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Isothermal and rapid detection of pathogenic microorganisms using a nano-rolling circle amplification-surface plasmon resonance biosensor

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ABSTRACT

Rolling circle amplification (RCA) of DNA is a sensitive and cost effective method for the rapid identification of pathogens without the need for sequencing. In this study, a surface plasmon resonance DNA biosensor based on RCA with a gold (Au) nanoparticle surface was established for isothermal identification of DNA. The probes included a specific padlock probe, a capture probe (CP), which is bound to biotin, and an Au nanoparticle-modified probe, which hybridizes with the RCA products. The CP was assembled on gold nanoparticles to increase its ability to bind and hybridize. The linear padlock probe, which was designed to circularize by ligation upon recognition of the bacterial pathogen-specific sequence in 16 S rDNA, hybridizes to fully complementary sequences within the CP. Upon recognition, each target gene DNA is distinguished by localization onto the corresponding channel on the chip surface. Then, the immobilized CPs act as primers to begin the in situ solid-phase RCA reaction, which produces long single-stranded DNA. The RCA products fixed on the chip surface cause significant surface plasmon resonance angle changes. We demonstrated that six different bacterial pathogens can be identified simultaneously and that 0.5 pM of synthetic oligonucleotides and 0.5 pg μl^{-1} of genomic DNA from clinical samples can be detected by this method with low background signals. Therefore, the multiplex diagnostic method provides a highly sensitive and specific approach for the rapid identification of positive samples.

1. Introduction

The accurate and fast detection and identification of pathogenic bacteria are increasingly important in clinical diagnostics. Traditional techniques used to identify pathogens involve the cultivation of bacteria, which is tedious and time-consuming (Bodrossy and Sessitsch, 2004; Eriksson et al., 2009). Cultivated pathogens are identified by biochemical testing or more advanced molecular methods like PCR, which can amplify pathogen-specific nucleic acid targets (Atkins and Clark, 2004; Cho and Brennan, 2007; Lopez et al., 2003; Mahony, 2008). These approaches are effective, but they target only a single pathogen per assay.

To improve the efficiency of detection, the development of a multiplex detection method for simultaneous identification of several pathogens is needed. Surface plasmon resonance (SPR) biosensors can enable highly parallel detection of diverse organisms. SPR is an optical detection technique that uses the reflection and refraction of light. For SPR biosensors, sample solutions that contain the target molecules are injected into a running buffer that flows continuously over the sensor surface with immobilized probes (Homola, 2008). Because of the high sensitivity of the optical device, the convenient operation and the real-time monitoring, SPR biosensors offer rapid, sensitive and on-site analysis (Cosnier and Mailley, 2008). In the past decade, SPR biosensors have been used for the detection of a wide variety of bimolecular interactions, including interactions between proteins, peptides, nucleic acids, bacteria and viruses (Chu et al., 2007; Mitchell and Lowe, 2009). For a SPR biosensor chip, the mass of the molecules bound to the surface of the gold film varies proportionally to the SPR angle. Target molecules bind capture probes (CPs) and change the SPR angle (Qavi et al., 2009). SPR biosensor technology has advanced from the detection of single analytes to the performance of high-throughput screening assays (Piliarik et al., 2009). In addition, short oligonucleotides have been directly detected by SPR biosensors at amounts in the femtomole range (Springer et al., 2010), or even lower if a more complex assay method is used (Okumura et al., 2005).

Recently, the superior utility of padlock probes (PLPs) (Landegren et al., 2003; Nilsson et al., 1994) has been demonstrated for the detection and amplification of sets of target nucleic

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acid sequences with high specificity and selectivity using a rolling circle amplification (RCA) mechanism (Fire and Xu, 1995; Liu et al., 1996). PLPs are long single-stranded oligonucleotide probes that contain three regions: the 5′ and 3′ ends of linear PLPs, which are designed to base pair adjacently on the target sequence; a unique sequence identifier, which binds the CP; and a universal primer binding site, which provides repeat sequences on the RCA product for Au nanoparticle-modified probe (AuNP-MP) binding. When properly hybridized adjacent to each other, the ends of PLPs can be enzymatically joined by DNA ligase. Thus, when both end segments recognize their target sequences correctly, PLPs form a circularly closed probe-target complex (Lizardi et al., 1998). Non-circularized PLPs can be removed by exonuclease treatment. The circularized PLPs can then be amplified by RCA, as observed for in vivo bacteriophage replication (Zhang et al., 2001). These circular PLPs can be amplified isothermally by DNA polymerase, which generates a long ssDNA molecule containing multiple repeats of the complementary sequence of the PLP that can reach up to several thousand-fold (Baner et al., 1998). Furthermore, the PLPs are capable of detecting single point mutations because of the necessity for precise base pairing (Nilsson et al., 1997). Consequently, the RCA technique has been applied successfully to the detection of different pathogens (Najafzadeh et al., 2011; Russell et al., 2014; Sun et al., 2011; Zhou et al., 2008).

Recently, there has been increasing interest in the use of Au nanoparticles (AuNPs) for the development of bioaffinity assays due to their unique physicochemical properties and high surface area (Manso et al., 2008). AuNPs allow stable immobilization of biomolecules due to their adhesion to metal surfaces via thiol groups (Contino et al., 2014). Notably, AuNPs can further enhance the signal amplification by RCA. AuNP-MPs that bind to RCA products strengthen the plasmon resonance of incident light excitation and improve the transduction of small refractive index changes on the chip surface, thereby increasing the sensitivity of SPR biosensors (Petryayeva and Krull, 2011). Because AuNPs serve as rapid, efficient and high-throughput platforms for detection (Su et al., 2010), they are used increasingly in SPR biosensors (Liu and Ye, 2013).

