Analytical Methods

COMMUNICATION

Cite this: *Anal. Methods*, 2016, **8**, 2330

Received 12th October 2015
Accepted 25th February 2016

DOI: 10.1039/c5ay02705e

www.rsc.org/methods

Analysis of the inhibition of nucleic acid dyes on polymerase chain reaction by capillary electrophoresis

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An integrated polymerase chain reaction (PCR) and capillary electrophoresis (CE) system can realize accurate quantification of the target PCR product by adding labeling dyes to the PCR reagents, because CE can discriminate all the subsequent nucleic acids, including the primers, non-specific and specific PCR products. Here we discuss the inhibition of labeling dyes on PCR by performing the PCR of *Porphyromonas gingivalis* (PG) with solutions containing Hoechst 33258, SYBR Green I, and SYBR Green II. Results demonstrated that Hoechst 33258 totally inhibited the PCR process, and PCR efficiency was highly dependent on the concentration of SYBR Green I/II. Such a study expands the capabilities of CE and contributes greatly to the development of hyphenated PCR–CE instruments for biological and medical diagnosis.

Introduction

Capillary electrophoresis (CE) remains a powerful tool for nucleic acid analysis[1–5] because of its numerous advantages, such as small sample volume consumption and high resolution for the separation of biomolecules within a short time. Furthermore, research on different factors (*e.g.*, high voltage applied, polymer solutions used, and migration process involved) that may affect the separation performance has made CE a very versatile method for bio-chemical applications.

During a CE experiment, the labeling dye is mixed with a sieving polymer in advance, and then DNA/RNA is introduced into the capillary without any pretreatment. During migration, the labeling dye is intercalated to the DNA/RNA sample, and subsequently the conjugated DNA/RNA–dye can be detected by fluorescence. The migration of DNA/RNA in the polymer is affected by the intercalating dye. For example, the separation of plasmid DNA was initially difficult because the plasmid DNA was originally packed itself. A representative intercalating dye for DNA, ethidium bromide (EtBr), was intercalated into the packing structure of the plasmid and loosened the plasmid DNA packing, and thus the plasmid DNA can be resolved by CE.[6] Therefore, intercalating dyes alter the structure of DNA.

Polymerase chain reaction (PCR) is an important method for biological diagnosis and detection.[7–11] The primer amplifies only the target DNA because of its high specificity and selectivity, and thus a particular species can be determined. For biological diagnosis, the combination of PCR and CE is quite useful because it can not only realize online detection of PCR products, but can also avoid cross-contamination. In the integrated PCR-CE system, both PCR solution and the background electrolyte for CE are necessary to be injected into the capillary. Once PCR is complete, the PCR products can be determined online through the integrated PCR–CE system. Such a system could address the drawbacks of being labor-intensive and time consuming, and the poor resolution of conventional biological assays.

An intercalating dye basically inhibits PCR because it intercalates into the double helix in DNA. For example, EtBr bonds to the hydrophobic structures in DNA, and Hoechst strongly bonds into adenine–thymine residues.[12–14] The labeling dye almost completely inhibits PCR when its concentration is high, while the inhibition is alleviated at a lower concentration of the labeling dye.[15] SYBR Green has been used for quantitative PCR.[16–21] For SYBR Green I, it intercalates into the double stranded structure of DNA[22] during PCR, so after a certain number of amplification cycles, the fluorescence intensity of conjugated DNA–SYBR Green is proportional to the concentration of the PCR products. However, SYBR Green, the same as almost all nucleic labeling dyes, intercalates any DNA. Almost all nucleic labeling dyes have low specificity, because they also intercalate into primers, especially long primers, and non-specific PCR products. Therefore, the fluorescence intensity is

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not always in accordance with the concentration of specific PCR products, indicating that the fluorescence intensity cannot always reflects the real concentration of specific PCR products. So knowledge of the inhibition of PCR due to nucleic acid dyes is important for developing integrated PCR–CE systems, especially for quantitative analysis.

In this paper, we analyzed the inhibition of nucleic acid dyes for PCR by CE. Such a study expands the capabilities of CE and contributes greatly to the development of integrated PCR–CE instruments for biological and medical diagnosis.

