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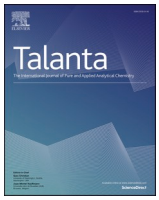

A multiplex and regenerable surface plasmon resonance (MR-SPR) biosensor for DNA detection of genetically modified organisms

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ABSTRACT

The continuous advancement of analytical technology has provided methods with increasing sensitivity and precision to detect genetically modified organisms (GMOs). Novel analytical strategy-based detection methods are alternatives to conventional polymerase chain reaction (PCR)-mediated assays, which are still the gold standard in this field. However, PCR primers and probes cannot be reused, which makes the technique uneconomical. Surface plasmon resonance (SPR) is an optical and label-free technique for studying ligand-analyte interactions, especially for DNA hybridization, and several SPR biosensors have been described for the detection of nucleic acids. Here, a multiplexed, regenerable and real-time SPR biosensor for the detection of GMOs is described. A biosensor was constructed for qualitative detection of *T-nos*, *CaMV35S* and *cry1A* and had good specificity and sensitivity. The limit of detection (LOD) of this biosensor was 0.1 nM without any signal amplification. Furthermore, our biosensor could be stably regenerated more than 100 times over at least 20 days and showed good reproducibility. This nucleic acid SPR biosensor has potential for application in other types of biological detection.

1. Introduction

Since the first genetically modified (GM) crop was commercially planted in 1996, genetically modified organisms (GMOs) have garnered increasing concerns from the government and citizens because of potential safety risks [1]. GMO detection technology is a requisite for GMO safety management. Many researchers have attempted to develop high-throughput, cost-effective, high accuracy tools to track and identify GMOs. Nevertheless, polymerase chain reaction (PCR) is the most accepted standard used to detect the presence of GMOs [2,3]. Common GMO testing consists of two steps: the initial screening step to confirm endogenous genes and genetic elements and the identification step of genetic modification events [4]. In the first step, various genetic elements need to be detected, thus multiplex PCR detection technology has been developed and extensively studied [5–7]. However, PCR methods have several drawbacks that inhibit their development, such as the inability to distinguish DNA fragments of the same size in multiplex reaction [8], false-positive results, contamination, matrix effects and the ability of reuse. Therefore, there is a need for a more efficient approach capable of covering a broader range of genetic elements and easily confirming the amplification sequences.

DNA sensors usually have one oligonucleotide used as a probe with one end bound to a suitable transducer, such as an electrode, surface plasmon resonance (SPR) chip, or a quartz crystal microbalance (QCM). SPR is one of the most attractive methods for surface-sensitive biomolecular interaction analysis (BIA) and detection of various molecules in real time [9]. In the last two decades, a large variety of SPR sensor platforms [10–13], including both laboratory systems for parallelized monitoring of molecular interactions [14–18] and compact SPR sensors for bioanalytical applications in the field [19–26], have become available. A Biacore analytical system based on SPR was developed by the Biacore AB Company in 1990. This optical analytical system monitors changes in the refractive index in the vicinity of the sensor chip surface. Such changes are directly proportional to the change in adsorbed mass,

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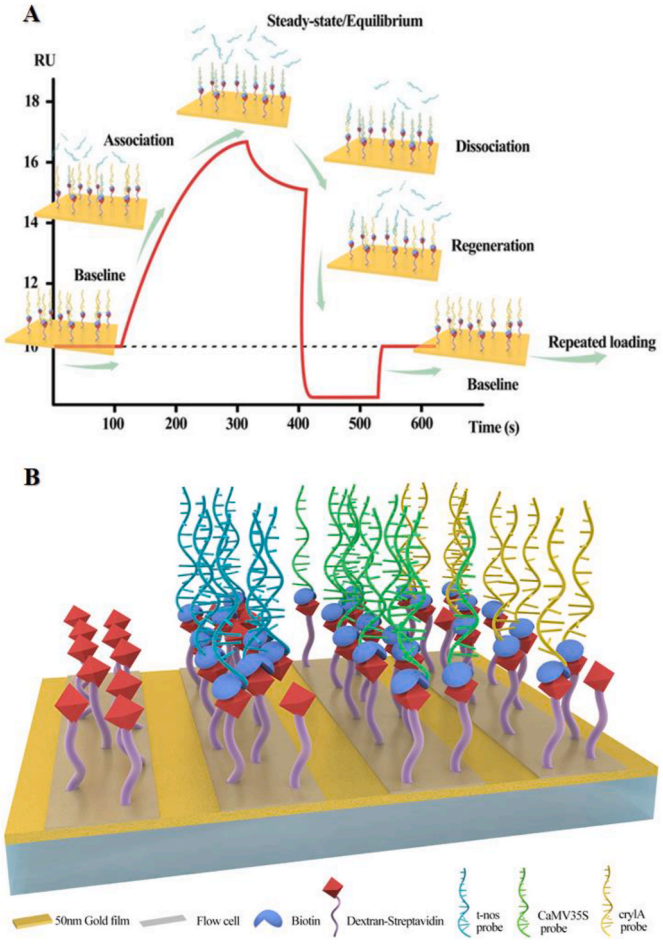
which makes this system suitable for the detection of biomolecules (Scheme 1). Most studies of gene interactions use a hybridization reaction between a DNA probe and the target. At the end of each assay, the sensor chip can be regenerated under suitable conditions. Therefore, there are economic benefits to this technique.

