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Sensitive detection of point mutation using exponential strand displacement amplification-based surface enhanced Raman spectroscopy

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ABSTRACT

Accurate identification of point mutation is particularly imperative in the field of biomedical research and clinical diagnosis. Here, we develop a sensitive and specific method for point mutation assay using exponential strand displacement amplification (SDA)-based surface enhanced Raman spectroscopy (SERS). In this method, a discriminating probe and a hairpin probe are designed to specifically recognize the sequence of human K-ras gene. In the presence of K-ras mutant target (C→T), the 3′-terminal of discriminating probe and the 5′-terminal of hairpin probe can be ligated to form a SDA template. Subsequently, the 3′-terminal of hairpin probe can function as a primer to initiate the SDA reaction, producing a large amount of triggers. The resultant triggers can further hybridize with the discriminating probes to initiate new rounds of SDA reaction, leading to an exponential amplification reaction. With the addition of capture probe-modified gold nanoparticles (AuNPs) and the Rox-labeled reporter probes, the amplified triggers can be assembled on the surface of AuNPs through the formation of sandwich hybrids of capture probe–trigger–reporter probe, generating a strong Raman signal. While in the presence of K-ras wild-type target (C), neither ligation nor SDA reaction can be initiated and no Raman signal is observed. The proposed method exhibits high sensitivity with a detection limit of 1.4 pM and can accurately discriminate as low as 1% variant frequency from the mixture of mutant target and wild-type target. Importantly, this method can be further applied to analyze the mutant target in the spiked HEK293T cell lysate, holding great potential for genetic analysis and disease prognosis.

1. Introduction

Point mutation represents a kind of general alterations in the human genome, and it may influence the function of encoded proteins through the transcription and translation processes (Bertina et al., 1994; Halushka et al., 1999; Syvanen, 2001). Recent researches demonstrate that many human diseases including cancer are associated with the point mutation in the particular genes such as single-base substitutions, deletions and insertions (Pharoah et al., 2004; Whibley et al., 2009). The K-ras gene mutations have been observed in many human tumors including lung cancer (Guo et al., 2013), gastric carcinomas (Wu et al., 2004), pancreatic adenocarcinoma (Ebert et al., 2001) and colorectal cancer (Khanna et al., 1999). Moreover, different types of point mutation can affect how human responds to pathogens, chemicals, vaccines and other agents (Evans and Relling, 1999). Therefore, the point mutation assay plays an increasingly important role in the medical diagnosis and the clinical therapy.

So far, a variety of methods have been developed for the point mutation assay, such as ligase-mediated detection (Landegren et al., 1988; Huang et al., 2009), primer extension-based method (Sokolov, 1990; Litos et al., 2007), flap endonuclease-based cleavage method (Lyamichev et al., 1999; Chen et al., 2005) and polymerase chain reaction (PCR)-based method (Hacia et al., 1998; Fujii et al., 2000; Germer et al., 2000). Among these approaches, PCR-based method dominates the field of point mutation analysis owing to its high amplification efficiency. However, PCR might introduce the false positivity from the nonspecific amplification due to the involvement of complicated thermal cycling steps (Su et al., 2010). Recently, various isothermal amplification reactions have gained increasing attention, among which the strand displacement amplification (SDA) is a representative isothermal technology because of its good specificity and simplicity (Van Ness et al., 2003; Tan et al., 2005; Wang et al., 2011). The SDA method can generate abundant target sequence of interest with the assistance of polymerase and endonuclease, and has been widely

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applied for sensitive detection of nucleic acids, proteins, and small molecules (Zhu et al., 2013; Zhang and Zhang, 2012; Li et al., 2008; Ye et al., 2014).

Herein, we develop a new method for sensitive detection of point mutation using exponential SDA-based surface enhanced Raman spectroscopy (SERS). SERS is an emerging and powerful optical technology that can provide a nondestructive and ultrasensitive detection even down to single molecule level (Kneipp et al., 1997; Nie and Emory, 1997; Li et al., 2010). SERS has distinct characteristics of significant signal enhancement, narrow band widths and alleviated photobleaching (Ni et al., 1999; Grubisha et al., 2003; Zhu et al., 2011). Although some SERS-based methods have been reported for point mutation assay (Mahajan et al., 2008; Huh et al., 2009), they usually involve complex chemical modification and thermal cycling with poor sensitivity. In our proposed method, the combination of exponential SDA reaction with SERS provides a simple platform for sensitive detection of point mutation in an isothermal condition. This method can detect human K-ras gene point mutation with a detection limit of 1.4 pM and a large dynamic range of 4 orders of magnitude, and it can even distinguish as low as 1% variant frequency from the mixture of mutant target and wild-type one. Moreover, the proposed method can be applied to analyze the mutant target in the spiked HEK293T cell lysate.

2. Experimental section

2.1. Materials and reagents

The oligonucleotides (Table 1) were synthesized and purified by Sangon Biotechnology Co. Ltd. (Shanghai, China). The *Escherichia coli* DNA ligase, the Klenow fragment polymerase, the nicking enzyme of Nt.BbVCI and the deoxynucleotide triphosphates (dNTPs) were purchased from New England Biolabs (Beverly, MA, USA). SYBR Gold was obtained from Xiamen Bio-Vision Biotechnology (Xiamen, China). All other reagents were of analytical grade.

