Capillary Electrophoresis with Electrochemiluminescent Detection for Highly Sensitive Assay of Genetically Modified Organisms

ARTICLE *in* ANALYTICAL CHEMISTRY · NOVEMBER 2009

Impact Factor: 5.64 · DOI: 10.1021/ac901510s · Source: PubMed

7 AUTHORS, INCLUDING:

Longhua Guo
Fuzhou University
80 PUBLICATIONS **978** CITATIONS

SEE PROFILE

Anal. Chem. **2009,** *81,* 9578–9584

Capillary Electrophoresis with Electrochemiluminescent Detection for Highly Sensitive Assay of Genetically Modified Organisms

Longhua Guo,[†,‡] Huanghao Yang,[†] Bin Qiu,[†] Xueyang Xiao,[†] Linlin Xue,[†] Donghwan Kim,[‡] and Guonan Chen*[,†]

Ministry of Education, Key Laboratory of Analysis and Detection for Food Safety, Fujian Provincial Key Laboratory of Analysis and Detection for Food Safety, Department of Chemistry, Fuzhou University, Fuzhou, 350002, China, and Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 637457, Singapore

A capillary electrophoresis coupled with electrochemiluminescent detection system (CE-ECL) was developed for the detection of polymerase chain reaction (PCR) amplicons. The ECL luminophore, tris(1,10-phenanthroline) ruthenium(II) ($Ru(phen)_3^{2+}$), was labeled to the PCR primers before amplification. $Ru(phen)_3^{2+}$ was then introduced to PCR amplicons by PCR amplification. Eventually, the PCR amplicons were separated and detected by the homemade CE-ECL system. The detection of a typical genetically modified organism (GMO), Roundup Ready Soy (RRS), was shown as an example to demonstrate the reliability of the proposed approach. Four pairs of primers were amplified by multiple PCR (MPCR) simultaneously, three of which were targeted on the specific sequence of exogenous genes of RRS, and another was targeted on the endogenous reference gene of soybean. Both the conditions for PCR amplification and CE-ECL separation and detection were investigated in detail. Results showed that, under the optimal conditions, the proposed method can accurately identifying RRS. The corresponding limit of detection (LOD) was below 0.01% with 35 PCR cycles.

A number of methods for genetically modified organism (GMO) detection have recently been developed as special concerns on the safety assessment of foods and food ingredients derived from GMOs. In general, GMOs can be detected by either exogenous DNAs or novel proteins.[1−3] Specific DNA sequencing by polymerase chain reaction (PCR) has been used more frequently than protein-based detection because of its higher stability and traceability of DNAs in both raw materials and processed food matrixes.[4−6] The most commonly used methods for PCR products detection involve agarose gel electrophoresis and ethidium bromide staining.[7−9] Capillary electrophoresis (CE) provides an attractive alternative to slab gel electrophoresis since it achieves better resolution and shorter separation time.[10−12] However, a major challenge for CE detection of GMOs is the poor detection limit due to short optical path length across the capillary and small sample volumes injected. Currently, most of the CE-based methods for GMOs detection use a fluorescence or laser-induced fluorescence (LIF) detector in order to obtain high sensitivity. However, the limitation of excited wavelengths of LIF and the expensive costs of the apparatus prevent its wide applications.[13−15] In addition, the high background resulted from free fluorescent probes or dyes as well as the reflection and scattering of light source is a fatal limitation for further improving sensitivity of fluorescent methodologies.

Coupling CE with a chemiluminescent (CL) detector seems a good alternative for pursuing higher sensitivity and lower cost due to its ultralow background and simple equipment. In a previous work, we developed an approach for simultaneous qualitative and quantitative detection of GMOs using CE coupled with an ultrasensitive CL detector.[16] This approach allowed us to reliably quantify Roundup Ready Soy (RRS) down to 0.1% with as small as 16 PCR cycles. CL luminophores (acridinium ester) had to be labeled to PCR products shortly before CE-CL detection because of its heat-sensitive characteristics. That requires CL

* To whom correspondence should be addressed. E-mail: gnchen@fzu.edu.cn. Fax: 86-591-83713866.

† Fuzhou University.

‡ Nanyang Technological University.

(1) Ahmed, F. E. *Trends Biotechnol.* **2002**, *20* (5), 215–223.

(2) Michelini, E.; Simoni, P.; Cevenini, L.; Mezzanotte, L.; Roda, A. *Anal. Bioanal. Chem.* **2008**, *392*, 355–367.

(3) Deisingh, A. K.; Badrie, N. *Food Res. Int.* **2005**, *38*, 639–649.

(4) Vollenhofer, S.; Burg, K.; Schmidt, J.; Kroath, H. *J. Agric. Food Chem.* **1999**, *47*, 5038–5043.

(5) Lipp, M.; Bluth, A.; Eyquem, F.; Kruse, L.; Schimmel, H.; Van den Eede, G.; Anklam, E. *Eur. Food Res. Technol.* **2001**, *212*, 497–504.

(6) Stefano, R.; Francesca, L.; Andrea, G.; Andrea, F.; Stefano, S.; Roberto, C.; Rosangela, M. *J. Agric. Food Chem.* **2007**, *55* (7), 2509–2516.

(7) Vollenhofer, S.; Burg, K.; Schmidt, J.; Kroath, H. *J. Agric. Food Chem.* **1999**, *47*, 5038–5043.

(8) Permingeat, H. R.; Reggiardo, M. I.; Vallejos, R. H. *J. Agric. Food Chem.* **2002**, *50*, 4431–4436.

(9) Lipp, M.; Brodman, P.; Pietsch, K.; Pauwels, J.; Anklam, E. *J. AOAC Int.* **1999**, *82*, 923–928.