Here, we present a new strategy for multichannel SPR biosensor assay detection of multiple pathogens in parallel that has increased signal due to the use of Au nanoparticle capture probes (AuNP-CPs) and Au nanoparticle rolling circle amplification (AuNP-RCA). We designed and characterized PLPs to target specific 16S rDNA sequences of six selected bacterial species: *Escherichia coli*, *Shigella dysenteriae*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus pneumonia* and *Enterococcus faecalis*. The specificity and sensitivity of the multiplex assay were established for the detection of individual and mixed target pathogen DNAs. The circular PLP mixture solution, which was ligated with DNA ligase, was applied to the detection channels for hybridization with the CP, which were immobilized on AuNPs of the chip film via specific complementary sequences. Because each detection channel only had one specific CPs, the CPs hybridized with the complementary circular PLPs, which flowed into the multichannel. The other CPs were mutually used as control group, to ensure the specificity of detection. AuNP-CPs and AuNP-RCA were developed together to amplify the detection signal thrice and decrease the detection limit. The sensitivity and specificity of the SPR biosensor method were evaluated, and to verify the applicability of this approach, the method was applied to the detection of clinical samples. Our results characterize a highly sensitive and specific approach for the rapid identification of positive samples.

2. Materials and methods

2.1. Materials

Single-stranded oligonucleotides were synthesized and purified by Sangon Biotech (Shanghai, China). The sequences of these oligonucleotides are provided in Table 1. Streptavidin (SA), polyethylene glycol, dimethyl sulfoxide and mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), *E. coli* DNA ligase, QuickCut™ HpaI, QuickCut™ EcoRI, QuickCut™ HindIII, QuickCut™ BamHI, TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver.3.0, exonuclease I and exonuclease III were purchased from Takara (Dalian, China). Deoxyribonucleosides (dNTPs) and Phi29 DNA polymerase were purchased from Fermentas (USA). Tween 20 was purchased from Sangon Biotech (Shanghai, China). AuNPs, which were synthesized

Table 1
Sequences of oligonucleotides used in this work.

Oligonucleotide	Target species	Sequence (5′-3′)
PLPs[a]	*E. coli*	5′-pCTGTTACCGTTCGACTTGCATG*AGCACCAATAGTCTTCAGTTC***ACATCAAATGCCAC**G**TTAAC**AGTCAGGCCT**AGCAAGCTTCTTC**-3′
	E. faecalis	5′-pGCAAATCTCTTAAAGCTTCTCT*ACTGGTATGCTGGTTTCATCA***ACATCAAATGCCAC**G**TTAAC**AGTCAGGCCT**CCGCGAGGTCAT**-3′
	S. dysenteriae	5′-pCATTCTGATCCACGATTACTAGCGA*TGCTTCTTCGGTGCCCAT***ACATCAAATGCCAC**G**TTAAC**AGTCAGGCCT**ACGTATTCACCGTGA**-3′
	S. pneumoniae	5′-pGAGTTGCGAACGGGTGAGTAA*TGTATTATCGTGTCTGCGTG***ACATCAAATGCCAC**G**TTAAC**AGTCAGGCCT**TTGCTTCTCTGGAT**-3′
	S. epidermidis	5′-pGGTCAGAGGATGTCAAGATT*ATACTTCACGACTCACCTCA***ACATCAAATGCCAC**G**TTAAC**AGTCAGGCCT**TATCTCTAGAGG**-3′
	S. aureus	5′-pAGCAAGCTTCTCGTCCGTTCGC*AGGTCATCAGTCATTCAAGTCA***ACATCAAATGCCAC**G**TTAAC**AGTCAGGCCT**GCTAACATCAGAGA**-3′
CPs	*E. coli*	5′biotin-CCCCCCCCCC*GAACTGAAGACTATTGGTGCT*-3′
	E. faecalis	5′biotin-CCCCCCCCCC*TGATGAAACCAGCATACCAGT*-3′
	S. dysenteriae	5′biotin-CCCCCCCCCC*ATGGGCACCGAAGAAGCA*-3′
	S. pneumoniae	5′biotin-CCCCCCCCCC*CACGCAGACACGATAATACA*-3′
	S. epidermidis	5′biotin-CCCCCCCCCC*TGAGGTGAGTCGTGAAGTAT*-3′
	S. aureus	5′biotin-CCCCCCCCCC*TGACTTGAATGACTGATGACCT*-3′
Target sequences	*E. coli*	5′- AGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGAAGCTTGCTTCTTTGCTGA-3′
	E. faecalis	5′-ATCCGAACTGAGAGAAGCTTTAAGAGATTTGCATGACCTCGCGGTCTAGCGACT-3′
	S. dysenteriae	5′-GAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGTCACGGTGAATACGTTCCCGGGCCT-3′
	S. pneumoniae	5′-CAGGTTACCTACGCGTTACTCACCCGTTCGCAACTCATCCAGAGAAGCAAGCTCCTCCTTC-3′
	S. epidermidis	5′-AACCTTACCAAATCTTGACATCCTCTGACCCCTCTAGAGATAGAGTTTTCCC-3′
	S. aureus	5′-TGCAAGTCGAGCGAACGGACGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGG-3′
AuNP-MP		5′HS-$(CH_2)_6$-**ACATCAAATGCCACG**-3′

[a] Abbreviations: PLPs, padlock probes; CPs, capture probes; AuNP-MP, AU nanoparticle-modified probe. The sequences underlined in PLPs are complementary to the sequences underlined in the targets (T_1 and T_2 in Fig. 1A), while the sequences italicized in PLPs are complementary to the italicized sequences in CPs (S in Fig. 1A). The universal primer binding site (G in Fig. 1A) is indicated by bold font, and the HpaI digestion site (R in Fig. 1A) is indicated by shading. The "p" represents a phosphate at 5′ end in order to ligate the PLPs. CPs are modified with biotin at 5′ end for immobilization on the AuNPs of the gold film; the AuNP-MP is modified with a thiol group at the 5′ end for hybridization with the RCA products.

by the citrate reduction of $HAuCl_4$ solution and possess an average diameter of 15 nm or 30 nm, were purchased from JY Biotech (Shanghai, China). Phosphate buffered saline (PBS) (0.15 M NaCl, 10 mM phosphate, pH 7.4) was prepared in our laboratory. Other chemicals utilized were of analytical grade. High purity deionized water was used in all of the experiments.