Materials and methods

Chemicals

HEC (1300k) was bought from Polysciences (Warrington, PA, USA). 10 000× SYBR Green I and II were purchased from Invitrogen (Carlsbad, CA, USA). 10× TBE (1× TBE = 89 mM Tris/89 mM boric acid/2 mM EDTA, pH = 8.4) buffer was bought from BIORAD (Hercules, CA, USA). SYBR Green I and SYBR Green II were bought from Takara. Hoechst 33258 was obtained from Wako (Tokyo, Japan). 0.5× TBE was prepared by mixing 10× TBE and distilled water with a ratio of 1 : 19. 1× or 3× SYBR Green I/II were obtained by diluting the 10 000× SYBR Green I/II to a final concentration of 1/10 000 or 3/10 000, respectively.

Sample collection and preparation of PG template

The *Porphyromonas gingivalis* (PG) template (ATCC 33277) was purchased from Microbiologics Inc. (217 Osseo Avenue North, St. Cloud, MN 56303, USA). The PG template was extracted from dried agar medium. The colony in the dried agar medium was washed with 1 ml of phosphate-buffered saline solutions (1× PBS), then the supernatant fluid of the PG solution was collected by centrifugation. The purity of PG was determined by a UV-VIS spectrometer, and the result showed that OD_{260}/OD_{280} was 1.798.

PCR of *Porphyromonas gingivalis*

PCR was performed with a Veriti™ 96-well thermal cycler (Applied Biosystems, USA). For all experiments, PCR was performed with optimized PCR solutions.[23,24] These consisted of 1.0 μl sample and 49 μl of reaction volume containing 5.0 μl 10× fast buffer I, 4.0 μl dNTP mixture (2.5 mM), 200 nM primers[25,26] (forward, TGTAGATGACTGATGGTGAAAACC; reverse, ACGTCATCCCCACCTTCCTC) (FASMAC Co., Ltd., Kanagawa, Japan), and 0.25 μl SpeedSTAR HS DNA polymerase. In order to investigate the inhibition due to the dyes, SYBR Green I, SYBR Green II or Hoechst 33258 was added into the PCR solution. For each dye, a series of concentrations was adopted. The concentration was in the range of 0.1× to 5× for SYBR Green I and SYBR Green II, and 0.1 μg ml^{-1} to 5 μg ml^{-1} for Hoechst. For PCR, the thermal-cycling program was 30 cycles of 95 °C for 10 s (denaturation) and 64 °C for 15 s (annealing and extension) with an initial cycle of 95 °C for 10 seconds. In order to confirm the specificity and selectivity of the primers, a genomic DNA strain of PG (ATCC 33277) was used as positive control. The size of the amplified products of PG was 197 bp. In each experiment, the positive control was performed simultaneously. The PCR product was used directly for CE without any further preparation. A positive control was carried out for PCR solutions without labeling dye, and a negative control was performed in the absence of both labeling dye and template.

Capillary electrophoresis

The experimental setup was previously described.[23] Briefly, a high-voltage power supply (HSR-25P(A), MATSUSADA, Japan) was employed to supply the high voltage for CE. The excitation wavelength from a mercury lamp was filtered to be 460–495 nm by an optical filter (U-MWIB-3, Olympus, Japan). The fluorescence emission was collected by a 60× objective (PlanApo/IR, Olympus), and then detected by a photomultiplier tube (R928, Hamamatsu Photonics, Japan). The applied voltage and data collection were controlled by LabVIEW software (National Instruments, Austin, TX, USA). A certain length fused-silica capillary (ID/OD = 75 μm/365 μm) was covalently coated with polyacrylamide.[27] The entire detection system was enclosed in a dark box. The PCR product was electrokinetically introduced into the capillary. After each run, the injection side of the capillary was flushed with sterilized water by a pump for 1 min. All separations were performed at 25 °C in a clean room controlled by an air conditioner, and 5 repetitions of data collection were performed for each experiment.

PCR efficiency

The PCR ratio was calculated by the following equation:

$$\text{PCR efficiency} = \frac{\text{fluorescence intensity of PCR product (at around 2.2 min)}}{\text{fluorescence intensity of primer (at around 1.6 min)}}$$

Error bars were determined by calculating the standard deviation of the five repeated CE experiments. The standard deviation of the noise was around 0.04 V.