In this paper, a multiple, selective, label-free, regenerable and real-time method for GMO detection based on an SPR platform is described. *T-nos*, *CaMV35S* and *cry1A*, typical genes of genetically modified plant elements, were chosen as target to detect using MR-SPR biosensor. The protocol is based on the immobilization of biotinylated single-stranded DNA (ssDNA) probes containing conserved sequences of the *T-nos*, *CaMV35S* and *cry1A* genes on a streptavidin (SA)-coated sensor chip with four flow cells. As *T-nos* and *CaMV35S* signals are positive that means the analyte contains ingredients of GMO. Otherwise, the analyte does not contain GMO. The multiplex and regenerable biosensor could achieve multiplex detection. The limit of detection (LOD) of this biosensor was 0.1 nM. A successful test of target gene in biological samples demonstrated that the proposed biosensor has immense potential for detection of DNA in GMO samples.



Scheme 1. The principle of multiple-reaction SPR. A, a sensorgram of a typical single strand DNA and probe interaction. Once the single strand DNA pass the surface and are bound to the immobilized probes the angle of reflection changes. These changes of SPR angle, that are proportional to mass absorption of the bound probe, are visualized in real-time as RU in a sensorgram. The classical interaction process consists of baseline, association, equilibrium, dissociation, and regeneration. B, modification of multiple-reaction chips. Plain gold surface of the chip was modified with a 50 nm-thick layer of carboxylated dextran-streptavidin. The biotin probes of *T-nos*, *CaMV35S* and *cry1A* were immobilized in three independent flow cells.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were purchased from GE Healthcare (Stockholm, Sweden) or Sigma (USA) and were purified as needed. Solutions were made with Milli-Q water obtained by purification with a Millipak® 40 filtration unit (0.22 μm, Millipore, USA). The HBS-EP + buffer used for immobilization contained 0.01 M HEPES, 0.15 M NaCl, 0.0034 M ethylenediaminetetraacetic acid (EDTA) disodium and 0.05% (v/v%) P20 and was adjusted to pH 7.4. The regeneration solution kit contained 50 mM NaOH and 10 mM glycine-HCl at pH values of 1.5, 2.0, 2.5, and 3.0. The HBS-EP + buffer and regeneration solution kits were acquired from GE Healthcare (Stockholm, Sweden). All chemicals were diluted in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.0005% (v/v%) Tween 20 and 15 mM $MgCl_2$, pH 7.4), which was purchased from Sigma (USA). The target oligonucleotides and biotinylated oligonucleotide probes were synthesized and purified by Sangon (Shanghai, China). The sequences are listed in Table 1.

2.2. Designation of DNA probes

Using the well-studied biotin-SA pair as a reference, biotin was added as a biomarker at the 5' end of nucleotide sequences, which were used as ssDNA probes. To explore the effect of the secondary structure of DNA sequences on detection sensitivity, three types of ssDNA probes with different secondary structures were designed. Probe design was performed using DNAMAN 6.0.