2.2. Apparatus

The SPR biosensor detection system was described in detail in our previous manuscript (Xiang et al., 2013). The instrument consists of a polarized light detection system, a micro-flow control system, a temperature control system, a sample automatic loading chamber and an eight-channel series connection detection groove. The sensor chips consist of glass prism substrate upon which a 50-nm-thick Au film has been deposited.

2.3. Preparation of AuNP conjugates

To prepare AuNP conjugates, a solution of AuNPs was incubated with SA (20 μg mL^{-1}) with gentle shaking for an hour, which allows SA to conjugate to AuNPs via noncovalent absorption (Nam et al., 2003). Next, about 110 μL of 1% polyethylene glycol was added to the solution of AuNP–SA conjugates. The mixture was incubated at room temperature overnight. The mixture solution was centrifuged twice at 4 °C for 20 min (12,000 rpm). Finally, the precipitate was dissolved in PBS solution and stored at 4 °C (Yan et al., 2012).

10 μl of biotin-CP (100 μM) were added to the AuNP–SA conjugates (200 μl), and the mixture was incubated with gentle shaking for 30 min at room temperature. The mixture solution was then centrifuged thrice for 20 min at 4 °C (12,000 rpm), and the precipitate was dissolved in PBS solution. The AuNP–SA–biotin CP conjugates were stored at 4 °C (Yan et al., 2012).

AuNP-MPs were prepared by the thiol method. First, a solution of AuNPs was incubated with the probe, which was hybridized with the RCA products for 16 h. Second, the supernatant was replaced with 0.1 M NaCl and 10 mM phosphate buffer (pH 7), and the mixture was incubated at room temperature for 40 h. Third, the solution was centrifuged for at least 25 min at 14,000 rpm to remove the excess reagents. The precipitate was washed with 5 ml of a stock of 0.1 M NaCl, 10 mM phosphate buffer (pH 7) and was centrifuged for at least 25 min at 14,000 rpm. Finally, the precipitate was dissolved in 5 ml of a 0.3 M NaCl, 10 mM phosphate buffer (pH 7), which was stored at 4 °C (Huang et al., 2007).

2.4. Clinical samples and DNA extraction

Fifty clinical samples including sputum, urine and feces samples, which were obtained from Southwest Hospital (Chongqing, China) were cultured in plate medium at 37 °C overnight. Depending on the source of the specimen, we selected different culture media. For example, for the sputum samples, we selected four kinds of culture medium: blood agar, chocolate agar, MacConkey agar and Sabouraud agar. Because each specimen may contain several pathogens, each specimen was inoculated onto different plates, and the pathogens grew according to their media preferences. Colonies that were selected for each culture plate were harvested and diluted in 0.9% NaCl buffer to a concentration of 10^8 CFU ml^{-1} using McBurney turbidimetric tube colorimetry. Bacterial DNA was extracted from 1 ml according to the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver.3.0. The extracted DNA was dissolved in PBS solution to obtain the 5 ng μL^{-1} bacterial DNA.

2.5. Preparation of SPR biosensor chips

The CPs were immobilized on the surface of gold film according to the following methods: Secondary antibody in PBS at 150 mmol L^{-1} was added to the designated aldehyde-activated channels of the sensor chip. After incubating at 4 °C overnight, the channels were rinsed with washing solution (150 mmol L^{-1}, 0.25% Tween 20) and blocked with blocking solution (150 mmol L^{-1}, 5% BSA, pH 7.4) for 30 min. Finally, the six AuNP–SA–biotin CP conjugate solutions were added to the six respective channels on the same SPR sensor chip, and the chips were incubated at 37 °C for 30 min.

2.6. Ligation and exonuclease treatment

Bacterial genomic DNA was fragmented by digestion using QuickCut™ EcoRI, QuickCut™ HindIII, and QuickCut™ BamHI for 5 min and used as target in the indicated amounts (Szemes et al., 2005). The target sequences were hybridized with the PLPs (100 nM) in a 20 μL reaction system (30 mM Tris–HCl pH 7.8, 4 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 1.2 mM EDTA, and 0.1 mM NAD). The mixture was incubated at 95 °C for 5 min and then cooled down to 4 °C. Then, the mixture was incubated at 60 °C for 30 min, and 5 U of *E. coli* DNA ligase and 0.05% BSA were added. The mixture was incubated at 37 °C for 40 min, a then 10 U each of exonuclease I and exonuclease III were added at 37 °C for 30 min to the ligation mixture to remove the unligated PLPs and excess linear target strands (Yan et al., 2004). The 30 μL reaction (20 mM Tris–HCl, pH 9.0, 10 mM $MgCl_2$, and 2 mM DTT) was terminated by heating at 95 °C for 5 min. For multiplex detection, targets were ligated with a mixture of the six individual PLPs.

2.7. SPR biosensor detection

For analyzing the samples, a 20 μL total reaction volume containing 10 μL of ligation products (circular PLPs) and 10 μL of hybridization buffer (0.15 M NaCl, 0.3 M KCl, 10 mM phosphate, 0.005% Tween 20, pH 7.2) was delivered to the detection well, which was hybridized with the CPs for 30 min at 37 °C. The SPR angle changes were recorded in real-time. After the hybridization, the surface of the chip was flushed using PBS buffer to wash away unhybridized oligonucleotides on the chip surface and to clear the pipeline. Then, 20 μL of RCA reaction mixture (0.5 U μL^{-1} Phi29 DNA polymerase, 1 mM dNTPs, 0.2 μg μL^{-1} BSA, and 5% dimethylsulfoxide) was delivered to the detection channel. After 35 min, the mixture solution was washed with PBS buffer for about 2 min to terminate the RCA. Finally, 20 μL of AuNP-MPs with different concentrations was hybridized with the RCA products for 25 min (Xiang et al., 2013).

