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Gold nanostar based biosensor detects epigenetic alterations on promoter of real cells

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ABSTRACT

Epigenetic changes, particularly in cancer suppressor genes, are novel biomarkers for cancer diagnostics and therapeutics. However, epigenetic studies should not only provide an estimation of the amount of 5-methylcytosine, but also examine the presence of epigenetic proteins to reveal the complete epigenetic alterations for downstream molecular process. The challenge of natural epigenetics is to unveil key factors of epigenetics in one assay, containing low concentrations. This would be valuable for the monitoring of early-stage cancer. On the basic of the nanoplasmonic biosensor, here we report a sensitive sensor to study epigenetics of DNA promoter. The results show detection limit for dual epigenetic biomarkers methyl-CpG group and methyl-CpG binding domain protein 2 (MBD2) are one 5-methylcytosine molecule and 125 fM MBD2. Moreover, DNA structure bending, steric competition under interaction of epigenetic proteins and transcription factors; and epigenetics-mediated suppression of transcription are observed during epigenetic alterations. This study provides a platform for full story of epigenetics, as compared with that of methylcytosine-based techniques only.

1. Introduction

Nanoplasmonics-based refractometric platforms are facilitating end-point and kinetic monitoring as next-generation novel biosensors because of their extreme sensitivity to changes of the active refractive index (RI), inside the surface plasmon decay length (δ_d) (Brolo, 2012). The sensors bring together two exceptional features. The first is high sensitivity in end-point detection, which can be determined at very low concentrations. The second is monitoring of the biological phenomena in δ_d of plasmonic nanoparticles (Song et al., 2013). Localized surface plasmon resonance (LSPR) is a coherent oscillation of the surface conduction electrons excited by electromagnetic radiation at a nanoscale, which strongly depends on the RI sensitivity of the nanoparticles, changes in RI, δ_d, and binding thickness. Moreover, plasmons of gold nanoparticles, which are collective oscillations of their conduction electrons, do not blink or photobleach, in contrast to fluorescent dye molecules (Sonnichsen et al., 2005; Reinhard et al., 2005). The plasmon due to coupling mode is sensitive to the vicinity between the particles in a distance-dependent manner (Reinhard et al., 2007). The resonant plasmon wavelength can be red-shifted from blue regions into infrared regions due to a reduction in the inter-particle distance. In particular, the LSPR transducer response is very sensitive to changes closer to the surface, which is required for the measurement of biomolecular kinetics.

Epigenetic changes play important roles in abnormal activation and silencing of genes that have been associated with cancer (Egger et al., 2004), chromosomal instability-linked syndromes (Robertson, 2002) and mental retardation (Penagarikano et al., 2007). Recent reports demonstrated sensor-based detection of methylation on single stranded DNA (ssDNA) (Wang et al., 2012; Hu and Zhang., 2012; Stains et al., 2006). However, real epigenetic change is a process that occurs on methylated double stranded (dsDNA), with the participation of epigenetic proteins directly influencing the transcription of a gene, in particular, in the promoter regions. Previous reports revealed that peptide nucleic acid (PNA) molecules hybridize and form stable complexes with dsDNA (Nielsen et al., 1991; De et al., 2013; Nguyen and Sim, 2014). Therefore, using PNA molecules to capture dsDNA is an applicable approach to then examine the number of 5-methylcytosine (methyl-CpG) sites, and to unveil epigenetics.

Here, we present a nanoplasmonic biosensor which was fabricated using PNA-functionalized gold nanostars (AuNSs) for epigenetic study of the p53 promoter. The sensor demonstrated the following results: a limit of detection of one methyl-CpG and 125 fM MBD2 for real measurement of epigenetic interactions; evidence of the bending of p53 structure and steric competition under interaction of transcription factors and epigenetic proteins; lower transcription of the epigenetically-mediated transcription of the p53 promoter. These results allowed to measure dual complementary

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biomarkers at low concentrations, eliminates the necessity of chemical modifications, and permits direct monitoring of the epigenetics on a specific gene as pivotal biomarkers for the early stages of epigenetics-driven cancer. The sensor can also be applied to the monitoring of drug development for epigenetic therapeutics, diagnostics for epigenetic changes of circulating tumor DNA.

2. Materials and methods

2.1. End-point detection for intimately associated dual biomarkers of epigenetics

Fabrication of the sensor was described in the supplementary method S2 (see Supplementary materials). A hybridization buffer (perfectHyb^{TM}, Sigma) containing the p53 promoters (300 ng) was injected into the fabricated biosensor chamber and incubated for 2 h at 52 °C, pH 5.4, to form a stable complex. The PNA probes complementarily hybridized to methylated p53, following the Watson–Crick Model (Nielsen et al., 1991). The temperature was set at 52 °C and pH at 5.4 to achieve optimal hybridization and minimize nonspecific binding (Supplementary Fig. S1). The temperature was then raised to 64 °C and the NaCl concentration was set to 100 mM to eliminate nonspecific binding. The conjugation of PNA on OEG_6COOH-functionalized AuNSs via EDC/NHS coupling was verified using FT-IR spectroscopy, and electrophoresis confirmed the presence of PNA-p53 promoter complexes on the AuNSs (Supplementary Fig. S2). For estimation of the detection limit of the LSPR-peak shift, a range of recombinant MBD2 concentrations (125×10^{-6} fM to 125×10^{6} fM) (New England Biolabs) were used. A range of methyl-CpG sites (1–4 sites) on the p53 promoter was used to measure the saturation of LSPR-peak shift. To check for nonspecific binding, the specificity was challenged corresponding with two characteristics: specificity for the p53 promoter, and specificity for methyl-CpG binding. For the p53 promoter, its PNA probes were used with several DNA sequences (Supplementary Table S1); for methyl-CpG binding, MeCP2, lysosome and p53 proteins were incubated with the mp53 promoter under the same conditions.