2.3. Immobilization of probes

Biotinylated probes targeting *T-nos, CaMV35S,* and *cry1A* were immobilized onto flow cells (Fcs) 2, 3, and 4, respectively, of the SA sensor chip. The SA sensor chip had 4 Fcs: Fc 1 was typically used as a reference surface, and no probes were immobilized to this cell; the other three probes were designed to detect analytes. The SA chip consists of a carboxymethylated dextran matrix pre-immobilized with streptavidin and ready for fast, high-affinity capture of biotinylated ligands. Biotinylated probes targeting *T-nos*, *CaMV35S*, and *cry1A* were diluted to 20 nM with the running buffer of HBS-EP+ (which contained 0.01 M HEPES, 0.15 M NaCl, 0.0034 M ethylenediaminetetraacetic acid (EDTA) disodium and 0.05% (v/v%) P20, pH 7.4.), then immobilized onto flow cells (Fcs) 2, 3, and 4, respectively. The immobilization of the biotinylated oligonucleotide (20 nM probe) on the sensor chip was performed at 25 °C. The flow rate was set at 5 μL/min.

2.4. Asymmetric PCR

Asymmetric PCR was used to produce a single-stranded PCR product. Asymmetric PCR amplification of plant genomic DNA was performed on a Veriti thermal cycler (Applied Biosystems, Waltham, MA). A pair of asymmetric primers (primer-F/primer-R = 10:1) was employed to generate as many ssDNA strands as possible. The following PCR cycling conditions were used: an initial denaturation step of 1 min at 95 °C,

Table 1
Nucleotide sequences used in this study.

Oligonucleotide	Sequence (5′-3′)
CaMV35S probe	biotin-GGCTATCGTTCAAGATGCCTCTGCC
CaMV35S target	GGCAGAGGCATCTTGAACGATAGCC
T-nos probe	biotin-GCAAGACCGGCAACAGGATTCAATC
T-nos target	GATTGAATCCTGTTGCCGGTCTTGC
cry1A probe	biotin-CAATTCAACGACATGAACAGCGC
cry1A target	GCGCTGTTCATGTCGTTGAATTG

followed by 40 cycles of 15 s at 95 °C, 20 s at 58 °C, and 15 s at 72 °C. The reaction system was further incubated at 72 °C for 10 min to extend any incomplete products. The PCR products were diluted with hybridization buffer to achieve predetermined concentrations. Additionally, the PCR amplification product was monitored by electrophoretic separation on a FlashGel system.

2.5. SPR measurements

SPR experiments were performed on a Biacore™ T200 analytical system (GE Healthcare, Sweden) at 25 °C. Sensorgrams were analyzed with Biacore™ T200 Control Software version 2.0 and Biacore™ T200 Evaluation Software version 2.0. Research-grade Series S sensor chips recoated with SA were obtained from GE Healthcare (Uppsala, Sweden).

2.6. MR-SPR sensor detection of GMOs

It was previously reported that a 70 bp PCR product could be used to detect multiple targets [27]. For practical detection of GMOs, asymmetric PCR was used to produce detection sequences for the model GMO genes. Using asymmetric PCR amplification, ssDNA targets of 75 bp were obtained and directly analyzed by 3% agarose gel electrophoresis. Synthetic oligonucleotides (targeting *CaMV35S*, *T-nos*, and *cry1A*), which were fully complementary to the immobilized probes, were used to characterize the biosensor. Detection with the target oligonucleotides was performed at 25 °C by injecting the oligonucleotide solution in PBS into the SPR flow cells; the flow rate was set at 10 μL/min. The interaction was monitored for 5 min, and the sensor chip was then washed with hybridization buffer to remove the unbound oligonucleotide. The analytical signal, reported as response units (RU), was the difference between the value after the interaction (binding) and the value recorded before sample injection (baseline). Both values were obtained with the same buffer solution so that the shift during the reaction only reflected compounds fixed to the sensor chip. Blank subtractions were performed in all the experiments by subtracting the signal obtained on experimental sensorgrams from injecting buffer into an empty flow cell. Surfaces were typically regenerated with 50 mM NaOH, which was injected for 30 s at a flow rate of 30 μL/min.