2.8. Regeneration of SPR biosensor chips

Upon the completion of the detection process, a 20 μL total reaction mixture containing 5 U of QuickCut™ HpaI was added for the digestion of products and regeneration of the CPs on the surface of the chip. Next, a series of regeneration buffers (20 μL pulses at 5 μL min^{-1}) was successively added: 10 mM acetate (pH 4.8), then 10 mM glycine (pH 3), and finally 10 mM glycine (pH 2) (Huang et al., 2007).

3. Results and discussion

3.1. Probe design

We sought to develop a sensitive and cost effective method for rapid identification of pathogens without the need for sequencing.

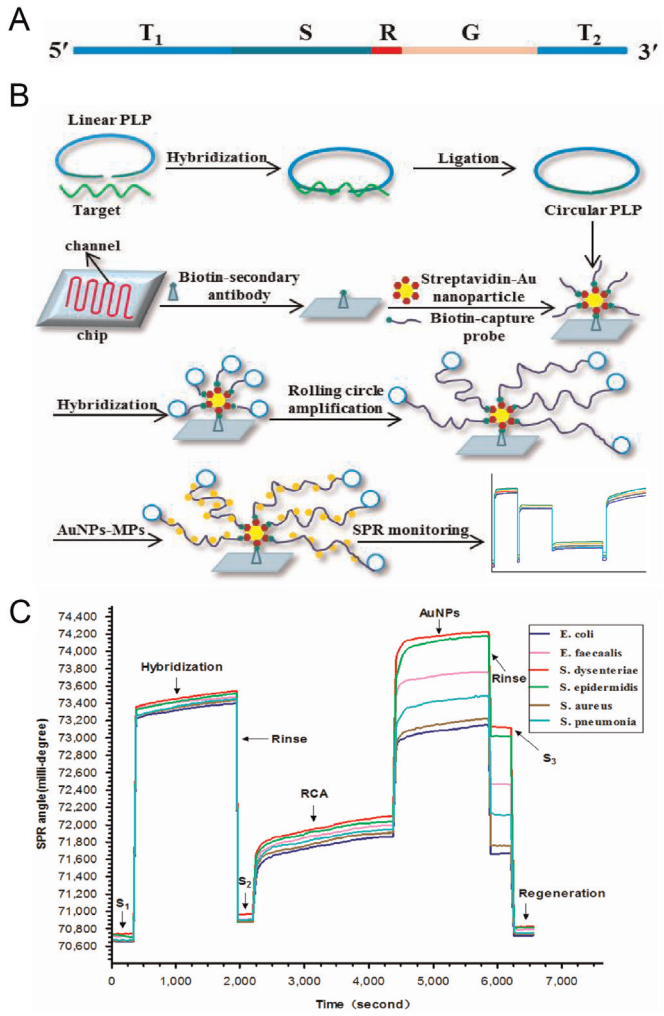

Fig. 1. AuNP-RCA-based SPR method. (A) The PLP design. The PLP contains target-complementary sequences at the 5′ and 3′ ends (T_1 and T_2); a sequence-specific region (S), which hybridizes with the CPs; a HpaI restriction endonuclease digestion sites (R) and a general region (G), which provides the repeat sequences on the RCA products for AuNP-MP binding. (B) Schematic representation of the proposed detection method. (C) Real-time detection of pathogenic microorganisms by SPR biosensor.

For simultaneous detection of pathogens, the high-discriminatory power of PLPs is extremely important. Related nucleic acid sequences for six different pathogens from GenBank were compared using Clustal X2, and detection sequences for the design of Padlock sections were identified. Each PLP was composed of a sequence-specific region that hybridizes with the CPs, a general region that binds a universal probe, a HpaI restriction endonuclease site and two target complementary sequences (target-specific sequences) located at the 5′ and 3′ arm regions (Fig. 1A). When the two arm regions of the PLP are perfectly hybridized with the complementary target sequences, the linear PLPs has the ability to be specifically ligated by *E. coli* DNA ligase (Long et al., 2011). The sequence-specific regions that hybridize with the CPs were designed with similar length (21 ± 1 base pairs) and melting temperature (Tm) (52 ± 2 °C), with care to avoid self-pairing, hairpin and dimer formation. For the efficient hybridization of the PLP to its target, we used the method of asymmetric design for two target-complementary sequences by lengthening the 5′ arm complementary sequence and shortening the 3′ arm complementary sequence (Szemes et al., 2005). According to this method, the Tm of the 5′ region of the PLP should be approximately 10 °C above the ligation temperature, while the Tm of the 3′ region should be below the ligation temperature. A hybridization temperature is selected that is above the Tms for both the 5′ and 3′ sequences (Zhang and Liu, 2003). To minimize secondary structures of the circular PLPs, we used MFold (http://mfold.rna.albany.edu/?q=DINAMelt/Two-state-folding) to predict the secondary structure. If necessary, we also adjusted the PLP end sequences to avoid strong secondary structures. Positioning a strongly destabilizing mismatch at the PLP 3′ region could ensure the specificity of detection (Luo et al., 1996). The CPs contained sequence complementary to the PLPs with an additional $(A)_{10}(C)_{10}$ space linker at the 5′end to improve the DNA hybridization and enhance the signal on the surface of the chip. The sequences of specific PLPs, CPs and target sequences are provided in Table 1.

3.2. The principle of the AuNP-RCA-based SPR biosensor for multiplex detection

The basic principle of the proposed method is presented schematically in Fig. 1B. For AuNP-RCA-based SPR biosensor detection, AuNP-RCA is initiated on the chip surface. First, biotin-secondary antibody is spotted and immobilized on the chip surface at room temperature. Non-immobilized antibody is removed with wash buffer, and the channel of the chip is blocked with blocking solution. After AuNP–SA conjugates are covalent bound with biotin-secondary antibody, the six biotin-CPs (recognizing the SA on the AuNPs) are respectively added to six different channels on the same SPR biosensor chip. The linear PLPs recognize the specific sequences on the DNA targets in solution and are ligated into circular PLPs, and then the circular PLPs hybridize directly with the CPs. Finally, the CPs act as primers to initiate the RCA reaction in situ with the addition of dNTPs and phi29 DNA polymerase. The RCA products are long ssDNAs. AuNP-MPs hybridize with each long ssDNA. Using the SPR biosensor, the amplification signal can be monitored online. By recording the angle change of the SPR biosensor, we can detect and identify the target sequences. Moreover, the amplified signal can be recorded continuously. Compared with linear PLPs connected on a chip surface, ligating in solution has better specificity and efficiency (Melin et al., 2008; Nallur et al., 2001). By using multiple PLPs targeting different sequences, biotin–SA and AuNPs can be used for multiplex signal amplification on the chip surface with significantly improved specificity and sensitivity. The SPR biosensor angle change of each step for monitoring multiplex detection is shown in Fig. 1C.

