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PCR-free quantitative detection of genetically modified organism from raw materials – A novel electrochemiluminescence-based bio-barcode method

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Abstract

Bio-barcode assay based on oligonucleotide-modified gold nanoparticles (Au-NPs) provides a PCR-free method for quantitative detection of nucleic acid targets. However, the current bio-barcode assay requires lengthy experimental procedures including the preparation and release of barcode DNA probes from the target-nanoparticle complex, and immobilization and hybridization of the probes for quantification. Herein, we report a novel PCR-free electrochemiluminescence (ECL)-based bio-barcode assay for the quantitative detection of genetically modified organism (GMO) from raw materials. It consists of tris-(2'2'-bipyridyl) ruthenium (TBR)-labeled barcode DNA, nucleic acid hybridization using Au-NPs and biotin-labeled probes, and selective capture of the hybridization complex by streptavidin-coated paramagnetic beads. The detection of target DNA is realized by direct measurement of ECL emission of TBR. It can quantitatively detect target nucleic acids with high speed and sensitivity. This method can be used to quantitatively detect GMO fragments from real GMO products.

Keywords

electrochemiluminescence; bio-barcode; TBR-labeled barcode DNA; gold nanoparticle; biotin-labeled DNA probe; genetically modified organism

INTRODUCTION

High-sensitivity nucleic acid detection is essential for clinical diagnosis, pathology, and genetics. Since polymerase chain reaction (PCR) was introduced in 1985 [1, 2], nearly all assays for DNA detection use PCR to amplify the target. PCR revolutionized the field by

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providing a reliable method for DNA detection with high sensitivity and stability. However, PCR has been criticized for its complex, expensive, time-consuming, and labor-intensive procedures. Furthermore, nonspecific amplification is a major drawback of PCR. In the past decade, various detection assays have been developed for nucleic acid detection [3–6]. However, few methods can approach the sensitivity of PCR. Mirkin *et al* reported a bio-barcode method [7–12] for nucleic acid and protein detection. This method has been shown to be as sensitive as PCR. It introduces two components: an oligonucleotide-modified gold nanoparticle (Au-NP) and a magnetic microparticle, which can sandwich a specific target. The target signal is amplified because barcode DNA, the species ultimately detected, greatly outnumbers capture DNA. However, this method also has some weak points. It still requires multiple complicated experimental procedures, including immobilization of oligonucleotides on a chip, repeated hybridization of barcode DNA probes with other oligonucleotides, release of the barcode probes from the target-nanoparticle complex, and light-scattering measurement [13–15].

Electrochemiluminescence (ECL), where light-emitting species are produced by reactions between electrogenerated intermediates, has recently become an important and powerful analytical tool [16–20]. An ECL reaction using tripropylamine (TPA) and tris-(2'2'-bipyridyl) ruthenium (TBR) has been demonstrated to be a highly sensitive method for quantifying amplified DNA in our previous studies [21–25]. Herein, we report a novel ECL-based bio-barcode method. This new method consists of TBR-labeled barcode DNA, nucleic acid hybridization using Au-NPs and biotin-labeled probes, and selective capture of the hybridization complex by streptavidin-coated paramagnetic beads. The quantitative detection of target DNA is realized by direct measurement of the ECL emission of TBR. Compared with the current bio-barcode method, this new assay introduces highly sensitive ECL into nucleic acid detection using TBR-labeled barcode DNA. This assay is simple, fast, and stable; it does not require the release and collection of barcode probes, the repeated hybridization of probes, and other time-consuming procedures. This method can be used to quantitatively detect genetically modified organism (GMO). This novel approach could make a significant contribution in nucleic acid and protein detection.

EXPERIMENTAL SECTION

Reagents and samples

Restriction endonucleases Fok I and BsrD II were purchased from New England Biolabs Company, USA. β -Mercaptoethanol was purchased from AMRESCO, Netherlands. Taq DNA polymerase, dNTP, and 100bp DNA Ladder were purchased from Shanghai Sangon Biological Engineering & Technology Services Co. Ltd. (SSBE), China. The streptavidin-coated paramagnetic beads were purchased from MACS, Germany. Au-NPs were gifts from Northwest University, China. TPA was purchased from Aldrich Chemical Company, USA. GM soybeans (*Brazil soybean No. 1*) were obtained from Guangdong Entry-Exit Inspection and Quarantine Bureau, China. Non-GM soybeans (*yu soybean No. 1*) were from China. GM papayas (*Huanong-1*), non-GM papayas (Suizhonghong), GM tobacco, and non-GM tobacco were donated by South China Agricultural University.