(10) Elenis, D. S.; Kalogianni, D. P.; Glynou, K.; Ioannou, P. C.; Christopoulos, T. K. *Anal. Bioanal. Chem.* **2008**, *329*, 347–354.

(11) García-Cañas, V.; Gonzáles, R.; Cifuentes, A. *J. Agric. Food Chem.* **2002**, *50*, 4497–4502.

(12) García-Cañas, V.; Gonzáles, R.; Cifuentes, A. *J. Agric. Food Chem.* **2002**, *50*, 1016–1021.

(13) Tao, L.; Kennedy, R. T. *Trends Anal. Chem.* **1998**, *17*, 484–491.

(14) Yang, B.; Tan, F.; Guan, Y. *Talanta* **2005**, *65*, 1303–1306.

(15) Lin, S.; Chang, G.; Lin, C. *J. Chromatogr., A* **2008**, *1192*, 198–201.

(16) Guo, L.; Qiu, B.; Chi, Y.; Chen, G. *Electrophoresis* **2008**, *29*, 3801–3809.

10.1021/ac901510s CCC: $40.75
Published on Web 11/10/2009

luminophores labeling for each sample after PCR amplification. In this study, we employed a heat-resistive electrochemiluminescent (ECL) luminophore for GMO labeling to simplify the operating procedures. Owing to the heat-resistive property of the ECL luminophore, it can be labeled to the primers prior to PCR amplification. The prior labeling of primers can be applied for high-throughput detection in which a large number of samples with the same primers are used.

ECL has been proved to be a powerful analytical tool as it combines the simplicity of electrochemistry with the inherent sensitivity and the wide linear range of the CL method.[17−19] CE-ECL has been widely exploited in the past decade, and a commercial CE-ECL system has become available.[17,19] This system focuses on the analysis of ECL coreactants in the presence of a constant concentration of luminophore (usually $Ru(bpy)_3^{2+}$), and various applications have been reported in the past few years.[20−38] However, application of this coreactant-based CE-ECL method has been limited due to lack of effective coreactants. Currently only a few analytes including oxalate, tripropylamine, and some other aliphatic tertiary amines are found to be effective coreactants so that one can obtain high sensitivity. A large number of biomolecules, such as proteins, DNAs, and peptides, either have no "coreactant functionalities" or have very poor ability to serve as coreactants,[17] making it hard to be detected by current CE-ECL methods.

This paper presents a new profile for application of CE-ECL, in which ECL luminophore was used as the label for the detection of PCR amplicons. The basic theory of this setup is similar to that of ECL immunoassay, which is based on that the emission intensity of ECL is proportional to the concentration of the emitter so that ECL can be used to analyze emitter-labeled analytes. The main difference between our new approach and the previous CE-ECL was the target analytes. Our approach focuses on the analysis of ECL labels while the previous one focuses on the analysis of ECL coreactants. Those biomolecules that cannot be detected by coreactant-based CE-ECL methods can be detected by this labeling-based approach.

On the other hand, this approach could also compete with the conventional CE-LIF due to its comparable sensitivity and relatively low equipment cost. From a recent review[39] it can be found that most CE-LIF approaches for GMO detection have the limit of detection (LOD) ranging from 0.1% to 0.01%. It was worth noting that the CE-LIF methods reported with a detection limit of ~0.01% used 40 PCR cycles for target DNA amplification,[11,40] whereas we used 35 PCR cycles in our experiments. It means that lower LOD could be achieved if we apply 40 PCR cycles for our samples. Moreover, in our setup, only a light-tight box and photomultiplier tube (PMT) were necessary for light detection, and the PMT could be put at any direction around the working electrode, which would provide sufficient options for system setup. We are sure that our equipment could have lower cost versus CE-LIF, not only because there is no need of a light source for our strategy, but also because of the simple design of the light detection route, which made the system simple and economic.

Though the analytes of this approach could be similar to that of the solid-phase ECL assay formats (e.g., ECL immunoassay and DNA hybridization assay), the separation mechanism and procedures of these two methods are quite different from each other. The distinctive advantages of the proposed CE-ECL method are its simplicity, low cost, and ability to conduct multitarget detection. As we known, solid-phase ECL assay involves two conjugation steps, which are the labeling of ECL luminophores to the biomolecules and the immobilization of recognition molecules to the solid substrate. However, the proposed CE-ECL approach only involves the labeling of ECL luminophores to the biomolecules. Therefore, the detection procedures of CE-ECL could be simpler than solid-phase ECL assay. The other limitation of the solid-phase ECL assay formats is that it usually can only recognize one target molecule in each well. However, the proposed CE-ECL approach could be used for multitarget detection by identifying the target molecules by their migration time. The multitarget detection in one specimen could be useful when multiple targets are needed to be identified in one sample, which is common in clinical diagnosis when there are several biomarkers related to one disease, or there are several diseases that need to be identified in one specimen. In comparison, the proposed method could have potential application to be plotted into a CE array in order to conduct high-throughput multitarget detection.

