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Rolling Circle Amplification and Circle-to-circle Amplification of a Specific Gene Integrated with Electrophoretic Analysis on a Single Chip

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We have developed an integrated platform for rolling circle amplification (RCA) and circle-to-circle amplification (C2CA) of circular probe (padlock probe) and subsequent microchip electrophoretic detection of a specific gene on a poly(methyl methacrylate) microchip. RCA and C2CA were successfully carried out at a steady temperature of 37 °C in the sample well of the microchip, and their respective product was detected on the same channel of the microchip, which was prefilled with a polymer separation matrix and fluorescent dye. Using a species-specific padlock probe for bacterial pathogen *V. cholerae*, a 25-ng bacterial genomic DNA could be detected in less than 65 min (including RCA and microchip electrophoresis) by this platform. Stable dsDNA C2CA product of genomic DNA for *V. cholerae* can be detected with the introduced integrated platform. Furthermore, the usefulness of this technique for the monitoring of RCA was demonstrated. This integrated platform provides a sensitive, fast, high-throughput, and reproducible method for signal amplification and detection of the padlock probes in the same microchip and is a promising tool for highly specific gene detection strategies.

Padlock probes are circularizing oligonucleotide probes with two complementary ends to the target DNA strand.[1] Following the perfect hybridization of target and padlock probe, the two adjacent ends could be joined by ligation. Padlock probes, coupled with rolling circle amplification (RCA), circle-to-circle amplification (C2CA), or both, have been reported as highly selective probes for detecting specific nucleic acids (DNA/RNA) variations and pathogen diagnoses, since these probes ensure high specificity and excellent selectivity for sequence variants at single nucleotide resolution.[2−4]

RCA is an effective linear-isothermal amplification method in which certain enzymes can be utilized to amplify a single-stranded DNA (ssDNA) minicircle target, such as a padlock probe. The method was discovered in the mid 1990s while investigating the replication of phage genomes.[5,6] The robustness, high potential, and simplicity of RCA soon made it a powerful DNA diagnostic technology among other isothermal amplification techniques for probe/signal amplification.[7,8] RCA product differ from other biological molecules in a way that the amplicon is a single-stranded tandem repeated copy of the circularized probe, typically including 1000 long units. To date, techniques for the analysis of RCA product have included radiolabeling,[7] UV absorbance,[8,9] conventional gel electrophoresis,[8,9] fluorescence,[10] and single molecule detection.[11−13] Microcapillary tubes have been used to detect RCA product using padlock probes, in which the primer was immobilized on the surface and the signal measurement was done with microfluorometry.[14]

C2CA is a robust and precise RCA-based amplification method for small DNA circles.[15] A billionfold amplification of reacted

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probes can be achieved with this highly controlled process. However, presently techniques for detection of C2CA have been limited to microarrays, real-time monitoring using molecular beacons, and denaturing acrylamide gel electrophoresis.[15] Although all of the above techniques are quite accurate, they are not practically suited for medical diagnostics because of extensive post-amplification handling and expensive labeling procedures.

Microchip electrophoresis (μ-CE) is a rapidly growing technique for the separation of biomolecules offering high speed, easy automation, low cost, and versatility for routine applications.[16−21] Combination of the highly specific gene amplification techniques and the high-throughput micro total analysis systems (μ-TAS) on disposable microchips would provide a powerful strategy for medical diagnostics. Recently we reported a reliable μ-CE detection method for C2CA product produced in an off-chip reaction.[20] In this paper, we developed a method for simple on-chip RCA and C2CA and downstream electrophoretic analysis directly after the reaction, which is strongly required for detection of genomic DNA variations and pathogens diagnoses.

EXPERIMENTAL SECTION

Chemicals. DNA fragments (HPLC purified) were ordered from Sigma Genosys. Φ29 polymerase, *Rsa*I, and DNA polymerase I Klenow fragment (Klenow fragment) were purchased from New England Biolabs. T4 DNA ligase was bought from Amersham Bioscience. Ampligase enzyme was ordered from Epicentre. SYBR Gold and TO-PRO-3 were purchased from Molecular Probes. Poly(ethylene oxide) (PEO) with different molecular weights (8×10^6, 1×10^6, 4×10^5, 1×10^5), Hydroxypropyl methyl cellulose (HPMC)-4000 and TBE 10× buffer (89 mM boric acid, 2.5 mM Tris-EDTA, pH 8.3) were purchased from Sigma-Aldrich.