3. Results and discussion

3.1. Verification of probes

In this study, the probe length was optimized by examining probes ranging from 5 nt to 35 nt, sequence information showed in Table S4. All probes were immobilized on SPR chips separately at the same concentration. Then, test samples at the same concentration were added to the MR-SPR reaction. The results are shown in Fig. 1. The 5 nt probe was too short to bind the target, resulting in a low signal. As the probe length increased, the signal increased; therefore, the ability and sensitivity of the detection method increased. However, as the length increased to 35 nt, the signal enhancement slowed, which may have been caused by formation of secondary structures that influenced interactions with the target sequence. Therefore, considering the cost of the reaction, the 25 nt probe was finally chosen as the optimal probe length.

3.2. Performance of the MR-SPR biosensor

3.2.1. Specificity and sensitivity

To confirm the specificity of the system, measurements with noncomplementary oligonucleotides (negative control) were performed. The interaction of the biosensor with noncomplementary DNA did not result in a measurable response (Fig. 2).

The sensitivity of the MR-SPR technique depends on several factors, including the refractive index of the analyte and its size. SPR has been shown to have adequate sensitivity to detect small amounts of unlabeled biomolecules [28]. Examples include SPR detection of sub-monolayer coverage of DNA probes on an SA surface and DNA probes with secondary structures. For the DNA sensor, the (G + C)% and secondary structure of the DNA probe are important factors that affect the DNA hybridization efficiency [29]. To explore the influence of secondary structure of DNA on sensor sensitivity, three types of DNA probes with

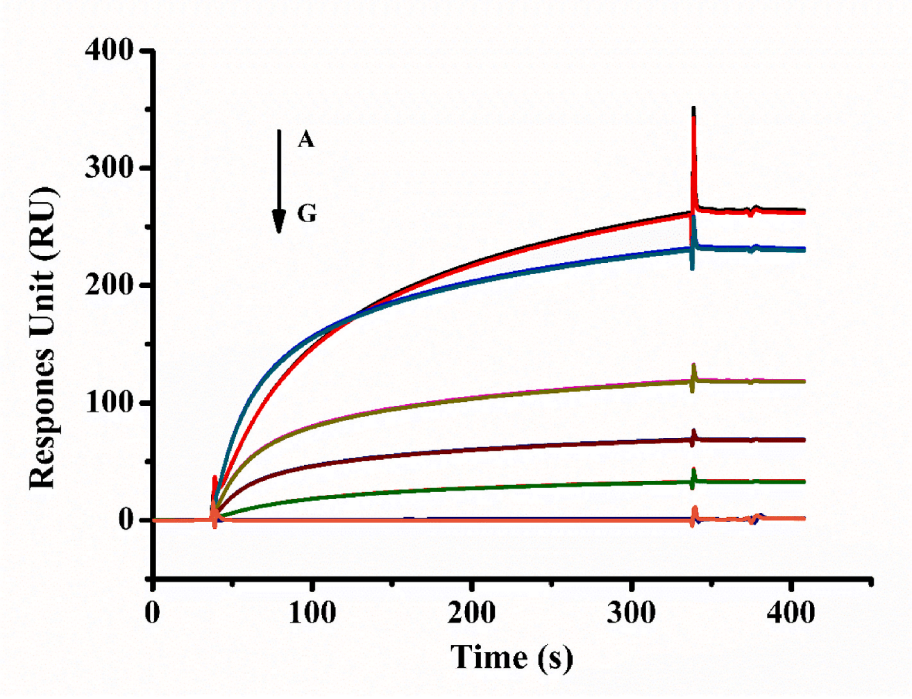


Fig. 1. Screening of probe length. A to G, Probes with lengths of 35, 25, 20, 15, 10 and 5 nt were tested in duplicate. The reaction was performed at 25 °C by injecting the oligonucleotide solution in PBS into the SPR flow cells; the flow rate was set at 10 μL/min. The interaction was monitored for 400 s, and the sensor chip was then washed with hybridization buffer to remove the unbound oligonucleotide.