Results and discussion

Before PCR, we tested the illumination activity of each individual solution for PCR, including enzyme solution, template solution, and dNTP solution mixed with each labeling dye. Results showed that each solution had no luminescence. Because of the hydrophilic aggregation of the dyes, each labeling dye had no effect or little effect on the components of the PCR solution.[19,21] Thus, each labeling dye simply affected the double-stranded DNA. We also performed PCR with various Mg^{2+} concentrations. The result was the same when Mg^{2+} concentrations varied from 1.0 to 3.0 mM. Therefore, we conclude that there are two possibilities for inhibition of PCR. One is inhibition of the enzyme itself and the other is inhibition of the construction of DNA double helixes. From this illumination experiment, inhibition of the construction of the double helix by the labeling dyes was more probable than inhibition of the enzyme.

In order to check the specificity and selectivity of the PCR primers, we have performed PCR positive and negative controls (without template), and then analyzed the PCR products in 0.5% HEC (1300k) containing 1× SYBR Green I. The electric field strength was 200 V cm^{-1}. The total length and effective length of the capillary were 10 cm and 4 cm, respectively. Results are demonstrated in Fig. 1a and e. They show that the peak corresponding to the PCR products of PG was at 2.2 min. Next, we prepared PCR solutions which were mixed with SYBR Green II, SYBR Green I, or Hoechst 33258, and then analyzed the PCR products under the same CE conditions, but the HEC solutions did not contain additional fluorescent dye. The concentrations of SYBR Green I and II were chosen as 1× (a 1 : 10 000 dilution of stock solution), and the final concentration of Hoechst 33258 was 1 μg ml^{-1}. Results showed that the PCR products of PG for the PCR solution containing SYBR Green II (Fig. 1b) and SYBR Green I (Fig. 1c) were consistent with the PCR positive control. Moreover, data in Fig. 1 demonstrate that the fluorescence intensity was weakened when the PCR solution was mixed with SYBR Green I, indicating that SYBR Green I partly inhibited the amplification of PG. In the case of the PCR solution mixed with Hoechst 33258, no electrophoretic peak corresponding to PCR product was observed.

Next, we examined PCR inhibition due to SYBR Green I by varying its concentration (Fig. 2). Prior to PCR, SYBR Green I was mixed into the PCR solution in advance. The CE conditions were the same as in Fig. 1. Data in Fig. 2 show that the fluorescence intensity was related to the concentration of SYBR Green I. The fluorescence intensity was the highest when the concentration of SYBR Green I was 0.5×, and the peak corresponding to the PCR products disappeared when the concentration of SYBR Green I was above 1×, indicating that the inhibition of PCR started at the concentration of 1× and PCR was totally inhibited at the concentration of 5×. The result is consistent with the conclusion in ref. 28, which is possibly because the high concentration of SYBR Green I increased the melting temperature of DNA during PCR. We repeated the same experiments of PG amplification for PCR solutions containing SYBR Green II, and analyzed the PCR products under the same CE conditions (Fig. 3). This showed that the fluorescence intensity of DNA reached its maximum at the concentration of 1×, because the fluorescence mechanism of SYBR Green II is the hydrophobic aggregation of the DNA. Then the fluorescence intensity corresponding to the PCR products of PG began to reduce. This result also supports that SYBR Green II is designed for RNA stains. We can deduce that SYBR Green II first hydrophobically bonded onto the backbone of the DNA, and gradually bonded to the bases and started to inhibit PCR.

