the changes in the refractive index are often small, which has impeded further application of SPR in the detection of trace targets in complex biological samples. Different methods to enhance the sensitivities in SPR analyses have been explored,<sup>6</sup> including metal NPs,<sup>7</sup> graphene,<sup>8</sup> and dendrimers<sup>9</sup> as labels to enhance the refractive index change. The metallic NPs, especially gold nanoparticles (AuNPs), have been used as labels for SPR amplification, which originated from the coupling between the

localized surface plasmon of the NPs and the surface plasmon wave.<sup>1a</sup>

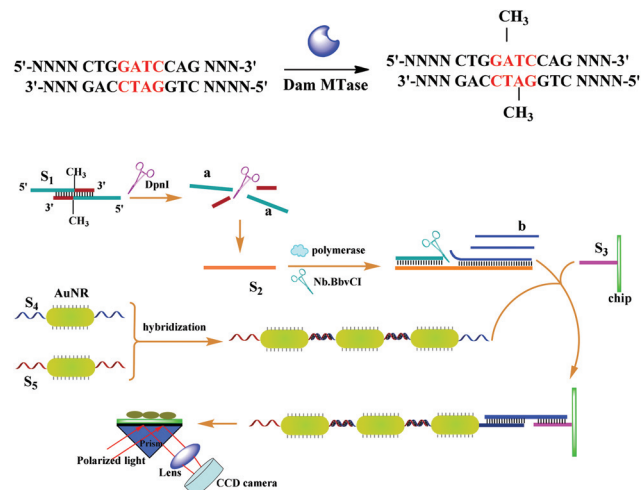
The sensitive and selective detection of biomarkers has great potential in biological studies, since it is essentially important for early diagnosis of tumors. A number of amplification strategies have attracted considerable attention, because of their striking improvement in the detection sensitivity.<sup>10</sup> In our previous work, we demonstrated a series of amplification methods, such as strand-displacement amplification (SDA), rolling-circle amplification (RCA), RNA ribozyme cyclic amplification, and the bio-bar-code technique.<sup>11</sup> Recently, we developed a SPR detection system based on a hybridization chain reaction (HCR) for amplified detection of DNA and small molecules.<sup>12</sup> In addition, there are some reports on the combination of enzyme cyclic signal enhancement methods with SPR detection, in which AuNPs were used as SPR labels.<sup>13</sup> However, a SPR detection system combining nanoparticle assemblies with a cyclic amplification strategy has not yet been reported.

DNA methylation is an important biological activity related to various pathogenic mechanisms of humans, such as some cardiovascular diseases and carcinomas.<sup>14</sup> Hence, detecting the DNA aberrant methylation level and the activity of methyltransferase (MTase) is imperative for rapid epigenetic evaluation and early cancer diagnosis. In the present work, an Au nanorod (AuNR) assembly-enhanced SPR system is developed for amplified detection of DNA methylation and adenine methylation (Dam) MTase activity assay. The palindromic duplex DNA probes were firstly methylated by DNA Dam MTase and then cut by methylation-sensitive restriction endonuclease Dpn I. The released sequence worked as a primer to induce polymerization and nicking reactions, producing a large amount of linker sequences. Subsequently, end-to-end (ETE) AuNR assemblies are linearly and periodically assembled to the Au chip through the specific interactions between capture probes and linker sequences. The enhancement of the SPR signal was achieved by increasing the refractive index of the surface, due to the high molecular weight of AuNR assemblies.