3.3. Optimization of the AuNP-RCA-based SPR biosensor experimental conditions

3.3.1. Effect of hybridization temperature of the PLPs

The success of the AuNP-RCA-based method mainly relies on the ligation reaction. However, the hybridization temperature of the PLPs is an important factor in the ligation reaction. To identify a hybridization temperature with high efficiency, a series of hybridization temperatures from 40 °C to 70 °C with an interval of 5 °C was evaluated using 10 nM target sequence and 100 nM PLP to achieve complete hybridization. As shown in Fig. 2A, the maximum SPR angle change was obtained at approximately 60 °C, while the control remained low with minimal change. Therefore, 60 °C was set as the hybridization temperature for all the subsequent experiments.

3.3.2. Effect of hybridization duration of circular PLPs and CPs

The time of hybridization of circular PLPs and CPs is an additional key factor in achieving higher amplification signals. To determine whether the amplification signal can be further

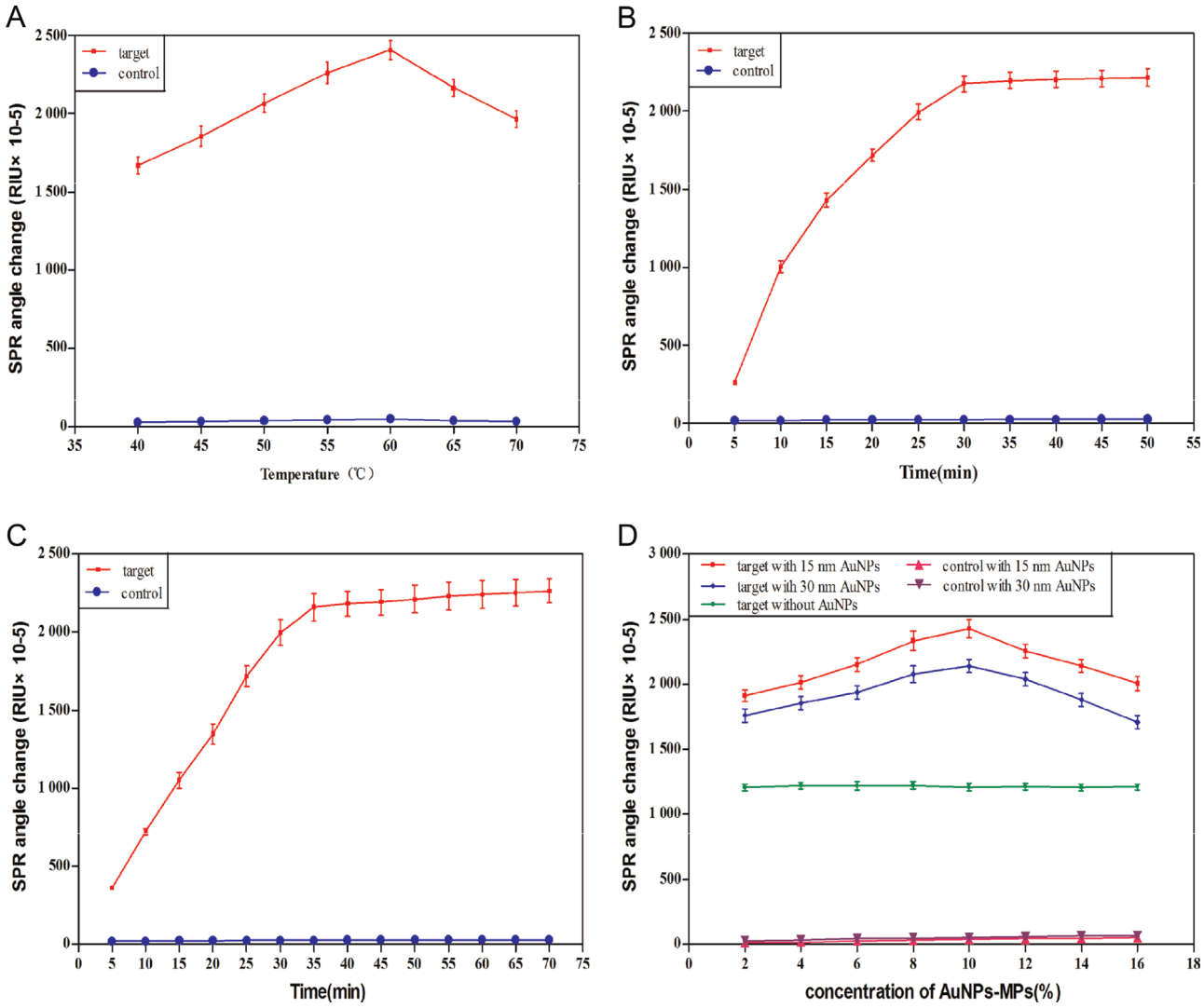

Fig. 2. Optimization of experimental conditions for AuNP-RCA-based SPR. (A) Effects of hybridization temperature between the PLPs and target on the SPR angle change. (B) Effects of hybridization duration between the circular PLPs and the CPs on the SPR angle change. (C) Effects of RCA duration on the SPR angle change. (D) Effects of AuNP concentration and size on the SPR angle change. In all experiments, the concentration of the PLPs and targets were 50 nM and 10 nM, respectively. RIU indicates the refractive index unit, 1 millidegree SPR angle $=10^{-5}$ RIU. Error bars indicate the standard deviation ($n=5$).

enhanced with differing durations of hybridization, we varied the hybridization time from 5 to 50 min with 5 min increments. As shown in Fig. 2B, the SPR angle increased significantly with a hybridization time of up to 30 min and remained high at times longer than 30 min but did not increase further. This could be because the hybridization between the circular PLPs and CPs reached saturation. In parallel assays with the control, the SPR angle signal remained low, suggesting that this reaction is specific. For practical purposes, we selected 30 min as the shortest optimal hybridization duration.