DNA sequences

All of the DNA sequences were synthesized by SSBE. A control DNA sequence was: 5'-GAATACTACGTTAGGATTCACGCGTGACGTACATGTACGTGCAGCAGTAATTCGTC CGATACGTAGTGGTCTGATGCGCAGCTCGATCGA-3'; the TBR-barcode DNA: 5'-TBR-CCAACGGTAA-SH-3'; the capture probe: 5'-GCCTTTCCTTTATCGCAATGGCAATC-SH-3', where underscore indicates the region of capture probe as a linkage; the biotin-probe: 5'-TGCTCCTCGTGGGTGGGGGTCCATCTT-biotin-3'. The TBR-NHS ester was synthesized by our laboratory according to Terpetsching's work [26]. The probes were labeled with biotin or alkanethiol modified by SSBE.

Apparatus

A custom-built ECL detection system was described in our previous studies [21–25]. It is composed of an electrochemical reaction cell, a potentiostat (Sanming Fujian HDV-7C), an ultra high-sensitivity single photon counting module (Channel Photomultiplier, PerkinElmer MP-962), a multi-function acquisition card (Advantech PCL-836), a computer and LabView software. The electrochemical reaction cell contains a working electrode (platinum), a counter electrode (platinum) and a reference electrode (Ag/AgCl).

DNA extraction

The cetyltrimethyl ammonium bromide (CTAB) method for sample extraction and purification reported by Lipp [27] was used in this study. The samples with or without GM components were minced with sterile surgical blades and dry samples in powder form were moistened with a three-fold amount of water. The DNA of the samples were then extracted with CTAB, precipitated, treated with chloroform, and precipitated with isopropanol to obtain a purified DNA matrix.

Digestion by restriction endonuclease and purification

DNA extracted from GM samples and non-GM samples were digested by the restriction endonucleases Fok I and BrsD II, and the digested products were subjected to electrophoresis in 2% agarose gel. The digested 169 bp DNA fragment in gel was sliced under ultra-violet lamp and extracted from agarose with QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA).

Preparation of oligonucleotide-nanoparticle conjugate

The procedures for the preparation of oligonucleotide nanoparticle conjugates used in the study were following Hill [10]. Solutions of Au-NPs and thiol-modified oligonucleotides were mixed in appropriate amounts; 4nmol of oligonucleotide was used per milliliter Au-NPs. The entire solution was brought to a concentration of 9mM in sodium phosphate buffer and 0.1% SDS, which was then wrapped in foil and placed on an orbital shaker for 30 min. The solution was then brought to a concentration of 0.3M in NaCl and allowed to stand at room temperature for 40 h. Then, the solution was centrifuged at 13000 rpm for 30 min. The supernatant was removed and the reddish solid at the bottom of the tube was dispersed in

0.3M NaCl, 9mM sodium phosphate buffer and 0.01% SDS (pH 7.0). This procedure was repeated.

Hybridization of target DNA fragment with probes

Three-strand complex was formed by mixing 20nmol of oligonucleotide-nanoparticle conjugate and biotin-probe with 1nmol of target DNA fragment, 0.3M NaCl, and 10mM sodium phosphate buffer in a total reaction volume of 25 μ l. The solutions were then heated to 94°C for 5 min, 65°C for 30 min, and incubated at 25°C for 3 h.

ECL-based bio-barcode detection

The solution of the three-strand complex was added into the electrochemical reaction cell and incubated with streptavidin-coated paramagnetic beads for 20 minutes. Unbound components, such as Au-NPs and DNA fragments, were removed by washing the beads in a magnetic field twice with TE buffer (pH 7.4). Then, the TPA was added to the reaction cell, where the magnetic beads were captured and temporarily immobilized on the working electrode by a magnet under it. A voltage of 1.25V was applied across the electrode and the photon signal was measured. Each sample was measured three times.