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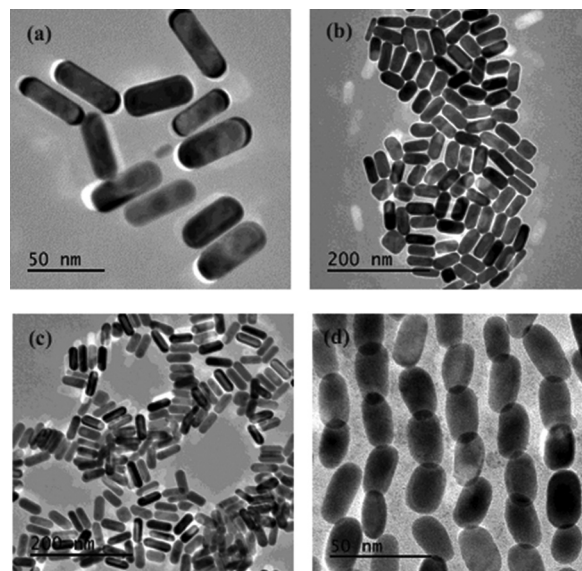


**Scheme 1** Schematic illustration of DNA methylation detection based on ETE AuNR assembly-enhanced SPR.

The configuration and operation principle of the DNA methylation detection and the Dam MTase activity assay by AuNR assembly-enhanced SPR are depicted in Scheme 1. The double-stranded DNA (dsDNA) probe was formed by the self-hybridization of  $S_1$  with a palindromic sequence of 5'-GATC-3', which was specifically recognized by Dam MTase and Dpn I. In the absence of Dam MTase, the reaction of DNA methylation has not been initiated, the dsDNA probes remaining intact. In the presence of Dam MTase, the palindromic sequence of 5'-GATC-3' was catalyzed to a methylated probe (5'-GAmTC-3') by Dam MTase with the help of *S*-adenosyl-L-methionine (SAM).<sup>15</sup> Then, this methylated dsDNA probe was cleaved into two parts by the methylation-sensitive restriction endonuclease Dpn I. The released part hybridized with the sequence  $S_2$ , which acted as a template. In the presence of polymerase and the nucleotide mixture (dNTPs), the polymerase-induced reaction replicated the template and yielded the double-stranded domain that associates Nb.BbvCI nicking endonuclease, resulting in the nicking of the replicated single strand. The cleaved single strand generated a new site for the initiation of replication.<sup>16</sup> Thus, a large number of sequences **b** were produced.

The solution containing **b** was introduced into the gold chip, on which the thiolated capture DNA was anchored *via* sulfur–gold affinity. Subsequently, AuNR assemblies were linearly and periodically assembled to the Au chip through the specific interactions between  $S_4$  and **b**. The enhancement of the SPR signal was achieved by increasing the refractive index of the surface, due to the high molecular weight of AuNR assemblies.

Fig. 1 shows the TEM images of the AuNRs in different forms. The free AuNRs have lengths and diameters of 50 nm and 18 nm, respectively, with an aspect ratio of 2.8 (Fig. 1a). TEM observation confirmed that the Au nanorods have uniform and well-defined structures, and this feature is well maintained in the DNA modified hybrid (Fig. 1b and c). Prefer-

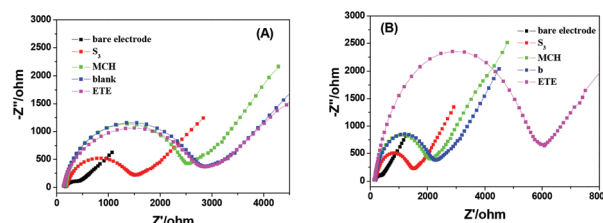


**Fig. 1** TEM images of the free AuNRs (a),  $S_4$ -AuNRs (b),  $S_5$ -AuNRs (c), and ETE AuNR assemblies (d).

ential binding of thiol-terminated primers to the end facets of the NRs allowed for the ETE growth mode for the NR chains (Fig. 1d).

UV-vis spectroscopy was employed to further characterize the assembly of the AuNRs. Gold nanorods are elongated nanoparticles whose extinction spectra exhibit two plasmon resonances, as shown in Fig. S1 in the ESI.† The transverse plasmon is the result of excitation across the nanorod diameter and is similar to plasmon resonances in spherical gold colloids. The longitudinal plasmon is due to excitation along the nanorod length. NR chains made from end-modified NRs can be approximated in terms of optical properties as nanowires (NWs).<sup>17</sup> The transverse plasmon for the ETE assembly changed very little, whereas the longitudinal peak underwent a red shift to 670 nm. Considering the geometry of the synthesized superstructure, one can consider their utilization as probes in surface-enhanced spectroscopy.