We also investigated the amplification of PG in PCR solutions mixed with Hoechst 33258, and then analyzed the PCR products under the above CE conditions (Fig. 4). This showed

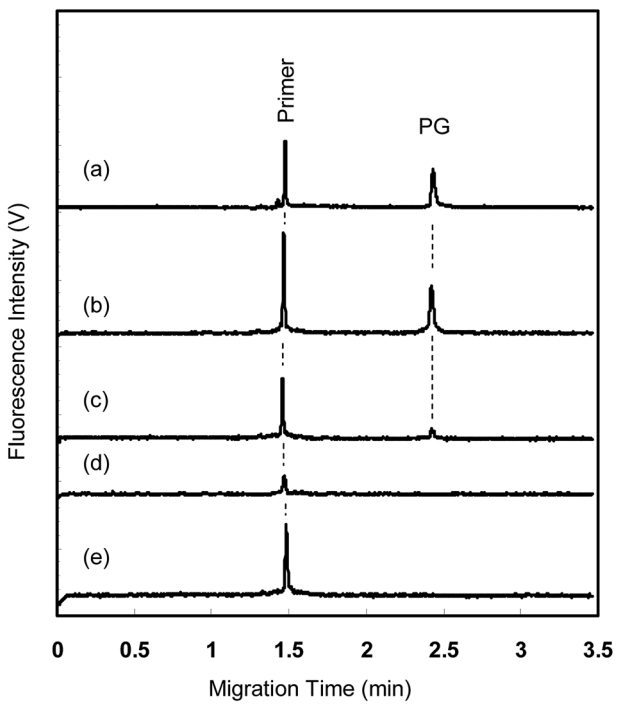

Fig. 1 The electropherogram of (a) positive control, (b) PG PCR product with SYBR Green II (1×), (c) PG PCR product with SYBR Green I (1×), (d) PG PCR product with Hoechst 33258 (1 μg ml^{-1}), (e) negative control. Electrophoretic conditions: effective length was 4 cm in a total length of 10 cm. Separation voltage: 2000 V; sieving polymer: 0.5% HEC (1300k)/(0.5× TBE); injections: 1000 V/1 s. For (a) and (e), the sieving polymer contained 1× SYBR Green I. For (b)–(d), the sieving polymer does not contain a fluorescent dye.

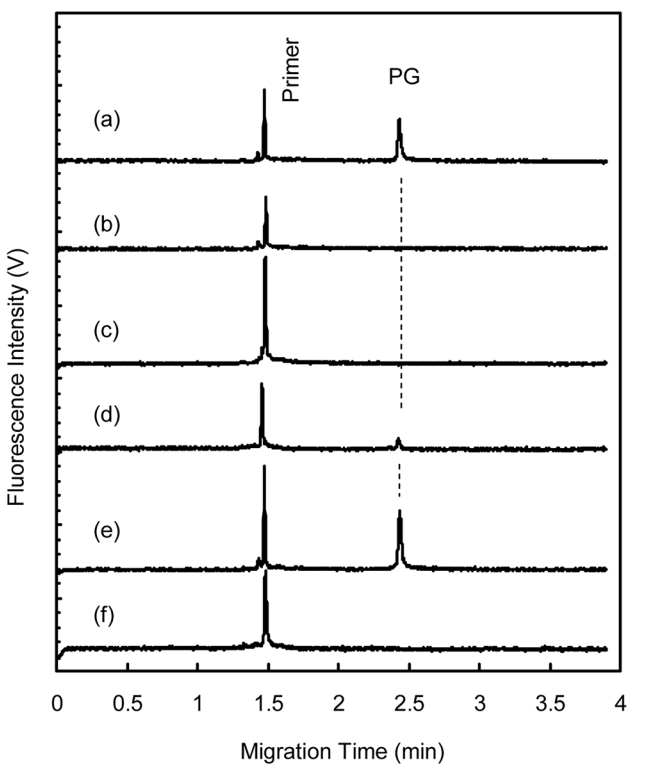

Fig. 2 The electropherogram of (a) positive control, (b) PG PCR product with 5× SYBR Green I, (c) PG PCR product with 2× SYBR Green I, (d) PG PCR product with 1× SYBR Green I, (e) PG PCR product with 0.5× SYBR Green I, and (f) negative control (without template). The electrophoretic conditions were the same as those in Fig. 1.