EXPERIMENTAL SECTION

Reagents and Samples. Dichlorotris(1,10-phenanthroline) ruthenium(II) ($Ru(phen)_3^{2+}$) was synthesized according to Wang's report.[41] Oligonucleotide primers (Table 1) were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). GeneFinder was from Bio-V Biotechnology (Xiamen, China). AmpliTaq Gold polymerase, including reaction buffer and $MgCl_2$, was from Perkin-Elmer (Madrid, Spain). Brilliant SYBR green QPCR master mix was from Stratagene (La Jolla, CA). Poly(vinylpyrrolidone) (PVP)

(17) Miao, W. J. *Chem. Rev.* **2008**, *108*, 2506–2553.
(18) Mark, M. R. *Chem. Rev.* **2004**, *104*, 3003–3036.
(19) Yin, X. B.; Dong, S.; Wang, E. *Trends Anal. Chem.* **2004**, *23*, 432–441.
(20) Ding, S. N.; Xu, J. J.; Chen, H. Y. *Talanta* **2006**, *70*, 403–407.
(21) Ding, S. N.; Xu, J. J.; Zhang, W. J.; Chen, H. Y. *Talanta* **2006**, *70*, 572–577.
(22) Qiu, H.; Yin, X. B.; Yan, J.; Zhao, X.; Yang, X.; Wang, E. *Electrophoresis* **2005**, *26*, 687–693.
(23) Yuan, J.; Wei, H.; Jin, W.; Yang, X.; Wang, E. *Electrophoresis* **2006**, *27*, 4047–4051.
(24) Wang, J.; Peng, Z.; Yang, J.; Wang, X.; Yang, N. *Talanta* **2008**, *75*, 817–823.
(25) Li, J.; Ju, H. *Electrophoresis* **2006**, *27*, 3467–3474.
(26) Yuan, J.; Yin, J.; Wang, E. *J. Chromatogr., A* **2007**, *1154*, 368–372.
(27) Gao, Y.; Tian, Y.; Wang, E. *Anal. Chim. Acta* **2005**, *545*, 137–141.
(28) Liu, Y. M.; Cao, J. T.; Tian, W.; Zheng, Y. L. *Electrophoresis* **2008**, *29*, 3207–3212.
(29) Sun, J.; Xu, X.; Wang, C.; You, T. *Electrophoresis* **2008**, *29*, 3999–4007.
(30) Ding, S. N.; Xu, J. J.; Chen, H. Y. *Electrophoresis* **2005**, *26*, 1737–1744.
(31) Wang, J.; Yang, Z.; Wang, X.; Yang, N. *Talanta* **2008**, *76* (1), 85–90.
(32) Peng, X.; Wang, Z.; Li, J.; Le, G.; Shi, Y. *Anal. Lett.* **2008**, *41*, 1184–1199.
(33) Zhou, X.; Xing, D.; Zhu, D.; Tang, Y.; Jia, L. *Talanta* **2008**, *75*, 1300–1306.
(34) Li, J.; Zhao, F.; Ju, H. *Anal. Chim. Acta* **2006**, *575*, 57–61.
(35) Li, H.; Liu, X.; Niu, W.; Zhu, S.; Fan, L.; Shi, L.; Xu, G. *Electrophoresis* **2008**, *29*, 4475–4481.
(36) Yun, W.; Xu, Y.; Dong, P.; Ma, X.; He, P.; Fang, Y. *Anal. Chim. Acta* **2009**, *635*, 58–62.
(37) Zhou, M.; Ma, Y. J.; Ren, X. N.; Zhou, X. Y.; Li, L.; Chen, H. *Anal. Chim. Acta* **2007**, *587*, 104–109.
(38) Ren, T.; Xu, J. Z.; Tu, Y. F.; Xu, S.; Zhu, J. J. *Electrochem. Commun.* **2005**, *7*, 5–9.
(39) Guo, L.; Qiu, B.; Xiao, X.; Chen, G. *Food Sci. Biotechnol.* **2009**, *18*, 823–832.
(40) García-Canas, V.; González, R.; Cifuentes, A. *Electrophoresis* **2004**, *25*, 2219–2226.
(41) Wang, P.; Zhu, G. Y. *J. Chem. Soc., Chem. Commun.* **1990**, *2*, 1457.

Table 1. Primers Used for PCR Application

primer	target	orientation	sequence (5′→3′)[a]	amplicon length (bp)
U-35S	35S	sense	CCGACAGTGGTCCCAAAGATG	158
D-35S		antisense	AGAGGAAGGGTCTTGCGAAGG	
U-NOS	NOS	sense	GAATCCTGTTGCCGGTCTTG	125
D-NO		antisense	GCGGGACTCTAATCATAAAAACC	
U-epsps	cp4-epsps	sense	GCAAATCCTCTGGCCTTTCC	145
D-epsps		antisense	CTTGCCCGTATTGATGACGTC	
U-lectin	lectin	sense	GGGTGAGGATAGGGTTCTCTG	210
D-lectin		antisense	GCGATCGAGTAGTGAGAGTCG	

[a] An amino group was labeled onto the 5′-terminal of each primer.