3.3.3. *Effect of AuNP-RCA amplification duration*

To further increase the sensitivity, the relationship between the amplification time of the AuNP-RCA and the SPR angle change were systematically investigated. The AuNP-RCA duration was varied from 5 to 70 min with an interval of 5 min. The SPR angle change increased rapidly with increasing AuNP-RCA reaction time up to 35 min, as shown in Fig. 2C. However, the SPR angle change did not increase linearly when the amplification reaction duration went beyond 35 min. This could be because the activity of the phi29 DNA polymerase was limited, the RCA products inhibited hybridization with the AuNP-MPs, or the AuNP-RCA reaction achieved equilibrium. In parallel testing, a minimal SPR angle change was consistently observed for the control regardless of the AuNP-RCA duration. These results suggest that higher sensitivity can be achieved by increasing the AuNP-RCA up to 35 min. Therefore, we selected 35 min as the optimum AuNP-RCA duration for subsequent assays.

3.3.4. *Effect of AuNP-MP concentration and different AuNP diameters*

Because the SPR technique displays a very high sensitivity to changes in the refractive index of the solution, the concentration and size of the AuNP-MPs could also improve the sensitivity of the SPR biosensor. To determine the optimal AuNP-MP concentration, we tested concentrations ranging from 2% (v/v) to 16% (v/v). In addition, we tested AuNPs of 15 nm and 30 nm diameter. As a control, AuNP-MPs without amplification and target without AuNPs were tested. As shown in Fig. 2D, different AuNP-MP concentrations had an effect on the SPR angle change, with a peak at 10% (v/v); however, the controls were consistently low. Because the SPR angle change is proportional to the mass of the molecules bound to the surface of the chip, one would expect larger signal amplification from the 30 nm AuNPs, which have the larger mass. Surprisingly, however, the 15 nm AuNPs had better amplification

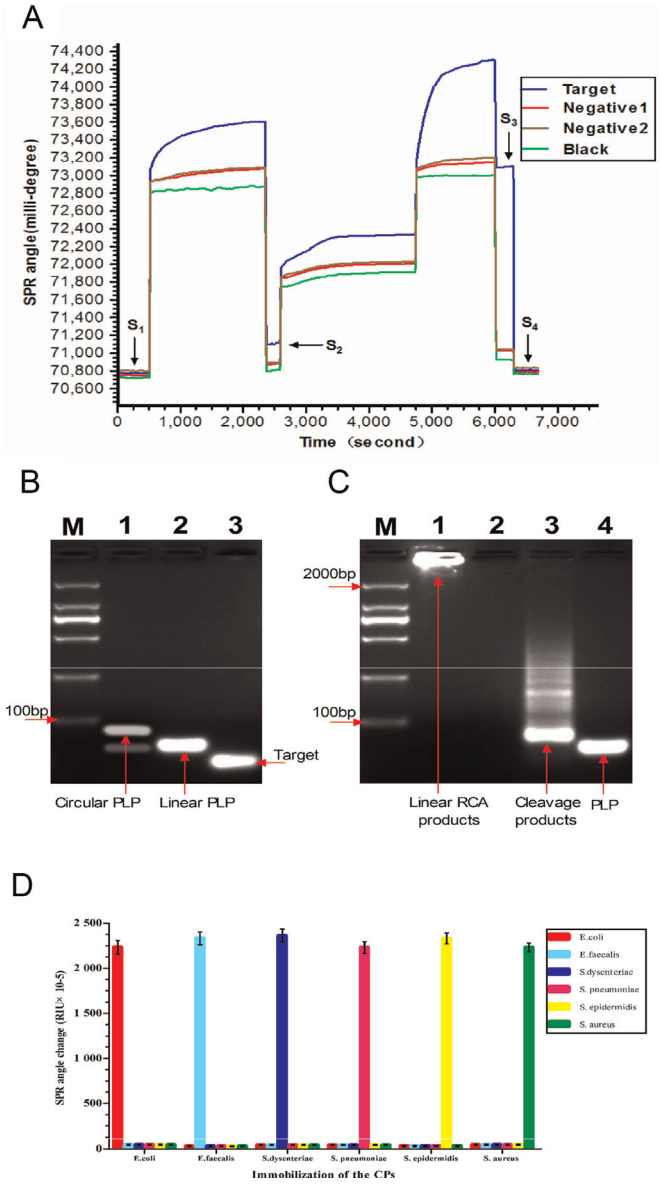

Fig. 3. Assessment of specificity of the AuNP-RCA-based SPR method. (A) SPR angle values corresponding to the target, two negative controls (negative 1 and negative 2) and a blank sample. (B) Agarose gel electrophoresis identification of the enzymatic ligation reaction. Lane M, DL2000 markers; Lane 1, ligated product from the specific target; Lane 2, ligated product from a target-free reaction; Lane 3, ligated product from a PLP-free reaction. (C) RCA products electrophoresed in a 3% agarose gel stained with SYBR Green dye. Lane M, DL2000 DNA markers; Lanes 1 and 2, RCA product derived from the specific target and the blank control, respectively; Lane 3, RCA products from the specific target digested with QuickCut™ HpaI restriction enzyme; Lane 4, PLP alone. (D) Cross-reaction of the circular PLPs with capture probes. 1, 2, 3, 4, 5 and 6 represent six capture probes, respectively. Error bars indicate the standard deviation ($n=5$).

signal. This could possibly be due to the particle–particle separation distance and size. Consequently, 10% (v/v) of 15 nm diameter AuNP-MP particles was selected for subsequent experiments.