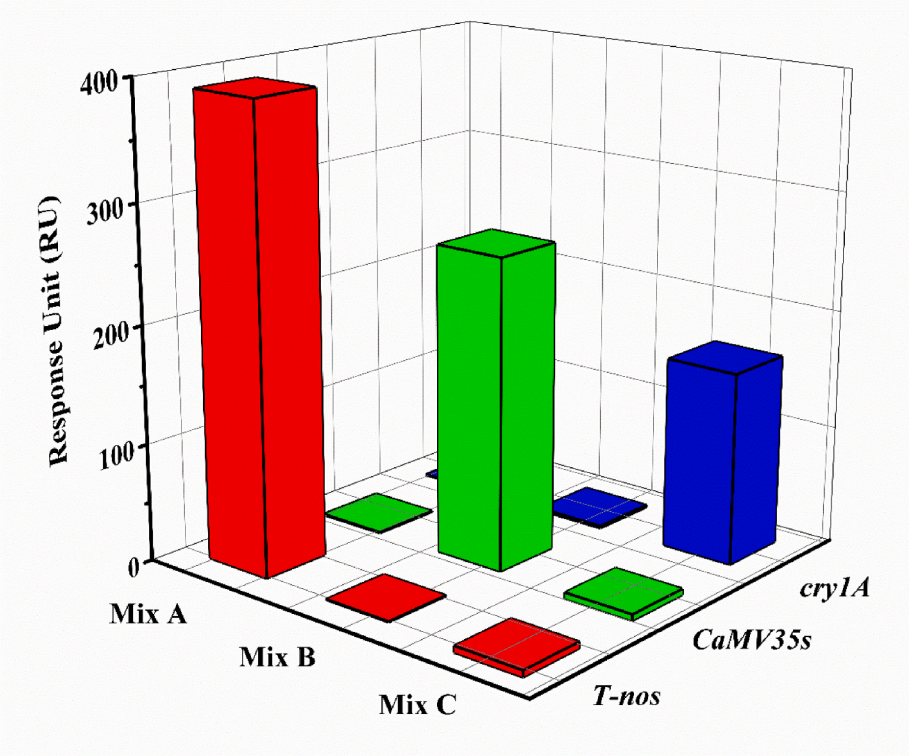


Fig. 2. Specificity of the MR-SPR sensor in multiplex detection. MixA contains *T-nos* gene, *CP4-EPSPS* gene, *vip3A* gene, *bar* gene and *NptII* gene. MixB contains *CaMV35s* gene, *CP4-EPSPS* gene, *vip3A* gene, *bar* gene and *NptII* gene. MixC contains *cry1A*, *CP4-EPSPS* gene, *vip3A* gene, *bar* gene and *NptII* gene. The flow rate was set at 10 μL/min at 25 °C.

the same (G + C) % contents but different secondary structures were designed. Then, a concentration gradient of the complementary target (10, 1, 0.1, and 0.0001 nM) was analyzed at different levels of immobilization (200, 400, 600, 800, and 1000 RU) to define the sensitivity of the different immobilization levels. As shown in Fig. 3, the LODs of the three targets decreased gradually with increasing surface immobilization levels. However, under the condition with the same amount of coupling, the LODs for the three targets were significantly different. For example, at the same immobilization level (1000 RU), the LOD of the *T-nos* target (minimum free energy: 0 kcal/mol) was 0.001 nM, but those of the *CaMV35S* target (minimum free energy: -1.60 kcal/mol) and the *cry1A* target (minimum free energy: -3.57 kcal/mol) were 0.1 nM. The signals of the *CaMV35S* and *cry1A* targets in the 0.1 nM systems were 1.37 RU and 0.97 RU, respectively (see Supplementary Table S1). This result indicated that for the SPR DNA sensor, coverage of DNA probes and secondary structures were the most important factors that affected the sensitivity of DNA interactions; therefore, for nucleic acid biosensor construction, coverage of DNA probes and secondary structures should be preferentially considered. Considering the detection efficiency and economic factor, 1000 RU (LOD 0.1 nM) was chosen as the immobilization level of the probe to detect practical samples.

3.2.2. Regeneration and stability

Effective regeneration is essential for data analysis. Effective regeneration refers to a condition that can completely remove analytes and maintain the activity and structure of the ligands or probes on the surface. After the regeneration cycle, the level of binding was approximately the same as that after the first injection, and the baseline recovered well.