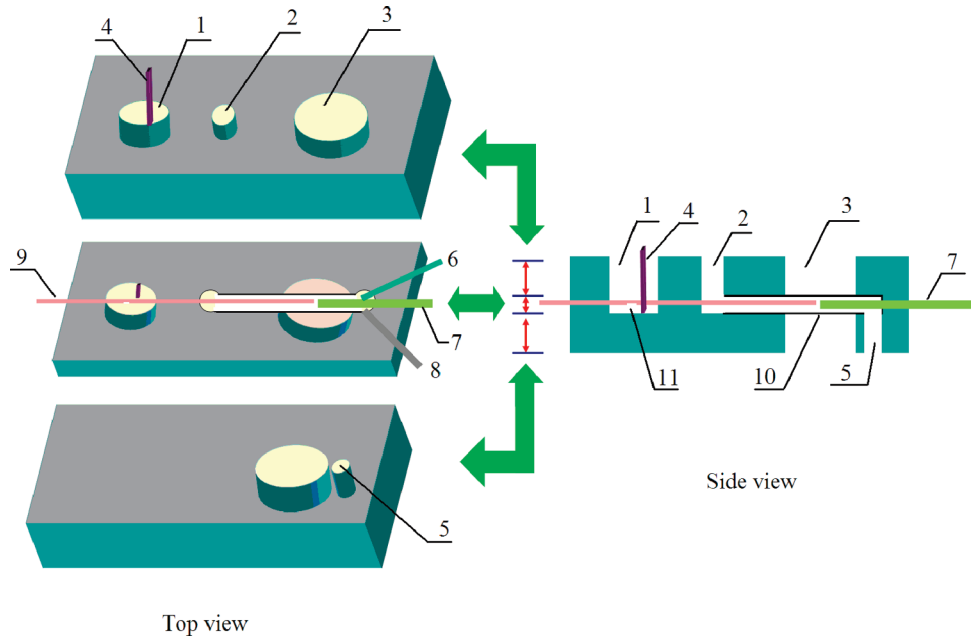

Figure 1. Schematic diagram of the homemade CE-ECL detection interface: 1, electrophoretic buffer reservoir; 2, coreactant injection entrance; 3, ECL detection window; 4, Pt grounding electrode; 5, waste exit; 6, silver wire quasi-reference electrode; 7, working electrode; 8, Pt counter electrode; 9, separation capillary; 10, detection capillary; 11, etched capillary porous joint.

with M_r = 1 300 000 was from Polyscience (Warrington, PA). DNeasy plant kit was from Qiagen, Inc. (Hilden, Germany). TIANquick oligo was from Tiangen Biotech (Beijing, China). The certified reference material (CRM) standards consisting of dried soybean powder with 0−5% GM Roundup Ready soybean materials produced by the Institute for Research Materials and Measurements (IRMM) were from Fluka Chemie GmbH (Buchs, Switzerland). The 100% GM Roundup Ready soybean samples imported from Brazil were kindly provided by Dr. Fang Yang from the Fujian Entry−Exit Inspection and Quarantine Bureau of the People's Republic of China. 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfo-succinimide sodium salt (sulfo-NHS) were from Shanghai Medpep Co., Ltd. (Shanghai, China). The water prepared using a Milli-Q equipment (Millipore, Bedford, MA) was used throughout.

Fabrication of the CE-ECL System. The high-voltage power supply (±30 kV, 0.3 mA) for CE was designed by the Shanghai Institute of Applied Physics (Shanghai, China). A WZS-50F6 microinfusion system (Medical Instrument Corporation of Zhejiang University, Zhejiang, China) was used to inject coreactant through a postcolumn mode. The ECL was generated by a HDV-7C electrochemical system (Fu Jian Chang Lian Electronic Co., Ltd., China). The light generated by the ECL reaction was detected using an ultraweak chemiluminescence analyzer (Institute of Biophysics Chinese Academy of Sciences, Beijing, China). The schematic diagram of the detection interface for the coupling of CE with the ECL detector is shown in Figure 1. Generally, it consisted of three functional components, an electric field decoupler, a coreactant injector, and an ECL detector. The detail description of this detection interface was reported in a previous paper.[42]

Genomic DNA Extraction. The DNeasy protocol for plant provided with the DNeasy plant kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from the CRM standards and samples. The DNA concentration was determined by measuring the UV absorption at 260 nm. The quality of DNA was evaluated by UV absorption ratios from 260 to 280 nm. Samples with a ratio of 1.7−1.8 were used in all assays.

Procedures for Primer Labeling. Primer labeling, in general, comprises three steps: activation, labeling, and purification (see Figure 2).

Activation of $Ru(phen)_3^{2+}$. An amount of 4 mg of EDC and 11 mg of sulfo-NHS was added to 1 mL of $Ru(phen)_3^{2+}$ DMF solution

(42) Guo, L.; Qiu, B.; Xue, L.; Chen, G. *Electrophoresis* **2009**, *30*, 2390–2396.

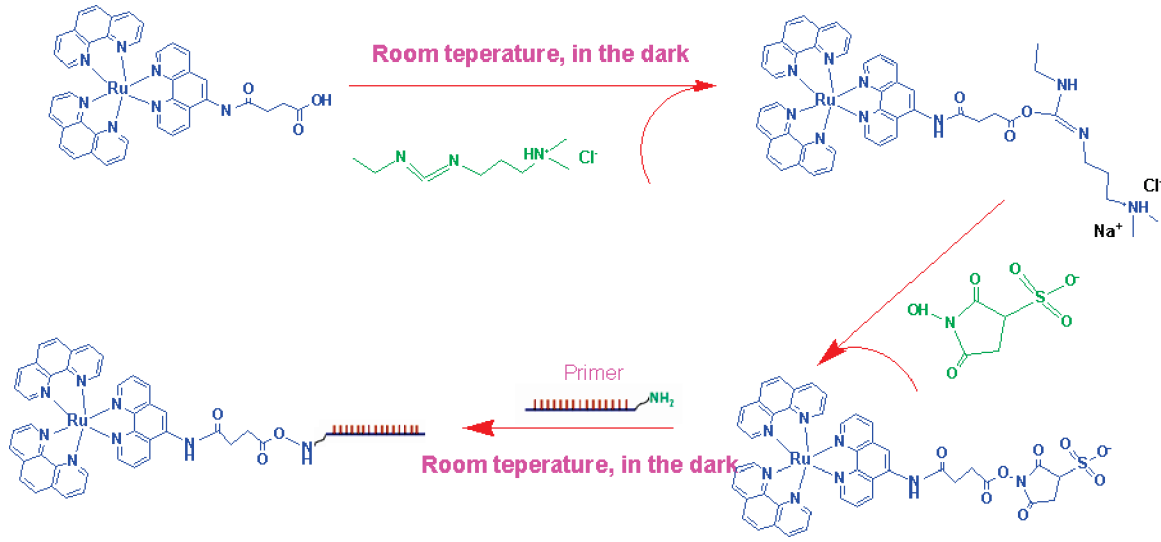

Figure 2. Schematic diagram of primer labeling.