RESULTS

Experimental approach

The basic principle of this method is outlined in Figure 1. The TBR-barcode DNA (10bp) is modified with 5'-TBR and 3'-alkanethiol. The capture probe (26bp) is modified with 3'-alkanethiol. Both are immobilized on the surface of Au-NPs by strong sulfur-Au adsorption. The oligonucleotide-nanoparticle conjugates and biotin-probe are hybridized with the target DNA at the same time. The hybridized product is separated through the highly selective biotin-streptavidin linkage; unbound components are removed by washing the beads in a magnetic field twice with TE buffer (pH 7.4). Then, TPA is added to the reaction cell, where the magnetic beads are captured and temporarily immobilized on the working electrode by a magnet under it. The photon signal is then measured. One target signal is transformed into multiple TBR-barcode DNA signals, hence amplifying the target signal.

Our assay target is the cauliflower mosaic virus 35S (CaMV35S) promoter, which is widely used in the commercial production of various GMOs.

Effect of the concentration of magnetic beads on ECL detection

The concentration of magnetic beads is crucial for the ECL detection. The biotin-labeled DNA probe is bound to the surface of streptavidin-coated beads through highly selective biotin-streptavidin linkage. The unlinked DNA fragments are then washed away. The appropriate amount of beads can capture the entire special hybridized complex, thus improving sensitivity. However, if excessive beads are used, the electrode would be overpopulated by the attached beads, hence reducing the reaction of TPA and TBR on the surface of the electrode. Figure 2(a) shows the effect of the concentration of beads on ECL intensity using GM soybean as target. Optimal ECL signal was observed at a concentration of 5×10^5 beads/ μ l.

Effect of the TBR-to-capture probe ratio on ECL detection

The oligonucleotide-nanoparticle conjugate is also an important factor for the ECL detection. Solutions of Au-NPs and two thiol-modified oligonucleotides should mix in appropriate amounts. Since optimal probe concentration provides maximum signal amplification, the number of TBR-barcode DNA conjugated to the surface of a nanoparticle should reach saturation while the number of capture-probes on a nanoparticle should be kept minimal. Figure 2(b) shows ratio-dependent ECL signals using GM soybean as the target sample; the optimal TBR-to-capture probe ratio is 70.

Capability of the ECL-based bio-barcode method

The ECL signal of blank control was 10.1 ± 3.2 cps, thus the detection threshold value was set as 19.7 cps (mean of blank control plus three times S.D.). To see the specificity of the ECL-based bio-barcode method, 10 pmol of control DNA (non-complementary to the capture-probe or the biotin-probe) in a volume of 25 μ l was detected by the ECL-based bio-barcode method. The detected ECL signal was 12.3 ± 3.9 cps, which was under the threshold value.

A calibration curve was obtained by measuring a series of purified GM soybean samples containing different quantities of CaMV35S molecules, as shown in Figure 3(a). The data show a linear relationship between the detected ECL intensity and the target molecule quantity from 1 fmol to 100 pmol in a volume of 25 μ l ($R^2=0.993$). Based on the calibration curve, a conservative estimate gives rise to a sensitivity of 1 fmol, below which the ECL intensity approaches the detection threshold value of 19.7 cps.

Quantitative GMO detection

The ECL-based bio-barcode method was applied to detect the CaMV35S sequence in a series of samples containing 0.1% to 100% genomic DNA from GM soybean, prepared by mixing the GM soybean DNA with appropriate aliquots of genomic DNA extracted from non-GM soybean. In order to avoid cumulated background signals, the assay started from low concentration to high concentration. The electrochemical reaction cell was cleaned by distilled water after each measurement. The experimental quantification limit, defined as the lowest concentration of the GM sample which gives rise to a signal-to-noise ratio of 4, was found to be 0.1%. A calibration curve using different concentration of GM component is shown in Figure 3(b). The dynamic range is from 0.1% to 100%. This wide dynamic range is useful in developing quantification assays.

Detection of purified GMO

Oligonucleotide-nanoparticle conjugates separated by magnetic particles emit ECL signals when they are injected into the electrochemical reaction cell. Figure 4(a) shows the results of the ECL-based bio-barcode method in the detection of purified GMO samples. The signals of non-GM soybean, tobacco and papaya were 12.4 ± 4.3 cps, 8.5 ± 4.3 cps, and 15.4 ± 5.6 cps; all of them were under the threshold value (19.7 cps). The signals of GM soybean, tobacco and papaya were 309.2 ± 23.1 cps, 282.1 ± 15.1 cps, and 322.1 ± 19.6 cps; all of them were over the threshold value. The signal-to-noise ratio of ECL detection for all GMO samples was

higher than 27.9. Therefore, GMO contents are clearly determined using the intensity of the ECL signal by this method.