Several regenerating buffers (10 mM glycine-HCl: pH 1.5, 2.0, 2.5 and 3.0; 10–50 mM NaOH) were tested in the experiment. A 50 mM NaOH solution could break down the hydrogen bonds between bases, and the analytes could then be removed from the surface without damaging the DNA probes. In an optimal regeneration buffer, the binding level should remain essentially stable compared with that after the first injection [30]. A bias of 10% is acceptable, as suggested by GE Healthcare. The results showed that 50 mM NaOH injected at a flow rate of 30 μL/min for 30 s was the optimal regeneration condition. The injection-regeneration cycle was performed six times successively under this condition, and the sensorgrams are shown in Fig. 4E; moreover, the relative standard deviation (RSD) of the six binding responses was 2.43%, as shown in Table 2, indicating that the sensor chip could be sufficiently regenerated under this condition. This treatment could be repeated five times every day for twenty days, totaling 100 uses, and the sensorgrams and tabulated data are shown in Fig. 4F and Table S2, respectively. Repeatability studies yielded an RSD ranging from 0.19 to 1.23% (n = 5), and the reproducibility RSD value was 1.15%.

3.2.3. Multiplex detection with the MR-SPR biosensor

The multiplex detection ability of our SPR sensor was tested with mixed samples A, B, C and D. Sample A contained *T-nos, CaMV35S* and *cry1A*. Sample B contained *T-nos* and *CaMV35S*. Sample C contained *T-nos* and *cry1A*. Sample D contained *CaMV35S* and *cry1A*. Each of the abovementioned samples was combined with other samples to produce 10 samples per group. The results are shown in Fig. 4. The mixed samples with two positive targets (Fig. 4B, C and D) and three positive targets (Fig. 4A) could be precisely detected. The signals of the Fc 2-1, Fc 3-1 and Fc 4-1 chambers indicated that only the *T-nos* target, *CaMV35S* target and *cry1A* target, respectively, were detected.

3.3. Practical application of MR-SPR biosensors

The MR-SPR biosensor was applied for practical sample analysis. The genomic DNA of a GMO was used as the practical sample. Asymmetric PCR was used to produce ssDNA from genomic DNA before MR-SPR detection. The correct fragments of *T-nos*, *CaMV35S* and *cry1A* were amplified (the amplicons are shown in Table S3), and the amplification products were then analyzed by MR-SPR. The asymmetric PCR products