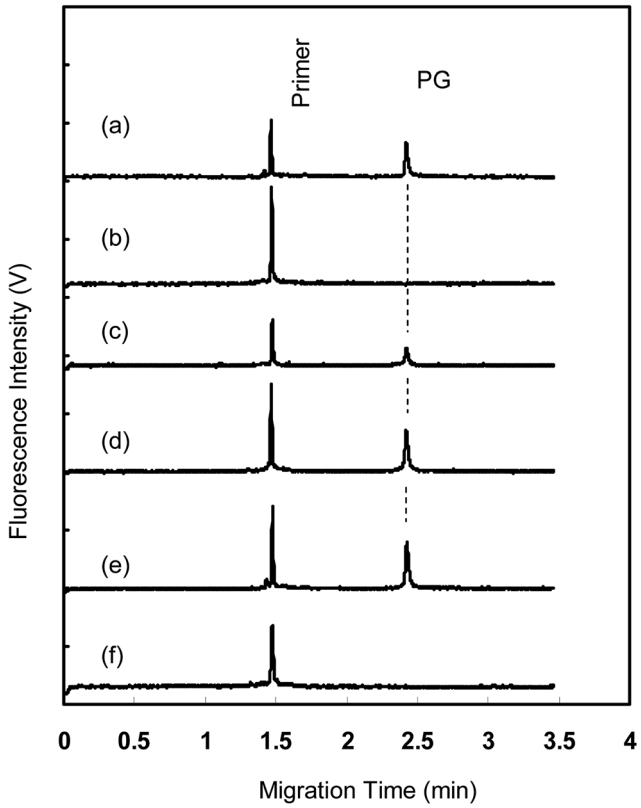

Fig. 3 The electropherogram of (a) positive control, (b) PG PCR product with 5× SYBR Green II, (c) PG PCR product with 2× SYBR Green II, (d) PG PCR product with 1× SYBR Green II, (e) PG PCR product with 0.5× SYBR Green II, and (f) negative control (without template). The electrophoretic conditions were the same as those in Fig. 1.

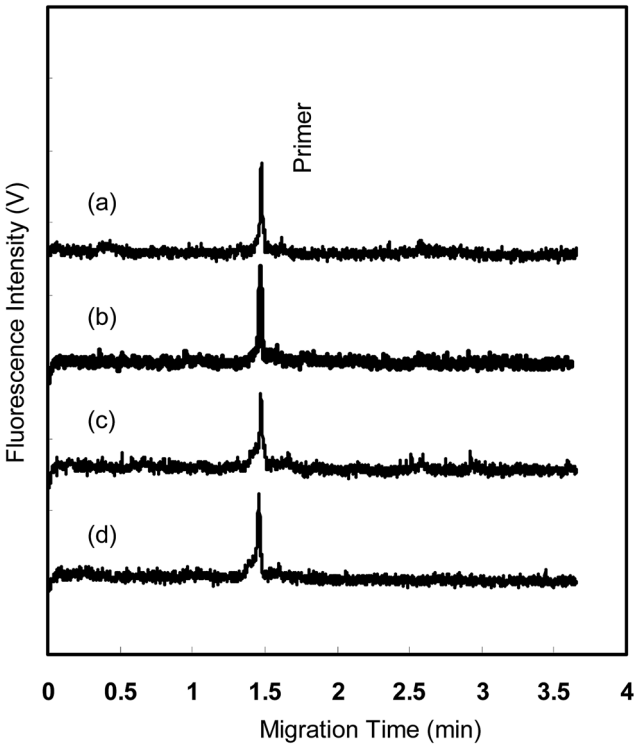

Fig. 4 The electropherogram of PG PCR product with (a) 5 μg ml^{-1} Hoechst 33258, (b) 1 μg ml^{-1} Hoechst 33258, (c) 0.4 μg ml^{-1} Hoechst 33258, (d) negative control (without template). The electrophoretic conditions were the same as those in Fig. 1.

that the PCR process was totally inhibited by Hoechst 33258, because no electrophoretic peak was observed. Furthermore, the inhibition of PCR was confirmed for each concentration of Hoechst. This is because Hoechst bonds to a specific DNA base pair, between adenine (A) and thymine (T), and therefore PCR was inhibited during the process of DNA extension and hybridization. Moreover, the reason that the peak intensity corresponding to primer in Fig. 4 appeared relatively small is because the fluorescence emission and excitation maxima are different between SYBR Green and Hoechst 33258.