(10 mM). The mixture was incubated in the dark for 120 min at room temperature. An amount of 10 μL of 2-mercaptoethanol was then added to quench the redundant EDC. The final solution was stored at −18 °C and used as the labeling solution in subsequent experiments.

Primer Labeling. One OD primer was dissolved in a suitable amount of phosphate buffer saline (PBS, 0.1 M, pH = 8.0) to obtain a 100 μmol/L solution, and then 25 times of $Ru(phen)_3^{2+}$−NHS (mol/mol) stock solution was added into the primer solution and the mixture was stirred for 30 min at room temperature in the dark.

Primer Purification. After ECL labeling, the primers were further purified by TIANquick oligo kit according to the specification. The purified $Ru(phen)_3^{2+}$-labeled primers were stored at 4 °C (for storage within 1 week) or −20 °C (for storage more than 1 week) before PCR amplification.

PCR Conditions. Multiple PCR (MPCR) was carried out on an EDC-810 gradient thermal cycler (Eastwin Life Sciences, Inc., Beijing, China). The reaction volume of 25 μL contained 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.5 mM dNTP, and primers (0.1 μM lectin; 0.3 μM 35S; 0.35 μM NOS; 0.5 μM epsps). Cycling conditions were as follows: denaturing of DNA at 95 °C for 10 min; 35 cycles of 30 s at 95 °C, 1 min at 59 °C, and 30 s at 72 °C; a final extension at 72 °C for 10 min.

The real-time PCR reactions were carried out on an Mx3000P QPCR system (Stratagene) using SYBRGreen I dye. The real-time PCR mix contained 12.5 μL of 2× brilliant SYBR green QPCR master mix (Stratagene), 0.1 μM upstream and downstream primers, and 50 ng of template DNA, making a final volume of 25 μL by adding the appropriate volume deionized water to the above mixture. The system runs an initial incubation at 95 °C for 10 min, 40 cycles of 95 °C for 30 s and 59 °C for 1 min, and a final elongation stage of 1 min at 72 °C. For the dissociation curve, incubate the reactions for 1 min at 95 °C to denature the PCR products, ramp down to 55 °C, ramp up the temperature from 55 to 95 °C at the rate of 0.2 °C/s, and collect fluorescence data continuously on the 55−95 °C ramp.

Agarose Gel Electrophoresis. Agarose gel electrophoresis was performed with a Langdun 552 visible electrophoresis system (Bio-V Biotechnology, Xiamen, China). The amplification products were separated using a 2.5% agarose gel in 1× TAE buffer stained with 1 μg/mL of GeneFinder (Bio-V Biotechnology, Xiamen, China). The running conditions were kept at 90 V in 1× TAE buffer. The separation process can be real-time observed through the observation window.

Capillary Electrophoresis. The self-assembled CE-ECL system was used for PCR amplicon detection. A 45 cm long bare fused-silica capillary with 75 μm i.d. (Reafine Chromatography Ltd., Hebei, China) was used as the separation capillary. The electrophoretic buffer contains 20 mM Tris−HCl, 2 mM EDTA, 1.5 M urea, and 2.5% (w/w) PVP at pH 8.0. The running electric field was −300 V/cm. The injection method was electrokinetic injection for 12 s at the running voltage. Between runs, the capillary was rinsed with 0.1 M HCl for 4 min, methanol for 4 min, and then refilled with the separation buffer by a 5 mL syringe. The coreactant injected by the microinfusion system was 20 mM 2-(dibutylamino)ethanol (DBAE) at pH 8.0, and the injection speed was 0.3 mL/h. The ECL was generated at 1.38 V (vs silver wire quasi-reference). After each CE run, the electrode was cyclic voltammetrically treated in the potential range from −0.5 to 0 V at a scan rate of 100 mV/s for 5 times to renew the electrode surface.

RESULTS AND DISCUSSION

Stability and PCR Amplification Efficiency of $Ru(phen)_3^{2+}$-Labeled Primers. ECL luminophores were labeled to primers before PCR amplification. The effect of ECL labeling to PCR amplification was investigated. Real-time quantitative PCR was used to investigate the PCR amplification plots before and after ECL labeling (see Figure 3). Figure 3 shows that the PCR amplification plots with ECL labeling are close enough to the one without ECL labeling, indicating that the ECL labeling event had no obvious effect to PCR amplification. Similar results were obtained when detecting other targets in RRS, such as NOS, cp4-epsps and lectin (data not shown).

The stability of primer sequences after ECL labeling and the stability of ECL luminophores after labeling to the primers were investigated. The stability of primer sequences can be tested by real-time PCR based on the fact that no specific PCR products can be observed if the sequence of primers was changed. Figure

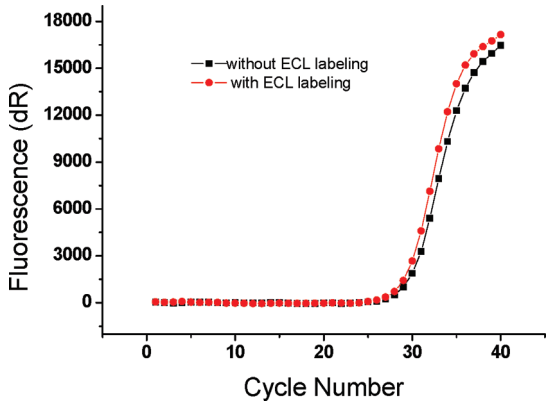

Figure 3. RT-PCR detection of RRS with primers (35S) labeled with (■) and without (●) $Ru(phen)_3^{2+}$.