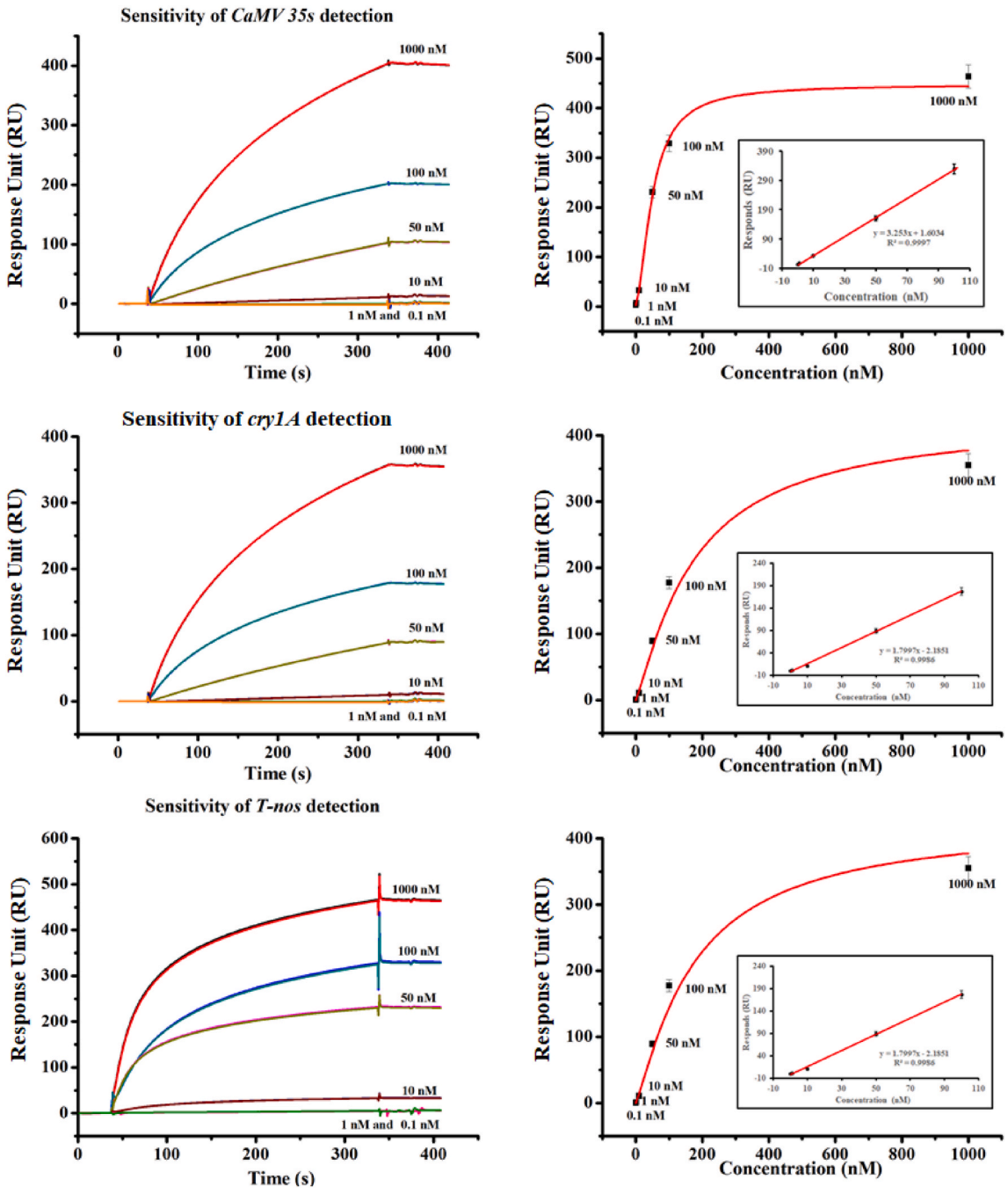


Fig. 3. Sensitivity of the MR-SPR system. Various concentrations of the target were tested ranging from 0.1 nM to 1000 nM. Each reaction was tested in duplicate. The response units of the minimum measurable concentration are presented in the inset graphs on the right.

were examined by agarose gel electrophoresis (Fig. 5A), which showed that ssDNA was clearly produced. Then, the asymmetric PCR products were mixed and used as the MR-SPR sample. As shown in Fig. 5B, the three targets could be precisely and simultaneously detected with the on-chip probes, successfully producing a good signal.

4. Conclusion

In this paper, BIA, employing SPR and biosensor technologies, was demonstrated as a rapid, sensitive, regenerable, label-free and real-time approach for screening GMOs. First, single-stranded biotinylated oligonucleotides containing the conserved sequences of the *T-nos*, *CaMV35S* and *cry1A* genes with different secondary structures were immobilized on three different flow cells of an SA sensor chip. Then, the efficiency and LOD of binding between the probe and target DNA as a function of secondary structure and immobilization level were determined. Second, suitable regeneration conditions are crucial for data analysis. Treatment of the sensor chip with 50 mM NaOH buffer for 30 s at a flow rate of 30 μL/min could maintain stability of the sensor in monitoring DNA-DNA interactions for more than 100 times over 20 days. The intraday RSD of the response at the binding site was 0.19–1.23% (n = 5), and the interday RSD was 1.15% (n = 20). Furthermore, the mass change on the surface could be monitored in the form of RUs in real time. Third, the developed DNA SPR biosensor achieved multiplex detection of three genes simultaneously. Compared with other SPR based method, the developed SPR biosensor was used to screen GMO based on specially