System Setup. A poly(methyl methacrylate) (PMMA) microchip (i-chip3; Hitachi) was used for carrying out both RCA/C2CA and μ-CE. The sample well of the microchip was used as a reaction chamber as described in the following. First, the channels were filled with an appropriate polymer premixed with dye (PEO mixed polymers were mixed with SYBR Gold for detection of RCA product; HPMC-4000 polymer was mixed with TO-PRO-3 or SYBR Gold for the detection of synthetic or clinical samples, respectively, through C2CA). Then, three buffer reservoirs were filled with the same polymer−dye mix as the channels and RCA or C2CA reaction mixtures were added to the sample well. Next, all the channels were covered by a costar PCR seal (Corning). The incubation was done in a block incubator (Astec). C2CA was carried out by opening the seal at the beginning of each addition step and after a new addition to the sample well and covering the well again; the incubation was continued as long as desired. The analyses of RCA of a synthetic and genomic DNA sample and C2CA of a genomic DNA sample were carried out using a Hitachi SV1100 μ-CE system, which was equipped with a blue-light emitting diode, emitted at 470 nm with a photodiode detector. DsDNA C2CA product of synthetic DNA sample were detected using a Hitachi SV1210 μ-CE system, which was equipped with a diode laser emitting at 635 nm.

Preparation of RCA and C2CA Reaction Mixtures. The bacterial specific padlock probe was designed at Uppsala University. For this design, the strain-specific target sequences were first chosen. Several alternative 30-bp target regions for *Vibrio cholerae* were identified using the probe design tool in ARB software (version 2.5b). The information for the 16S rRNA of the chosen strain is available in Genbank (DQ068935, Z21729). The target sequence candidates were queried against all completely sequenced bacterial genomes using BLASTN 2.2.10 as well as the 16S rRNA sequences in Genbank. Finally, the padlock was designed for detection of *V. cholerae*. The designed padlock probe could be hybridized to all known 16S rRNA sequences. We used two kinds of DNA targets: a synthetic DNA target that is described in this section and a target from a clinical sample that is described in the next section. The DNA circle was produced by ligation of a 100 nM phosphorylated padlock probe (5′ P-TAG GTT GAG CCC AGG GAC TTC TAG AGT GTA CCG ACC TCA GTA GCC GTG ACT ATC GAC TTG CGT CTA TTT AGT GGA GCC CAA ATG CGA TTC C 3′) circularized on 600 nM of the target (5′ CCC TGG GCT CAA CCT AGG AAT CGC ATT TG 3′). A 4 mu/μL T4 DNA ligase was used in a ligation mixture (1 mM ATP, 0.2 μg/μL BSA, 5 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $(NH_4)_2SO_4$, 0.4 mM DTT), and this reaction was done in a microtube. All RCA and C2CA described here were done in the sample well of the microchip, and all the reaction conditions were for an on-chip reaction. C2CA has been previously reported for an in-tube reaction.[15] In this report, we scaled down the previously reported reaction volume by 5-fold to be able to carry out the reaction in the space of the microchip sample well. The DNA circle prepared in the above-described ligation reaction was serially diluted in PBS buffer and then was used as initial circle. A 10 nM final concentration of DNA circle was used in the replication by the following method. One microliter of a 1/100 dilution of circle (in PBS buffer) was added to 9 μL of replication mix containing 100 μM dNTP mix, 10 nM replication oligonucleotide (RO-), 4 mu/μL φ29 polymerase and 1× φ29 polymerase buffer (0.2 μg/μL BSA, 5 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM $(NH_4)_2SO_4$, 0.4 mM DTT) in a total volume of 10 μL. The sequence of RO- was as follows: 5′-TAC TGA GGT CGG TAC ACT CT 3′. The time-course RCA experiment (using a synthetic DNA target) was carried out for 0, 15, 30, 45, and 60 min polymerization times at 37 °C followed by a 10-min incubation at 65 °C to inactivate enzyme. RCA for genomic DNA detection was carried out at a constant temperature of 37 °C for 60 min.

C2CA was started with RCA using the above-described method. For both the monomerization and duplication steps, a 1-μL addition volume was used. The monomerization was carried out by mixing 182 nM replication oligonucleotide (RO+, 5′ AGA GTG TAC CGA CCT CAG TA 3′), 0.2 μg/μL BSA, and 10 units *Rsa*I restriction enzyme, in 1× φ29 polymerase buffer followed by incubation at 37 °C for 10 min. The duplication mixture in a total volume of 1 μL was made as follows: 0.2 μg/μL BSA, 1× φ29 buffer, 40 mM dNTP mix, and 5 units of Klenow fragment enzyme were mixed

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and then added to the monomerized product followed by an incubation at 37 °C for 10 min.