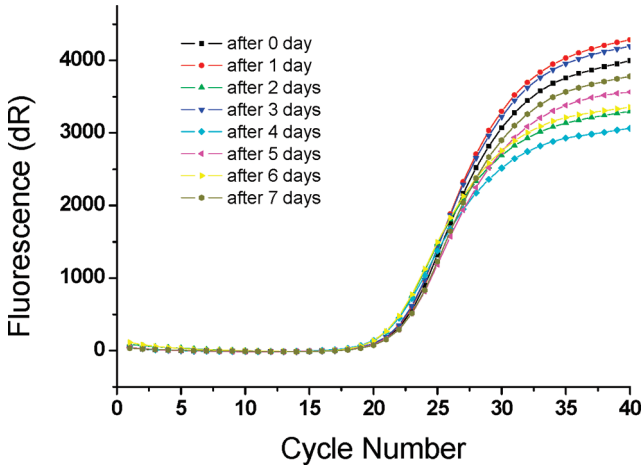

Figure 4. Primer stability evaluation by RT-PCR: 1% RRS certified reference materials was used; 0.1 μM of primer after ECL labeling for 0 (▲), 1 (●), 2 (▼), 3 (◆), 4 (○), 5 (■), 6 (□), and 7 (*) days was used for these detection.

Table 2. CE-ECL for Stability Evaluation of Primers Labeled with $Ru(phen)_3^{2+}$ [a]

time after labeling (day)	migration time (s)[b]	ECL (count)[b]
0	834 ± 10	8367 ± 103
1	829 ± 9	8218 ± 136
2	833 ± 11	8257 ± 122
3	835 ± 8	8244 ± 146
4	822 ± 12	8167 ± 149
5	828 ± 11	8259 ± 131
6	837 ± 10	8217 ± 124
7	831 ± 13	8266 ± 157

[a] The sense primer of lectin (U-lectin) was tested in these experiments. CE conditions were as follow: 45 cm long bare fused-silica capillaries with 75 μm i.d. were used; the buffer contained 20 mM Tris−HCl, 2.5% (w/w) PVP, 2 mM EDTA, and 1.5 M urea at pH 8.0; the running electric field was −300 V/cm; samples were electrokinetically injected for 12 s at the running voltage. [b] Values represent the means of three tests.

4 shows the amplification plots of lectin gene in 1% RRS. After ECL labeling, no obvious amplification efficiency change was observed within 7 days under a storage temperature of 4 °C. However, for long-term storage, −20 °C is recommended even for primers without ECL labeling in order to prevent decomposition of DNA sequences. By our experience, $Ru(phen)_3^{2+}$-labeled

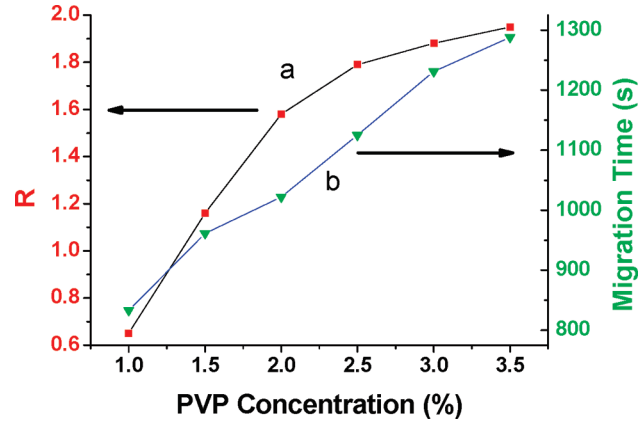

Figure 5. Optimization of PVP concentration: 45 cm long bare fused-silica capillaries with 75 μm i.d. were used; the buffer contained 20 mM Tris−HCl, 2 mM EDTA, and 1.5 M urea at pH 8.0; the running electric field was −300 V/cm; samples were electrokinetically injected for 12 s at the running voltage. (a) The effect of PVP concentration to the resolution of two DNA fragments of 145 and 158 bp. (b) The effect of PVP concentration to the migration time of DNA fragments of 145 bp.

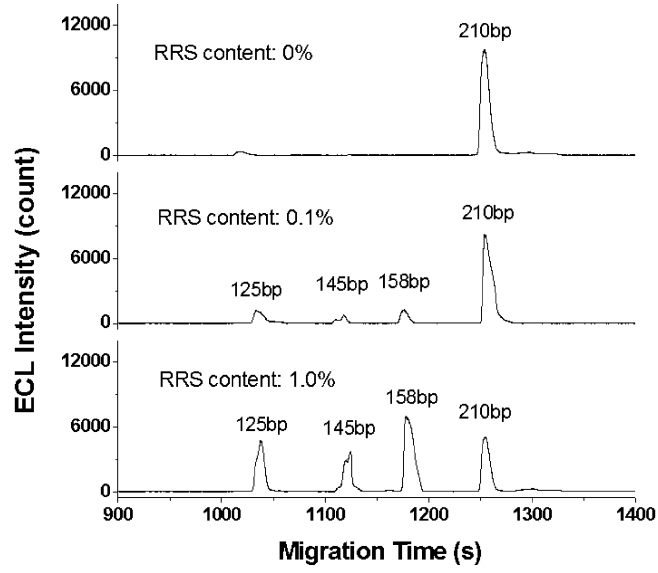

Figure 6. Electropherograms of certified reference material (CRM) standards of RRS. The electrophoretic buffer contained 20 mM Tris−HCl, 2.5% (w/w) PVP, 2 mM EDTA, and 1.5 M urea at pH 8.0; the running electric field was −300 V/cm; samples were electrokinetically injected for 12 s at the running voltage.