Direct detection of GMO from raw materials

Finally, the ECL-based bio-barcode method was applied to detect GMO components in a number of raw materials including soybean, tobacco and papaya. DNA extracted from GMO samples and non-GMO samples were digested by the restriction endonucleases Fok I and BsrD II, then the digested products were directly detected by the ECL-based bio-barcode approach without purification. The results of the detection of CaMV35S sequences are presented in Figure 4(b). The signal of blank control was 9.2 ± 2.2 cps. Thus the detection threshold was set as 15.8 cps (mean of blank control plus three times S.D.). The signals of non-GM soybean, tobacco and papaya were 9.6 ± 2.1 cps, 10.8 ± 2.4 cps, and 8.1 ± 2.9 cps; all of them were under the threshold value. The signals of GM soybean, tobacco and papaya were 40.8 ± 3.3 , 42.7 ± 2.7 , and 34.1 ± 3.0 ; all of them were over the threshold value. These results demonstrate the feasibility of the ECL-based bio-barcode method in detecting GMOs without sample purification.

DISCUSSIONS

Traditional GMO detection methods require PCR amplification [28], which are time-consuming and often lead to false identification. Mirkin and co-workers have pioneered a PCR-free bio-barcode method, making a significant progress in overcoming the major limitations of non-specific amplification of PCR [7–10]. The standard bio-barcode assay can be used for GMO detection with high accuracy and sensitivity [10]. However, the standard bio-barcode method requires a two-stage signal amplification. The current reported ECL-based bio-barcode method is simpler and faster. It does not require complicated instrumentation and lengthy experimental procedures. Only simple mixing and one-step separation of the hybridized target-probe complex are required to determine targets without using a microarrayer and other complicated signal amplification steps such as enzymatic amplification and silver enhancement. This method relies on two different hybridizations which significantly limit false positives. Furthermore, without PCR amplification, a small amount of false positives due to nonspecific binding would not negatively impact the final detection of target molecules. The sensitivity of the ECL-based bio-barcode method, 1 fmol as shown by data in Figure 3(a), is not as high as the standard bio-barcode method (500 zeptomolar for purified DNA samples) [9], or as the real-time quantitative PCR (2 pg transgenic DNA per gram starting sample) [29]. However, this new method is sufficient for GMO detection, since it can detect GMO contents at a concentration of 0.1%, as shown by data in Figure 3(b), while the international standard only requires a GMO detection sensitivity of 1%.

To ensure the highest sensitivity in detection of target DNA fragments, the ECL-based bio-barcode method has been optimized using different concentrations of streptavidin-coated magnetic beads and different TBR-to-capture probe ratios to detect the CaMV35S fragments.

The high affinity streptavidin-biotin binding system is widely utilized in clinical diagnostic applications [30–31]. It is of special importance because it has one of the largest free energies of association currently available for noncovalent binding of a protein and small ligand in aqueous solution. The bond formation between streptavidin and biotin is rapid and essentially non-reversible, unaffected by most extremes of pH values, organic solvents, and denaturing reagents. Thus, the streptavidin-biotin interaction has been extensively used as a research tool. In this novel method, we used the streptavidin-biotin system to separate target DNA fragments from other components. Therefore, the concentration of streptavidin-coated beads was an important factor to be optimized. As shown in Figure 2(a), by adjusting the concentration of streptavidin-coated beads, we found that the ECL intensity reached a maximum at a bead concentration of 5×10^5 beads/ μ l, which was adopted as the optimal concentration in this study. This optimal bead concentration was determined using a fixed target concentration. For optimal performance, magnetic beads should capture the entire special hybridized complex, without overpopulating the electrode. Therefore, the optimal bead concentration should be dependent on the target concentration. However, without a detailed study to determine the relationship between the two concentrations, we only used one target concentration to demonstrate the functionality of our method, without loss of generality.