Clinical Sample Preparation. The bacterial sample was isolated from a patient in Nagoya University Medical School Hospital. The bacterial sample was identified as *V. cholerae* O1 serovar, Ogawa. The strain properties were confirmed by a Japanese health facility center. Genomic DNA from *V. cholerae* was purified with a genomic DNA purification kit (Promega) according to the manufacturer's instructions. The concentration of genomic DNA was measured as 372 ng/μL using a Nanodrop ND-1000 UV−vis spectrophotometer from (Nanodrop Technologies Inc.). One microliter of extracted DNA was diluted in 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA). Then 25 or 700 ng of the above diluted genomic DNA was used for RCA and C2CA, respectively. Genomic DNA was digested in a reaction containing 10 units of *Rsa*I, 1× NEB2 buffer, and Milli-Q water in a final volume of 20 μL at 37 °C for 1 h, and then the enzyme was inactivated at 65 °C for 10 min. The digested genomic DNA was introduced to the ligation reaction to be ligated to the padlock probe. Ligation was carried out by the following method: 5 μL of digested genomic DNA was mixed with 1 μL of 4 nM phosphorylated padlock probe, 2.5 units of Ampligase, 0.2 μg/μL BSA, 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, and 0.01% Triton X-100 in a total volume of 10 μL. The reaction was carried out at 95 °C for 10 min and 55 °C for 3 h. A 5-μL portion of the above ligation reaction was used for subsequent RCA or C2CA. The reagent concentrations used for RCA and C2CA of the clinical sample were the same as those used for the synthetic target. RCA, or C2CA for the negative control was done by using Milli-Q water instead of DNA circle.

Polymer Preparation and Microchip Electrophoresis Condition. Preparation of polymer solution was performed using the method as described before.[22] Briefly, a mixture of PEOs having four different molecular weights (MWs) was mixed with the percentages of 0.01% (MW 8×10^6), 0.02% (MW 1×10^6), 0.15% (MW 4×10^5), and 0.2% (MW 1×10^5) in TBE 1× buffer. The mixed polymer solution was stirred for 1 h for a complete dissolving, and it was then kept at 4 °C. The 1.5% HPMC-4000 solution was prepared in TBE 1× buffer. Detection of RCA product using the SV1100 μ-CE system was carried out using the following program. Sample injection was done by applying 300 V at the sample waste reservoir and grounding the other three reservoirs for 80 s. Separation was done by applying 150 V at the sample and sample waste reservoirs, while the buffer reservoir was grounded, and the analysis reservoir was set at 800 V for 400 s. Detection of C2CA product of a clinical sample using the SV1100 μ-CE system was carried out using the following method: Sample injection was carried out by applying 300 V at the sample waste reservoir and grounding the other three reservoirs for 100 s. Separation was carried out by applying 130 V at the sample and sample waste reservoir, while the buffer reservoir was grounded, and the analysis reservoir was set at 750 V for 200 s. The electrophoretic injection for analysis of C2CA product of a synthetic sample using the SV1210 was done for 80 s by applying a 300-V injection voltage at the sample waste reservoir while the other reservoirs were grounded. The separation was accomplished for 140 s by applying 130 V at the sample and the sample waste reservoir while the buffer reservoir was grounded and the analysis reservoir was set at 750 V.

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RESULTS AND DISCUSSION

Principal of RCA. RCA is an amplification method for small DNA circles and can very favorably be combined with padlock probes (as templates for RCA) in a highly specific manner, since only circularized probes can be amplified using RCA. Briefly, in an RCA reaction using padlock probes, a specific linear padlock is first hybridized and ligated to its target in a ligation reaction, forming a DNA circle. A DNA polymerase can then start RCA by extending a RO, which is complementary to a part of the circle. The enzyme continuously progresses around the circle while it displaces the amplified fragment and produces a long ssDNA concatemer, containing several complementary copies of the original circle. (φ29 polymerase, with its high strand displacement activity, is a perfect choice for this reaction.) A schematic illustration of hybridization and ligation of the padlock probe to the target plus RCA is shown in Figure 1A and B, and in the first part of Figure 1C.