primers could be stable at least 3 months by aliquot storage at −20 °C and recovered to room temperature shortly before using. The results indicate that the ECL-labeled primer can be stable at least 7 days. The other concern was the stability of ECL luminophores after labeling to the primers. This can be identified by CE-ECL detection. If the luminophores had been hydrolyzed (or partially been hydrolyzed), the ECL intensity with the same migration time would be decreased. Table 2 shows the CE-ECL detection results of $Ru(phen)_3^{2+}$-labeled primers (U-lectin) in 7 consecutive days. The results show that both migration time and ECL intensity of the primers have no obvious change, indicating that the $Ru(phen)_3^{2+}$-labeled primers could be stable at least 7 days at 4 °C. However, for

Table 3. Reproducibility of Analysis Time and Peak Height for the Detection of RRS Standard by the Proposed CE-ECL Approach (n = 15, for 3 Consecutive Days)[a]

		peak height		migration time	
objective gene	size (bp)	average (count)	RSD (%)	average (s)	RSD (%)
NOS	125	4653	3.77	988	2.36
cp4-epsps	145	3879	4.23	1125	2.79
35S	158	6732	4.67	1240	1.87
lectin	210	5087	3.87	1312	1.91

[a] Values determined by repeated injection of AE-labeled MPCR products of 1.0% standard RRS.

long-term storage, −20 or −80 °C is recommended in order to prevent decomposition of DNA sequences.

Optimization of PCR Conditions. Four target DNA segments were coamplified by MPCR in our experiments. In order to allow the four targets to be amplified and detected simultaneously, diverse conditions for MPCR, such as the concentration of primers, DNA templates, Mg^{2+}, dNTP, and the anneal temperature, were optimized.

Primer concentration and ratio is of vital importance for MPCR. At the beginning, the four pairs of primers were used with the same concentration (0.1 μM), and the amplicons were tested with agarose gel electrophoresis. The primer concentration of those "weak genes" was then increased, and similar procedures as above were repeated until the amplicons of the four targets reached a close concentration. The optimal conditions were as follows: 0.1 μM U-lectin, D-lectin; 0.3 μM U-35S, D-35S; 0.35 μM U-NOS, D-NOS, and 0.5 μM U-epsps, D-epsps.

The template DNA concentration was tested by adding 50, 75, 100, 125, and 150 ng of template DNA in a 25 μL reaction solution, respectively. Results showed that 100 ng of template DNA could obtain the best amplification results. Lower template concentration would lead to the significant decreasing of some amplicons, whereas higher template concentration brought obvious background disturbing.

The concentration of Mg^{2+} and dNTP was also investigated. Some of the Mg^{2+} will bind to dNTP and DNA template. However, only the dissociative Mg^{2+} will affect the activity of TaqDNA polymerase so that change the PCR amplification efficiency. Therefore, the concentrations of Mg^{2+} and dNTP were co-optimized to minimize the error. The concentration of Mg^{2+} ranging from 0.5 to 4.5 mmol/L and dNTP ranging from 0.1 to 0.6 mmol/L and their different combinations were investigated. Results showed that best amplification could be obtained when the concentration of Mg^{2+} and dNTP was 1.5 and 0.5 mM, respectively.

Anneal temperature was another important factor which affects MPCR significantly. The temperature gradient function of the PCR apparatus was used to optimize the anneal temperature. The temperature was set from 55 to 65 °C with a gradient of 1 °C, and the MPCR results were checked with agarose gel electrophoresis. Results showed that best amplification can be obtained when the anneal temperature was set at 59 °C.

Optimization of CE Conditions. The CE conditions including sieving matrix, running buffer, applied voltage, injection time, etc. were optimized in order to get satisfactory separation of the four amplicons. PVP has been proven to be an effective dynamic capillary coating matrix with very low viscosity at moderate concentrations.[43] Consequently, in this paper PVP (M_r = 1 300 000) was used as a capillary inner wall dynamic coating matrix as well as sieving matrix for DNA segments separation. The concentration of PVP was optimized. Because the target amplicons were 125, 145, 158, and 210 bp DNA segments, and the amplicons of 35S (145 bp) and cp4-epsps (158 bp) were two segments with the closest size, therefore, these two segments were used to optimize the PVP concentration. Figure 5a shows the effect of PVP concentration to the separation of these two segments. The resolution of the two segments increased with the increasing of PVP concentration and they could be completely separated when the PVP was more than 2.0%. On the other hand, the migration time was also increased with the increasing of PVP concentration (see Figure 5b). In addition, PVP solution with high concentration had a high viscosity which would make the operation difficult. In view of the separation efficiency, separation time, and the convenience of operation, we chose 2.5% PVP as the sieving matrix for a compromise. The concentration and pH of electrophoretic buffer were also investigated. The concentration range of Tris−HCl buffer from 10−50 mmol and pH range from 5.0−11.0 were investigated in detail. Results showed that best separation could be obtained when 20 mmol/L Tris−HCl at pH

Table 4. Qualitative Detection of Standard Soybean and Simulative Samples by the Proposed Method[a]

sample	RRS content (%)	125 bp	145 bp	158 bp	210 bp	result
IRMM standard	0	−	−	−	+	non-GM soybean
	0.1	+	+	+	+	RRS soybean include
	0.5	+	+	+	+	RRS soybean include
	1	+	+	+	+	RRS soybean include
	2	+	+	+	+	RRS soybean include
	5	+	+	+	+	RRS soybean include
simulative samples	blank	−	−	−	−	no soybean include
	non-GM soybean	−	−	−	+	non-GM soybean
	0.01	+	+	+	+	RRS soybean include
	0.1	+	+	+	+	RRS soybean include
	0.5	+	+	+	+	RRS soybean include
	1	+	+	+	+	RRS soybean include
	2	+	+	+	+	RRS soybean include
	5	+	+	+	+	RRS soybean include
	100	+	+	+	+	RRS soybean include

[a] +, the corresponding DNA fragment exists; −, the corresponding DNA fragment does not exist.