3.4. Assessment of the specificity of optimized AuNP-RCA-based SPR biosensor detection

Using optimized experimental conditions, we conducted assays to verify the specificity of the PLPs. The SPR angle changes were assessed using 100 nm of PLP, 10 nM of synthetic target sequence and 10 ng μl^{-1} DNA sequence for samples in which the absence of the six pathogenic bacteria. Corresponding negative and blank experiments were also performed under the same experimental conditions. Our results showed that the average SPR angle change obtained from the target sequence was $(2314.9 \pm 90.6) \times 10^{-5}$ RIU, which was significantly higher than that of the negative control (only $[34.9 \pm 5.8] \times 10^{-5}$ RIU after subtraction of the background [blank control]) (Fig. 3A). These data suggest that the proposed method could be used to detect and identify multiple pathogens in parallel. The small SPR angle changes of the negative control are ascribed to limited RCA-free extension from unligated PLPs and small amounts of nonspecific accumulation of biological molecules. To validate these findings, the ligation reactions were analyzed by 4% agarose gel electrophoresis. The ligated product migrated slower than the linear PLP, which verifies that linear PLP was ligated into a ring only in the presence of target (Fig. 3B).

Next, we added exonuclease I and exonuclease III to digest the linear oligonucleotides in the ligation mixture. Exonuclease treatment removes the unreacted probes so that they cannot cause self-ligation amplification, which could lead to a background signal (Hafner et al., 2001). The resulting products of RCA were validated by 3% agarose gel electrophoresis. Digestion with HpaI endonuclease caused a shift of the product to the same size as the PLP (Fig. 3C). This verifies that the RCA products were long ssDNA molecules consisting of multiple tandem repeated copies of the complementary sequence of PLP.

Finally, we evaluated the ability of the optimized method to detect several pathogens in parallel. Six synthesized target sequences were used to ensure that the corresponding individual linear species-specific PLPs were ligated into a circular form. The cross-reaction between the circular PLP and the non-specific CPs was also assessed in order to verify the specificity of the method for application of multiplex detection. The SPR angle change was obvious between specific base pairing and non-specific base pairing ($p < 0.01$), but no cross-reaction was observed (Fig. 3D). These results further validate the specificity of the CPs on the chip surface.

3.5. Assessment of the sensitivity of AuNP-RCA-based SPR biosensor detection

To assess the sensitivity of the method, experiments using different concentrations of target were performed with invariable 100 nM PLP in the RCA-based SPR biosensor. Under optimized conditions, the SPR angle changes showed a linear relationship with the logarithm of the target concentration from 10^{-13} M to 10^{-8} M ($y=503.5x+6126.0$) (Fig. 4A). The detection limit of the method was set at 3-fold standard deviations (SD) more than the blank. According to the standard, the threshold value was confirmed as $(51.6 \pm 7.2) \times 10^{-5}$ RIU with a concentration of 5×10^{-13} M. The SPR angle change increased significantly when the concentration of target DNA was increased from 5×10^{-13} M to 5×10^{-7} M. When the concentration was higher than 5×10^{-7} M, however, the SPR signal no longer increased. This could be because the PLPs in solution were fully ligated, or because hybridization between the higher concentration of circular PLPs and the CPs on the chip reached saturation.

The sensitivity of the current assay (0.5 pM) is comparable with or even greater than similar reported methods, such as the RCA-based DNA biosensor (0.1 nM) (Zhang et al., 2009); DNA hybridization detection using SPR biosensors (10 pM) (Ananthanawat et al., 2009); hybridization of fully match DNA detection (20 pM) (Milkani et al., 2010); SPR detection of oligonucleotide sequences (5 nM) (Rachkov et al., 2011); detection for both the SPR and FBAR (1 nM) (Auer et al., 2011) and detection using a spectral SPR imaging system (5 nM) (Yang et al., 2013). The high sensitivity of the assay might be attributed to the following factors: first, the AuNP–SA–biotin CP conjugates on the chip surface improve the