2.2. Preparation of capture probe-modified AuNPs

Gold nanoparticles (AuNPs) were prepared by citrate reduction of chloroauric acid following the reported protocol (Brown et al., 2000; Hu and Zhang, 2010). The maximum absorbance wavelength of AuNPs locates at 526 nm, and the concentration of AuNPs is estimated to be about 1.4 nM by assuming the reduction of all Au^{3+} to Au^0 and the particle size of 25 ± 4 nm (see Fig. S1). The capture probe-modified AuNPs were prepared according to a published protocol with minor modifications (Hurst et al., 2006). Firstly, 1 OD (6.1 nmol) capture probe was added to 1 mL of AuNP solution (9.1 nM), and incubated at room temperature for 16 h. Then the concentration of phosphate (NaH_2PO_4/Na_2HPO_4) was adjusted to 10 mM by adding 0.2 M phosphate buffer (pH 7.0), and the concentration of NaCl was adjusted to 0.1 M, followed by standing for 40 h. Lastly, the AuNPs were centrifuged three times to remove the excess capture probes, then resuspended in 234 μL of PBS buffer (10 mM phosphate, 0.1 M NaCl, pH 7.0) and stored at 4 °C before use. In this capture probe-modified AuNPs solution, the concentration of AuNPs was estimated to be 38.9 nM, and the DNA concentration was estimated to be 19.1 μM (see Supplementary information).

Table 1
Sequences of the targets and the probes.[a]

Note	Sequence (5′–3′)
Mutant target	AAG GCA CTC TTG CCT ACG CCA TCA GCT CCA ACT ACC ACA AGT TT
Wild-type target	AAG GCA CTC TTG CCT ACG CCA CCA GCT CCA ACT ACC ACA AGT TT
Discriminating probe	CTT GAC TAG CTA CGA *GCT GAG G*CT TGA CTA GCT ACG AGC TGA AGG TAG TTG GAG CTG A
Hairpin probe	PO_4-TGG CGT AGG CAA GAG ACA TCG GCC TTT TTT TTT TGG CCG ATG
Capture probe	SH-$(T)_9$CTT GAC TAG C
Reporter probe	TAC GAG CTG A-Rox
Synthesized trigger	TCA GCT CGT AGC TAG TCA AG

[a] The underlined letters in the mutant target and the wild-type target indicate the point mutation. The italic region in the discriminating probe indicates the recognition sequence of Nt.BbvCI. The Rox in the reporter probe indicates the carboxy-X-rhodamine.

2.3. Ligation and SDA reaction

The ligation reaction was performed in 10 μL of reaction mixture containing 1 × ligase buffer (30 mM Tris–HCl, 4 mM $MgCl_2$, 1 mM DTT, 26 μM NAD^+, 50 μg/mL BSA, pH 8.0), 10 nM discriminating probe, 10 nM hairpin probe, 0.2 U/μL ligase and different concentrations of targets, and incubated at 16 °C for 1 h. After the ligation reaction, the SDA reaction was performed in 10 μL of reaction mixture containing 1 μL of ligation products, 0.75 μL of discriminating probe (1 μM), 0.4 μL of Nt.BbvCI (10 U/μL), 0.1 μL of polymerase (5 U/μL), 0.25 μL of dNTPs (10 mM), 1 μL of 10 × NEB buffer 2 (100 mM Tris–HCl, 500 mM NaCl, 100 mM $MgCl_2$, 10 mM dithiothreitol, pH 7.9), and 6.5 μL of H_2O at 37 °C for 30 min, followed by incubation at 80 °C for 20 min to inactivate the enzymes. The amplification products were kept at 4 °C for subsequent analysis.

2.4. Sandwich hybridization reaction and Raman measurement

The sandwich hybridization reaction was carried out in the solution containing 10 μL of amplification products, 5 μL of capture probe-modified AuNPs and 5 μL of Rox-labeled reporter probe (10 μM) at the room temperature for 2 h. The solution was then centrifuged at 8000 rpm for 20 min to remove the excess reporter probes. After the removal of the supernatant, the red precipitate was washed three times with 50 μL of 10 mM phosphate buffer (pH 7.0, 0.1 M NaCl) and resuspended in 2 μL of PBS buffer. The droplet was dripped on the silico pellet and air-dried at room temperature before the Raman measurement. The SERS spectra were measured by a LabRAM HR Raman spectrometer with a 632.8-nm laser (HORIBA Jobin-Yvon, France). The laser power at the sample location was 15.7 mW, and the resolution of SERS spectra over 900–1800 cm^{-1} was about 0.65 cm^{-1}. The collection time for each spectrum was 10 s.

2.5. Gel electrophoresis

The amplification products was analyzed using a 10% non-denaturating polyacrylamide gel electrophoresis (PAGE) in 1 × TBE buffer (9 mM Tris–HCl, pH 7.9, 9 mM boric acid, 0.2 mM EDTA) at a 100 V constant voltage for 50 min with SYBR Gold as the fluorescent indicator. The stained gel was scanned by a Kodak Image Station 4000 MM (Rochester, NY, USA). The band intensities in the gels were analyzed by *Quantity One* software.

2.6. Preparation of cell lysate

The HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were removed from the substrate by trypsinization, washed