Then we summarized the PCR efficiency (%) in Fig. 5. The PCR efficiency, resulting from the positive control of conventional PCR, was distributed between 0.46 and 0.54. This distribution was attributed to the uncertainty of PCR and the reproducibility of CE. Moreover, data in Fig. 5 show that each labeling dye inhibited PCR when its concentration was high. The threshold concentration of each dye was different. When the concentration of SYBR Green I and II was 1×, the PCR efficiency of the solution mixed with SYBR Green II was higher than that mixed with SYBR Green I. PCR was strongly inhibited by Hoechst with concentrations between 0.1 μg ml^{-1} to 5 μg ml^{-1}.

Finally, we carried out fluorescence measurements for the PCR product by comparing with the solution before PCR (Table 1). A simple fluorescence measurement was convenient for the analysis of PCR products. This measurement was basically performed by a fluorescence spectrometer in a quantitative PCR system. In our measurements of fluorescence intensity, the

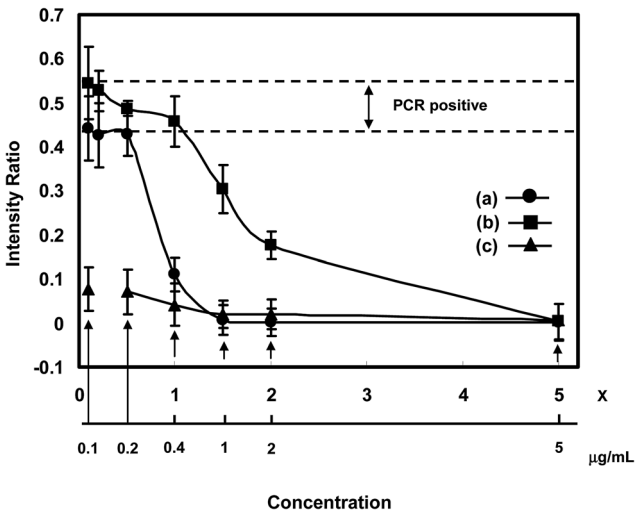

Fig. 5 Summary of the PCR intensity ratio with labeling dyes: (a) SYBR Green I, (b) SYBR Green II, and (c) Hoechst 33258. The efficiency of PCR in the positive control included the noise of the detector and the reproducibility of PCR and CE. The *x* axes denote the concentration of SYBR Green I and II (upper axis) and the concentration of Hoechst (lower axis).

Table 1 Direct fluorescence intensity detection of PCR products. Voltage was detected by a photo-multiplier (PMT) with amplicons of 200. The concentrations of SYBR Green I, SYBR Green II and Hoechst 33258 in PCR solutions were 1×, 1× and 1 μg ml^{-1}, respectively. The range was calculated from the standard deviation of the measurement repeated five times

Dye	Before PCR (V)	After PCR (V)	Difference (V)
SYBR Green I	0.30	0.62	0.32 ± 0.04
SYBR Green II	0.25	1.13	0.84 ± 0.05
Hoechst 33258	0.35	0.40	0.05 ± 0.02

PCR product with SYBR Green II was the most effective at 0.25 V before PCR and at 1.13 V after PCR.

Conclusion

To realize a hyphenated system of PCR and CE, the fluorescent dyes need to be mixed into the PCR solutions in advance. Therefore, the inhibition of the dyes on PCR is of great concern. In this study, we investigated the inhibition of nucleic acid labeling dyes on PCR by CE, and found that the inhibition was highly dependent on the concentration of the fluorescent dyes. Results demonstrated that SYBR Green II was more adequate for a hyphenated analytical system of PCR and CE. Such fundamental research is beneficial for the development of successful biological systems of PCR and CE, which may bring great advances for biological and medical diagnosis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We gratefully acknowledge support from the National Natural Science Foundation of China (No. 21205078) and Research Fund for the Doctoral Program of Higher Education of China (No. 20123120110002), and this project was partly supported by Grand-in-Aid for Scientific Research (Houga) (No. 25600049 (Y. Y.)), JSPS, Japan. We also thank Mr Tsuji at Yamato Esulon Co., Ltd (Osaka, Japan) for the technical assistance in this experiment.

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