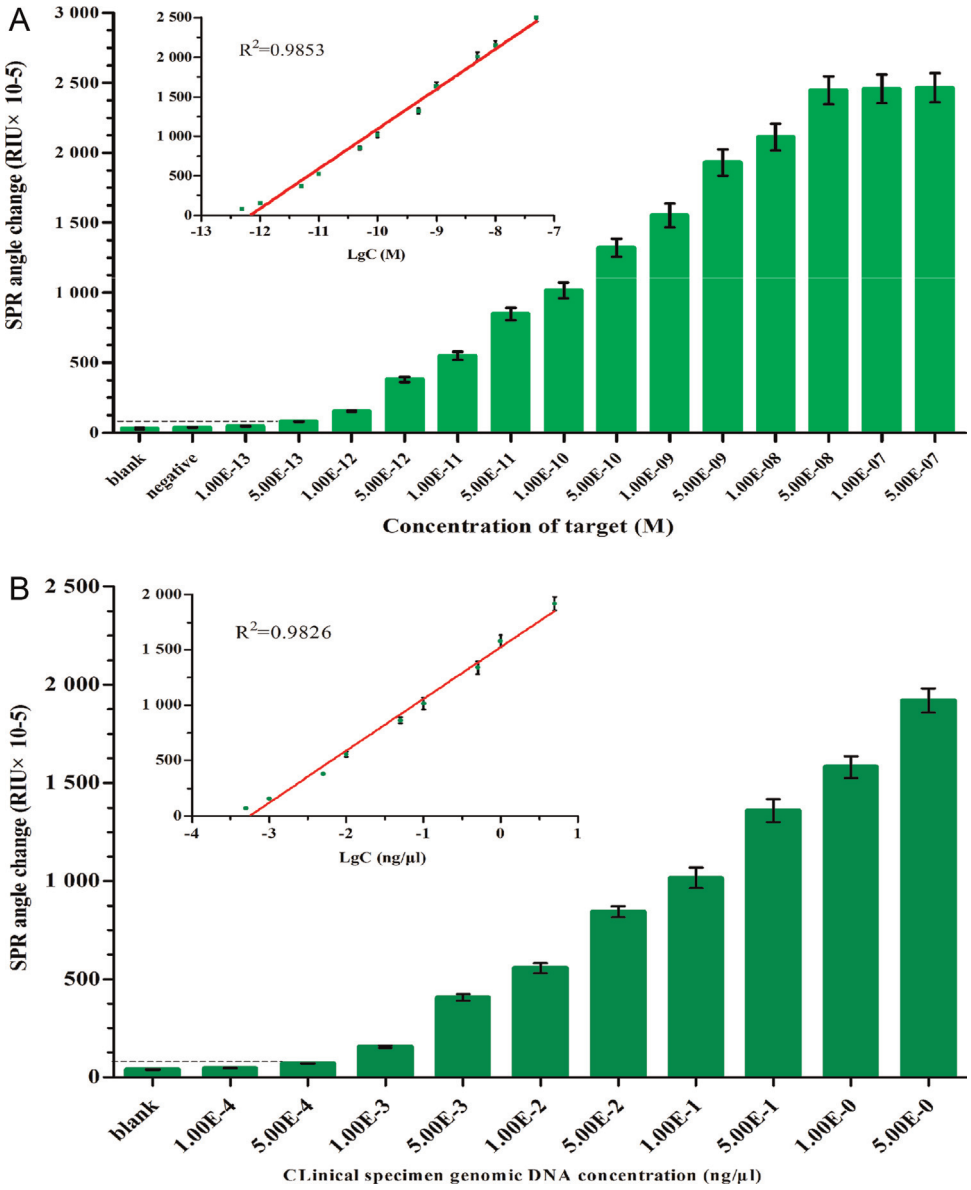

Fig. 4. Assessment of the sensitivity of the AuNP-RCA-based SPR method. (A) Sensitivity assessment of the SPR biosensor method was applied to target DNA detection. (B) Sensitivity assessment of the SPR biosensor method was applied to the detection of genomic DNA from clinical specimens. The dashed lines represent the threshold values.

efficiency of hybridization between the CPs and the circular PLPs; second, AuNP-RCA produces large ssDNA sequences with thousands of tandem repeats, which are immobilized on the chip surface to increase the SPR signal; third, a large number of AuNP-MPs can hybridize with the ssDNA products, which increases the mass of the chip surface and enhances the refractive index of the SPR biosensor. Thus, the sensitivity is improved significantly.

3.6. Detection of bacterial genomic DNA from clinical samples

To test the applicability of this method for mixed samples, the AuNP-RCA-based SPR multiplexing assay was applied to the detection of six genomic DNAs from clinical samples. There was no uniform standard for bacteria DNA extraction of all the clinical samples, because the samples included sputum, urine and feces samples. Therefore, the samples were cultured in plate medium at 37 °C overnight. Each sample was divided into two parts before detection. One part was used for identification by traditional clinical identification methods (Dade Behring MicroScan automatic microbial analysis system and Biomérieux ATB automatic microorganism identification method), and the other was used for AuNP-RCA-based SPR biosensor detection. Under the optimized condition, the detection limit was 0.5×10^{-3} ng μl^{-1} genomic DNA (Fig. 4B), which is adequate for detecting clinical samples. This compares to the detection of genomic DNA by hyperbranching rolling circular amplification (HRCA) combined with magnetic bead-based electrochemiluminescence (ECL), which is reported to have a sensitivity of 2×10^{-4} ng μl^{-1} (Long et al., 2011). Real-time PCR microfluidic devices with electrochemical detection also have a high sensitivity of 0.8 pg μl^{-1} (Fang et al., 2009). However, the AuNP-RCA-based SPR multiplexing detection still has the advantages in some aspects, such as the multiplex detection, isothermal reaction, label-free conditions and real-time monitoring.

To assess the clinical application of this method, we applied it to the detection of 50 clinical samples (Table 2). One case of *E. coli* and one case of *S. epidermidis* were not detected. This might be due to the failure of DNA extraction or low concentrations of the

Table 2
Comparison of clinical methods and SPR biosensor method for parallel detection of pathogenic bacteria.

Clinical methods (Dade Behring MicroScan and Biomérieux ATB)	SPR biosensor												Total
	E. coli		*E. faecalis*		*S. dysenteriae*		*S. pneumonia*		*S. epidermidis*		*S. aureus*		
	P	N	P	N	P	N	P	N	P	N	P	N	
P	25	1	4	0	8	0	18	2	22	0	13	1	
N	2	22	0	46	0	42	1	29	1	26	0	36	
Total	27	23	4	46	8	42	19	31	23	27	13	37	50

P: Positive, N: Negative.

target in the sample. Additionally two cases of negative samples were inconsistently detected by the AuNP-RCA-based SPR multiplexing assay, possible due to degradation or contamination before detection. However, according to the Kappa test, $p < 0.01$, Kappa > 0.75, the two methods had a remarkable overall consistency.

4. Conclusion

We demonstrated that the AuNP-RCA-based SPR method is useful for detecting several pathogens in parallel. By taking advantage of the high amplification efficiency of AuNP-RCA and the intrinsically high sensitivity of the SPR biosensor, as low as 5 pg μl^{-1} genomic DNA was detected by this assay with high specificity. The RCA reaction does not require variable temperatures (but instead uses an isothermal reaction at 37 °C). With the combined use of AuNPs and the SPR biosensor, we showed that the method can be easily expanded for rapid, simple, sensitive, specific and multiplex detection. We applied this method to the detection of pathogens in clinical samples. Six individual species-specific PLPs were used to identify bacteria, and the results had good consistency with standard clinical assays. Future research may improve the multiplex capabilities of the SPR biosensor to immobilize more CPs on the chip surface. Eventually, we hope to expand this technique to the detection of a variety of pathogenic microorganisms (including plant pathogens). Nevertheless, this study provides the foundation for future applications of AuNP-RCA-based SPR.

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