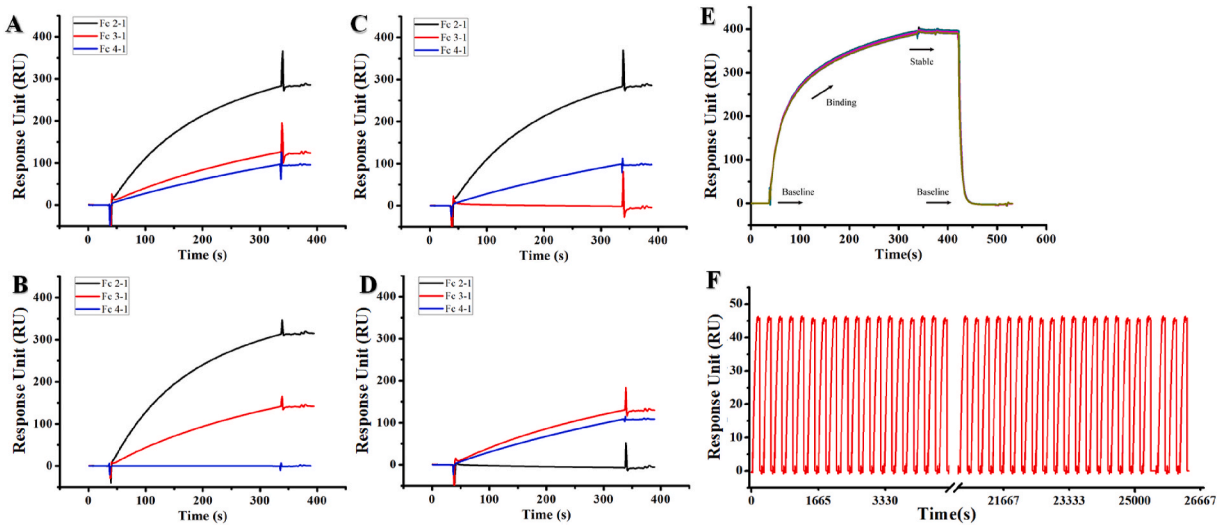


Fig. 4. Multiplex detection with the MR-SPR sensor and stability of the sensor. A, sample A contained *T-nos*, *CaMV35S, cry1A* and 7 other samples. B, sample B contained *T-nos, CaMV35S* and 8 other samples. C, sample C contained *T-nos*, *cry1A* and 8 other samples. D, sample D contained *CaMV35S, cry1A* and 8 other samples. Chamber Fc 2-1 was immobilized with the *T-nos* probe. Tunnel Fc 3-1 was immobilized with the *CaMV35S* probe. Tunnel Fc 4-1 was immobilized with the *cry1A* probe. E, six injection-regeneration cycles were performed successively with 50 mM NaOH buffer. F, stability of the SPR system (sensorgrams of consecutive injections of one sample 100 times over 20 days with 50 mM NaOH regeneration buffer).

Table 2
Stable binding responses.

Cycle	1	2	3	4	5	6	RSD
Binding (RU)	375.82	389.51	396.47	376.19	389.90	396.86	2.43%

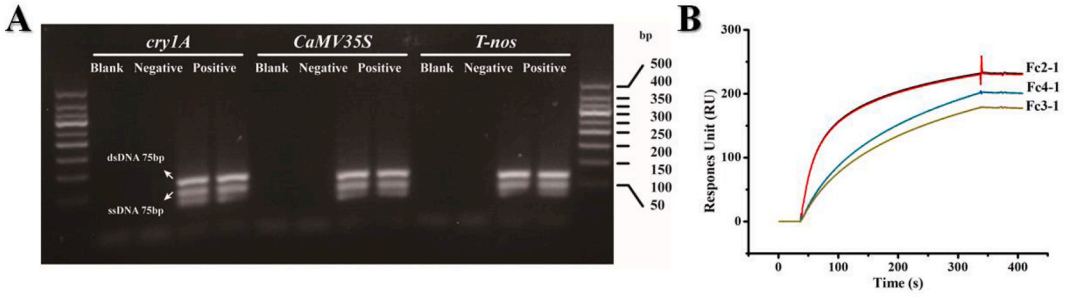


Fig. 5. Practical detection of MR-SPR. A, agarose gel electrophoresis of products of asymmetric PCR of *cry1A*, *CaMV35S* and *T-nos*. ssDNA (75 nt) fragment migrated faster than dsDNA (75 bp). B, real-time results of MR-SPR over 400 s. Fc2-1 indicates the detection results of *T-nos*, Fc3-1 indicates the detection results of *CaMV35S*, Fc4-1 indicates the detection results of *cry1A*.

binding between DNA probe and target. In addition, this biosensor provided a platform to achieve screen detection and quantitative detection simultaneously. Compared with other DNA SPR sensors used for GMO detection, the capacity for genetic screening has been largely improved. Therefore, sensor chips with several flow cells used for simultaneous analyte detection have potential in clinical applications.

Credit author statement

Na An: Validation, Writing- Original draft preparation. Kai Li: Data curation, Writing - Review & Editing, Methodology. Yukun Zhang: Visualization, Investigation. Tingting Wen: Supervision, Project administration. Weixiao Liu: Software. Gang Liu: Writing- Reviewing and Editing. Liang Li: Resources, Conceptualization, Methodology. Wujun Jin: Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122361.

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