Principal of C2CA. C2CA, which is an RCA-based amplification, was originally used for highly specific amplification of padlock probes and can be performed in a few (up to three) cycles. Each cycle can be done via the three steps of replication, monomerization, and ligation. Figure 1C shows the steps of C2CA. Usually the starting DNA circle is considered as positive polarity, and the polarity of the complement is judged based on that. Briefly, in each cycle, the tandem repeated complement of DNA circle (RCA product, negative polarity) is generated in the replication step by extending RO- complementary to the circle. RCA product is then subjected to restriction enzyme and RO+ to be cut into linear monomer (monomerization). This monomer can then be directed to the ligation step, which mediates generation of circular ssDNA monomer, and be continued to the next cycle (the last ligation step is not shown in this figure).

We modified C2CA by adding an alternative step for ligation, which termed "duplication" (Figure 1D). This means that the last cycle's monomerized product can be used for ligation or otherwise, as we described, for duplication. In this step, the monomerized product is directed to make double-stranded (dsDNA) product using the Klenow fragment, ensuring the generation of blunt-end dsDNA. In more detail, in this reaction first, intact (uncut) RO+ that is in excess hybridize to both ends of the monomerized product, creating a transient open circle. The polymerization reaction then starts from the 3′ end of the attached RO+ or the monomer. The Klenow fragment will then displace the 5′ end of the attached RO+, and the ssDNA fragment will be filled to make blunt-end dsDNA product. The 3′ end filling activity of this enzyme will result in an increase in the size of the final dsDNA product, based on the length of the overhang sequence between RO+ and monomer. Finally, stable dsDNA C2CA product will be formed for further electrophoretic analysis.

Integration of RCA and C2CA with Microchip Electrophoresis. RCA and C2CA can be carried out at a constant temperature of 37 °C, and the highest temperature in the whole dsDNA C2CA formation is 65 °C for the enzyme inactivation at the end of the replication and monomerization steps. The ability to carry out the reaction at a constant low temperature makes the PMMA chamber a suitable environment for them, because it accords very well with the PMMA glass transition temperature.