8.0 was used. The influence of separation voltage and injection time on separation of the four DNA segments was also studied. Results indicated that increasing the separation voltage gave shorter migration time for all analytes but decreased the resolution of the analytes at the same time. The experimental results indicated that −300 V/cm could be selected as the optimum separation voltage to accomplish a good compromise. The effect of injection time on peak height was investigated by varying injection time from 3 to 30 s with an injection voltage at −300 V/cm. The peak height was proportional to the injection time from 3 to 12 s. When the injection time was longer than 12 s, the peak height increased slowly and peak broadening became severe. Therefore, 12 s was selected by considering both the sensitivity and selectivity.

Detection of RRS. Figure 6 shows the typical electropherogram for the detection of soy samples with different RRS content under the optimal CE conditions. Nontransgenic soy only had one peak located at 210 bp, which was related to the PCR amplicons of lectin; besides the peak at 210 bp, the transgenic soy also had three other peaks located at 125, 145, and 158 bp, which were corresponding to the amplicons of NOS, cp4-epsps, and CaMV, respectively. Therefore, the peaks located at 125, 145, and 158 bp can be used to the qualitative detection of RRS. Figure 6 also shows that the peak height of exogenous genes (located at 125, 145, and 158 bp) of sample with RRS content of 0.1% is much lower that those with RRS content of 1%, indicating that the proposed method also had the potential application for quantification.

The repeatability of peak heights and migration times was tested by repeated injection of sample solutions (1.0% RRS) in 3 consecutive days ($n = 15$), and the results are shown in Table 3. The RSD of migration time of the four DNA segments was less than 2.8%, and the RSD of peak heights was less than 4.7%.

RRS standards and simulative samples with different RRS contents were detected with the proposed method in order to demonstrate its reliability. The results are shown in Table 4. Results showed that the proposed method can accurately detect soy samples with RRS more or equal to 0.01%. It was worth noting that, when detecting soy samples with RRS content of 0.01%, the signal-to-noise ratio (S/N) of all the three exogenous genes of RRS was larger than 5 (see Figure 7), which meant that the LOD of the proposed method could be lower than 0.01% based on S/N = 3.

CONCLUSIONS

In summary, we reported a sensitive method for PCR amplicons detection using labeling-based CE-ECL. ECL luminophore was labeled to PCR primers before amplification. The stability of ECL-labeled primers and the effect of ECL labeling to PCR efficiency were investigated in detail. Results showed that ECL-labeled primers can be stable for more than 7 days at 4 °C, and no obvious amplification efficiency decreasing was observed after ECL labeling. The proposed method was used to detect RRS, and the corresponding detection limit was found to be 0.01% (S/N > 5) with 35 PCR cycles. We believe the labeling-based CE-ECL approach reported in this paper represents an advantage in this area because it changed the targets from conventional coreactant-based analytes to labeling-based analytes so that it could be a good complement to the conventional methods.

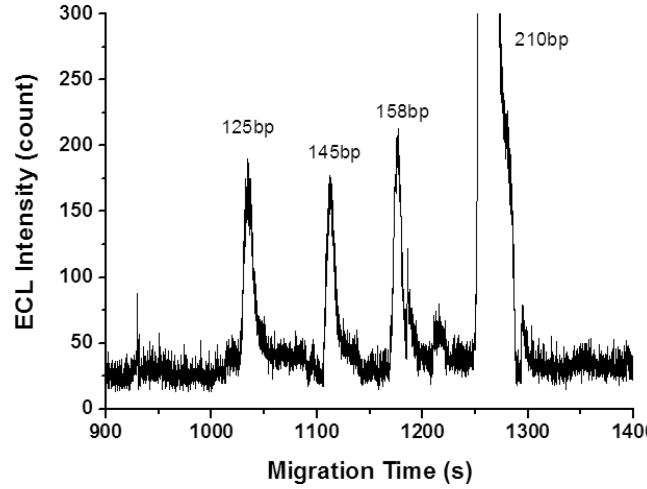

Figure 7. Electropherogram of simulative soybean sample with RRS content of 0.01%. Conditions were the same as in Figure 6.

ACKNOWLEDGMENT

This project was supported by the National Basic Research Program of China (No. 2010CB732403), NSFC (20735002, 20877019), the Key Natural Sciences Foundation of Fujian Province, China (D0520001), the Key Program of Science and Technology Department of Fujian Province, China (2007Y0026), NTU-MOE Academic Research Funds (RG65/08), and the National Research Council of Singapore (NIG/0060/2009).

NOTE ADDED AFTER ASAP PUBLICATION

This manuscript was originally published ASAP on November 10, 2009. Corrections were made to three of the references, and the corrected manuscript was published ASAP on December 1, 2009.

Received for review July 7, 2009. Accepted October 26, 2009.

AC901510S

(43) Gao, Q. F.; Yeung, E. S. *Anal. Chem.* **1998**, *70*, 1382–1388.