The fabrication process of the SPR aptasensor was characterized by electrochemical impedance spectroscopy (EIS), and the resulting voltammograms are displayed in Fig. 2. The bare



**Fig. 2** Nyquist diagrams for the electrochemical impedance measurements of the gold electrode at different modification stages. (A) shows the addition of blank and (B) shows the addition of sequence **b**.

gold electrode exhibited a very small semicircle domain with an electron transfer resistance ( $R_{\text{et}}$ ) of  $\sim 400 \Omega$ . After immobilization of the capture probe **S**<sub>3</sub> on the electrode, a big semicircle with a  $R_{\text{et}}$  of  $\sim 1.5 \text{ k}\Omega$  was obtained. 6-Mercapto-1-hexanol (MCH) treatment induced a further increase in the electron transfer impedance. The addition of a buffer solution and ETE probes produced no further impedance increase (Fig. 2A). Nevertheless, due to the introduction of sequence **b** and ETE probes, a significantly large impedance was obtained (Fig. 2B).

SPR was used to study the methylation process from the planar substrate, with the injection of the methylation reaction solution and the end-to-end AuNR assemblies into the fluidic channels separately, and a typical SPR response curve of this process is shown in Fig. S2 (ESI†). Compared with the bare Au substrate, the modification of **S**<sub>3</sub> induced the increase of the SPR angle due to the coverage of **S**<sub>3</sub> onto the Au chip. After the **S**<sub>3</sub> functionalized substrate was incubated with product **b** for 30 min, the SPR angle increased from 69.616 degrees to 69.632 degrees as a result of the hybridization process. Then the ETE AuNR probes were introduced into the flow cell, and the SPR angle further increased by 0.782 degree, corresponding to the loading of the ETE probes. In order to subtract the background signal such as the bulk refractive index change or the environmental noise, two fluidic channels were used by serially flowing the ETE solution into a reference channel (no Dam MTase added) and the analytical channel. As shown in Fig. S2,† a small binding signal in curve **a** was expected, might be due to the nonspecific adsorption of methylation product and the ETE probes. This background was subtracted in the following experiment.

In order to carry out the experiment in a short time, the methylation reaction solution and the ETE AuNR assemblies were mixed and injected at the same time, as described in the experimental section (see the ESI†). To demonstrate the necessity for the coexistence of Dam MTase and Dpn I, SPR measurement of the reaction was performed with the involvement of two enzymes. As shown in Fig. S3,† in the absence of either Dam MTase or Dpn I, the palindromic dsDNA probe cannot be cleaved and no signal primer was produced even though it is in the presence of the nicking enzyme. As a result, sequence **b** was not produced and no SPR signal was observed (blue and green lines). In contrast, in the presence of Dam MTase and Dpn I, the SPR angle increased with the reaction time in a sigmoidal fashion (red line).

To demonstrate the analytical performance of the proposed method, we measured the Dam MTase at various concentrations under the optimal conditions (see the ESI† for optimization). As shown in Fig. 3A, the SPR signal of the sensing system increased with the increasing Dam MTase concentration. This is in accordance with the fact that at higher concentrations of Dam MTase, more sequences **a** are released, producing more **b**. The SPR signal was proportional to the Dam MTase concentration from 0.5 to  $120 \text{ U mL}^{-1}$ . The regression equation is  $y = 0.0049x + 0.1527$  with  $R^2 = 0.9875$ , in which  $x$  is the concentration of Dam MTase and  $y$  is the SPR angular shifts. A detection limit of  $0.2 \text{ U mL}^{-1}$  is estimated for