It has also been concluded that the TBR-to-capture probe ratio can greatly affect the sensitivity of the assay. As shown in Figure 2(b), ECL intensity from GMO targets changes with the TBR-to-capture probe ratio; the optimal ratio is 70 in this study.

The two different hybridizations in the ECL-based bio-barcode assay should significantly limit the false positives. Furthermore, without PCR amplification, a small amount of false positives due to nonspecific binding would not negatively impact the final detection of target molecules. Thus, the method is supposed to be high specific. However, some studies on two-hybridization events have shown the cross-reactivity between the two probes and thus causes false positives result [32]. To evaluate the true specificity of our method, the control DNA that is non-complementary to the capture-probe or the biotin-probe was detected by the assay. The result shows that the signal of the control DNA is under the threshold value, demonstrating a high specificity of the method. Moreover, the method shows a wide detection range with a linear relationship between target DNA quantity and ECL intensity over a 5 orders of magnitude, as shown in Figure 3(a).

We determined the detection limit of the ECL-based bio-barcode method by detecting GMO of different concentrations in soybean samples. The ability of this method was demonstrated by the measurement of the 169bp CaMV35S fragment, as shown by the calibration curves in Figure 3(b). The detection limit of this method for GMO components could be as low as 0.1%. We noted that regulations in European Union and Japan have set a threshold of 1% and 5%, respectively, for contamination of GM material in a non-GM product [33–34]. Therefore, the limit of detection of this novel method (0.1%) is more than adequate for screening GM components.

At present, the increasing number of GMO-derived products has led many countries to require labeling of grains and foodstuffs that contain GM components in order to allow

consumers an informed choice. A rapid and simple method, which can directly detect GM components from raw materials without complex sample purification, is increasingly important for commerce as well as for ordinary life. Hence, this method was applied to determine a number of real samples derived from soybean, tobacco and papaya without purification. The samples were collected and assayed only by means of extraction and digestion using restriction endonuclease. Figure 4 shows the results of GMO components with and without purification detected by the ECL-based bio-barcode method. Although the purified samples resulted in high ECL signals (Figure 4a), the non-purified samples resulted in sufficient ECL signals for unequivocal determination of GMO using raw materials (Figure 4b).

In summary, a simple, rapid, quantitative, PCR-free method with high specificity and sensitivity was developed, and its capability for GMO detection was demonstrated using raw materials without additional purification.

CONCLUSION

Compared with current bio-barcode method, the ECL-based bio-barcode method is faster and simpler. It avoids multiple hybridizations and washing steps used in current assays, by avoiding the use of PCR and special instrumentation. This method can directly detect target DNA fragments from raw materials and does not require sample purification. Quantitative information for GMO components in the samples can be obtained by the calibration curve.

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Figure 1.
A schematic of the ECL-based bio-barcode method for quantitative detection of GMO.

Figure 2.

(a): Effect of the concentration of streptavidin-coated beads on ECL intensity. Streptavidin-coated beads of 1 μ l was added to 25 μ l of three-strand complex. The concentration of beads is $1\text{--}7 \times 10^5$ beads/ μ l. **(b):** Effect of the TBR-to-capture probe ratio on ECL intensity. The TBR-to-capture probe concentration ratio is in the range of 40 to 100 (40 μ mol/l, 1 μ l).

Figure 3.

Calibration curve for the ECL-based bio-barcode assay. **(a):** The calibration curve was obtained by measuring a series of purified GM soybean samples containing CaMV35S molecules with a quantity ranging from 1 fmol to 100 pmol in a volume of 25 μ l. **(b):** The calibration curve was obtained by measuring a series of samples containing 0.1% to 100% genomic DNA from GM soybean.

Figure 4.

(a): ECL signals of three purified GMO samples (solid bars), in comparison with three non-GMO samples (white bars). The DNA samples were digested by restriction endonucleases. Then, the digested products were subjected to electrophoresis in 2% agarose gel and the 169 bp DNA fragments of CaMV35S were collected and detected by the ECL-based bio-barcode assay. The dashed line represents the threshold value for purified GMO samples detection.

(b): ECL signals of three GMO samples from raw materials (solid bars), in comparison with three non-GMO samples (white bars). The DNA samples were digested by restriction

endonucleases and directly detected by the ECL-based bio-barcode assay. The dashed line represents the threshold value for non-purified GMO samples detection.

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