2.2. Characterization of bending of the methylated p53 promoter and MBD2-mediated steric competition

Plasmonic coupling was applied to measure the bending of the p53 promoters under epigenetic conditions. 213 bp forward strands (F) (100 nM) and 213 bp reverse strands (100 nM) of the p53 promoter (R) with methylation (mp53 promoter) and without methylation (p53 promoter) were bound with 45-nm AuNSs. After immobilization of F-AuNSs, the R-AuNSs were injected into the chamber and incubated in hybridization buffer. The inter-particle distance was about 72.4 nm (213 bp, 3.4 nm for 10 bp), which is outside the plasmon band (Brolo, 2012). 200 µl of HeLa nuclear extracts (HeLaScribe®, Promega) were injected into the chamber and incubated at 37 °C for 1 h. Bending of the p53 promoters induced by transcription factors was monitored in the plasmonic coupling mode, based on inter-particle distance, plasmon-coupled mode occurring as collective excitation between the nanoparticles. The adsorption and coupling mode phenomena were different from the peak shape of the LSPR spectra (Reinhard, et al., 2005).

In the experiment for MBD2-mediated steric competition, 300 ng PNA probes capturing methylated fragments transcription binding sites and 125 fM MBD2 were injected into the biosensor chamber, and then incubated for 2 h at 37 °C. 100 pM of each of the TFs was subsequently injected and incubated for 2 more hours. To determine the steric competition, the LSPR values of each binding were recorded. The binding to the surface after each step was washed by using the regeneration buffer (pH 5.4/ PBS 0.01% SDS solution). To check MBD2 saturation on the sensor surface, the surface was treated with 100 pM of anti-MBD2 antibody after incubation with MBD2.

2.3. Epigenetics-based suppression of transcription

The sensor was used to identify epigenetics-mediated transcription of the mp53, p53, He-p53, and Hk-p53 promoters. The p53 promoter sequences contained 243 nucleotides, including the region of promoter basic activity (213 bp) and an extended part of exon 1 (from +1 to+30) for the transcription. The status of genomic methylation of HeLa and HEK293 cell lines was examined by Imprint® Methylated DNA Quantification (Sigma-Aldrich) (Supplementary Fig. S3A). The nuclear extracts were divided into two parts, with the MBD2 molecules in the second part eliminated (e.MBD2) by an anti-MBD2 antibody affinity column (Supplementary Fig. S3B). For measuring epigenetics-mediated transcription, two conditions were measured: (i) MBD2-free conditions and (ii) MBD2-dependent conditions. In (i), the p53 promoters on the sensor surface were overlaid with 200 µl of MBD2-free HeLa nuclear extracts (190 µl HeLa nuclear extract in 1 × transcription buffer, 10 µl 25 mM $MgCl_2$) and incubated for 2 min before the addition of 1x ribonucleotides mixture (0.4 mM rATP, 0.4 mM rCTP, 0.4 mM rUTP, 16 µM rGTP) in 1 × transcription buffer. When the ribonucleotide solution reached the sensor, the LSPR spectra were immediately measured with exposure time 0.2 s at 40 °C. For MBD2-dependent measurements, the same procedure was conducted in the presence of MBD2 molecules. The transcription reaction was stopped after 20 min, and the RNA products in the solution were alternatively measured with a Qubit RNA assay kit (Invitrogen, USA) and a spectrofluorophotometer (Shimadzu, Japan).

3. Results and discussion

3.1. Sensor fabrication and data analysis

The sensor was fabricated based on the refractive index sensitivity of AuNSs. The plasmon resonances of AuNSs were reported extreme sensitive to the local dielectric environment at single structure compared with that of other shapes (Rodríguez–Lorenzo et al., 2012; Nehl et al., 2006). For sensor fabrication (Fig. 1A), AuNSs (45 nm) were synthesized from a surfactant-stabilized seed (Nehl et al., 2006) (Fig. 2A), immobilized on a glass support, and functionalized with PNA as the bioreceptor. Each AuNS plays the role of a biosensor element. AuNSs were functionalized with PNA to catch the region of basic promoter activity of p53 promoter (NCBI J04238), for identification of methylation on a 213-bp sequence, and to analyze the native p53 basic promoter activity region, for revealing epigenetics. First, binding of MBD2 into the methyl-CpG sites was assessed and subsequent analysis of the steric competition between MBD2 and transcription factors was performed by measuring the LSPR-peak shift due to RI changes. Second, the stiffness of mp53 promoters was monitored by analyzing the structural bending of the p53 promoter with an *in vitro* transcription system (HeLaScribe®, Promega). Bending of the structure due to the transcription factors was detected by plasmon coupling mode between two nanoparticles on the sensor platform (Fig. 1A (ii and iii)). Plasmon shift of a coupled pair of dimers depends on the inter-particle distance in the nuclear extract medium (RI 1.6) (Sonnichsen et al., 2005; Reinhard et al., 2005). Third, epigenetically-mediated suppression of transcription was monitored by measurement of the changes of local RI in real time, within δd (Fig. 1A (i) (green arrow)). Dark-field microscope showed plasmon scattering in end-point detection, DNA stiffness and epigenetics-mediated transcription (Fig. 1B).