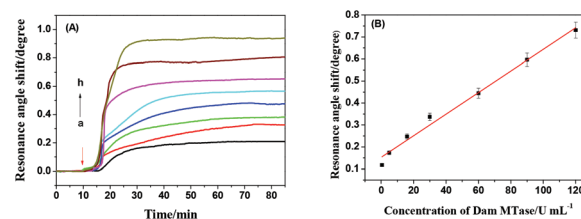


Fig. 3 (A) Real-time resonance angle responses of the biosensor for Dam MTase detection with ETE AuNR assembly probes. The concentration of Dam MTase from (a) to (h): 0, 0.5, 5.0, 15, 30, 60, 90, and  $120 \text{ U mL}^{-1}$ . (B) Calibration curve of the determination of Dam MTase with ETE AuNR assembly probes.

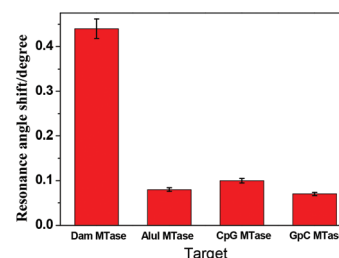


Fig. 4 Selectivity of the proposed method. The concentration of all DNA MTase is  $60 \text{ U mL}^{-1}$ . Error bars show the standard deviation of three experiments.

Dam MTase from the slope and the standard deviation of the zero level ( $3 \times \text{SD zero/slope}$ ), and is comparable to previously reported results with the lowest LOD of  $0.1 \text{ U mL}^{-1}$ .<sup>18</sup> The detection limit is 5 fold lower than that with the monomer AuNR as a probe (see the ESI†).

In order to evaluate the specificity of this assay, the SPR response of the sensing system to other DNA MTases such as AluI MTase, CpG MTase and GpC MTase was also investigated. All the results are displayed in Fig. 4. It was found that  $60 \text{ U mL}^{-1}$  Dam MTase could induce a significant SPR increase, whereas all other DNA MTases at the same concentration did not induce an obvious SPR enhancement (the signal for other DNA MTases was 16–23% of Dam MTase), demonstrating that our sensing system has a high specificity for Dam MTase.

To investigate the applicability of the method in the real biological environment, the detection of Dam MTase in the cytosol was performed. The HeLa cell cytosol spiked with different concentrations of Dam MTase was detected with our method. As presented in Table S2,† a series of five parallel measurements of samples yielded a relative standard deviation less than 8.2%, and recoveries were obtained with 93.4–112%. These results indicated that the proposed assay showed good applicability under practical conditions.

Dam MTase has become a promising target for antimicrobial drug development, since Dam MTase inhibitors have a broad antimicrobial action.<sup>19</sup> For testing the potential of the proposed system to evaluate and screen the inhibitor of Dam

MTase, four kinds of antibiotics and two kinds of anticancer drugs as model inhibitors had been chosen as models.<sup>18a</sup> As shown in Fig. S7 (ESI†), benzylpenicillin sodium, gentamycin sulfate, and fluorouracil could distinctly inhibit the methylation. The inhibition by 5-fluorouracil was the most serious one, which may decrease the activity of Dam MTase by about 46%. Nonetheless, the rest of the antibiotics and anticancer drugs had hardly any effect on the methylation.

In conclusion, we have demonstrated an SPR platform for sensitive DNA methylation detection and Dam MTase activity assay based on the cyclic amplification strategy of polymerization and nicking reactions and of SPR signal amplification with ETE AuNR assemblies. The Nb.BbvCI nicking endonuclease assisted cleavage process can produce a large number of cleaved **b** fragments with the initiation of a few methylation products, which then act as linkers to introduce the AuNR assemblies onto the Au chip. Furthermore, the AuNR assemblies functioned as a signal-enhancing scaffold, inducing significant enhancement of the SPR signals. The biosensor showed a good linear relationship between the SPR angle shift and the Dam MTase concentration and the LOD can reach 0.2 U mL<sup>-1</sup> level, thus it has great potential to be further applied in early clinical diagnosis.

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