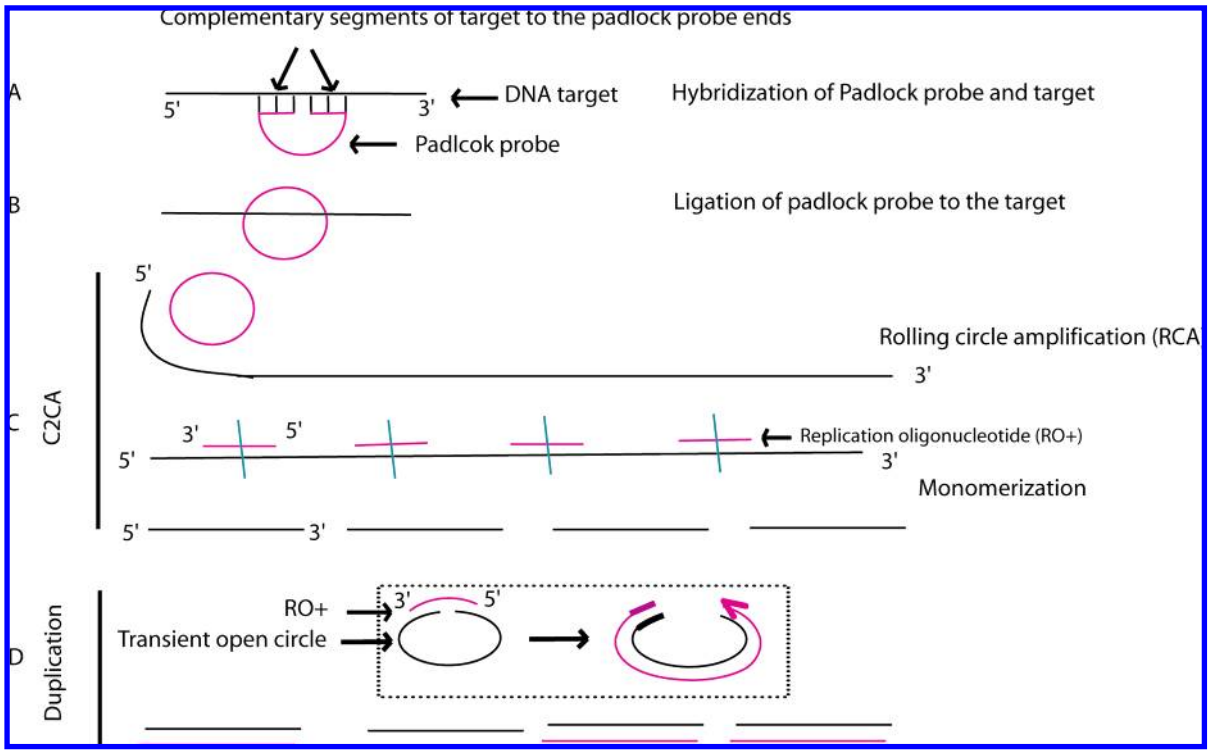

Figure 1. Schematic illustration for the principal of RCA and C2CA and generation of dsDNA C2CA product. Positive and negative polarities are shown in pink and black, respectively. (A) Padlock probe hybridization to target, (B) Ligation of padlock probe to target. (C) C2CA. RCA is the starting point of C2CA. C2CA then can procede to the monomerization step, in which monomer appear upon restriction digestion with the aid of RO+, (D) Duplication step added to C2CA for converting ssDNA to dsDNA product.

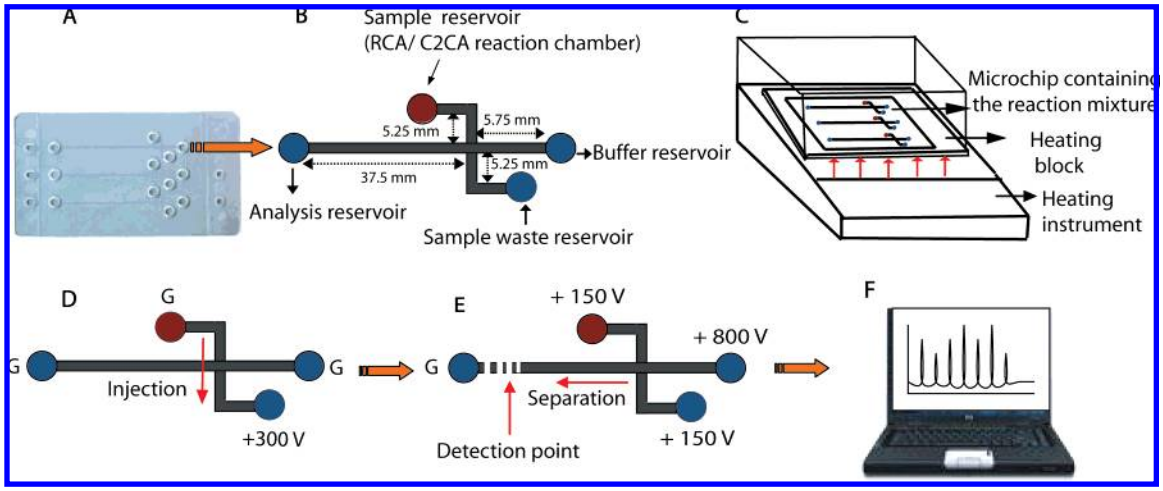

Figure 2. System design for RCA/C2CA and μ-CE integrated platform. (A) An image of the microchip having channels with 100-μm width, 30-μm depth, and effective separation length of 30 mm. (B) Schematic view of a single channel and its reservoirs. The distances from the cross-channel to the reservoirs are indicated. (C) Putting the microchip containing the RCA/C2CA reaction mixtures in a block incubator. (D) and (E) The injection and electrophoretic separation, respectively. The indicated separation voltages were related to RCA. (F) Analysis of the results.

Figure 2 shows system design for the integrated platform for RCA and C2CA. The PMMA microchamber was compatible with all reagents used for RCA and C2CA and the enzymes showed good activity. As described in the Experimental Section, the channels and reservoirs should be filled with polymer solution before adding the reaction mixture to the sample well. This strategy not only facilitates the subsequent electrophoretic analysis but it also prevents evaporation, so the choice of an appropriate polymer is crucial for optimal sample analysis.

Linear polymers have been reported to have great potential for DNA separation in μ-CE analysis.[16,18] Use of low-viscosity polymers is advantageous for loading into the microchip channels due to their easy injection properties. The resolving power of low molecular weight matrixes has proven insufficient for resolving a wide range of DNA sizes. However, mixing of low molecular weight polymers with high molar mass polymers has enhanced resolution for DNA separations. A wide-range dsDNA separation (1−40 kbp) using a low-visosity mixed PEO solution with percentages of 0.01% (MW 8×10^6), 0.02% (MW 1×10^6), 0.15% (MW 4×10^5), and 0.2% (MW: 1×10^5) in TBE 1× buffer was introduced previously.[22] Considering RCA product, which also include a large

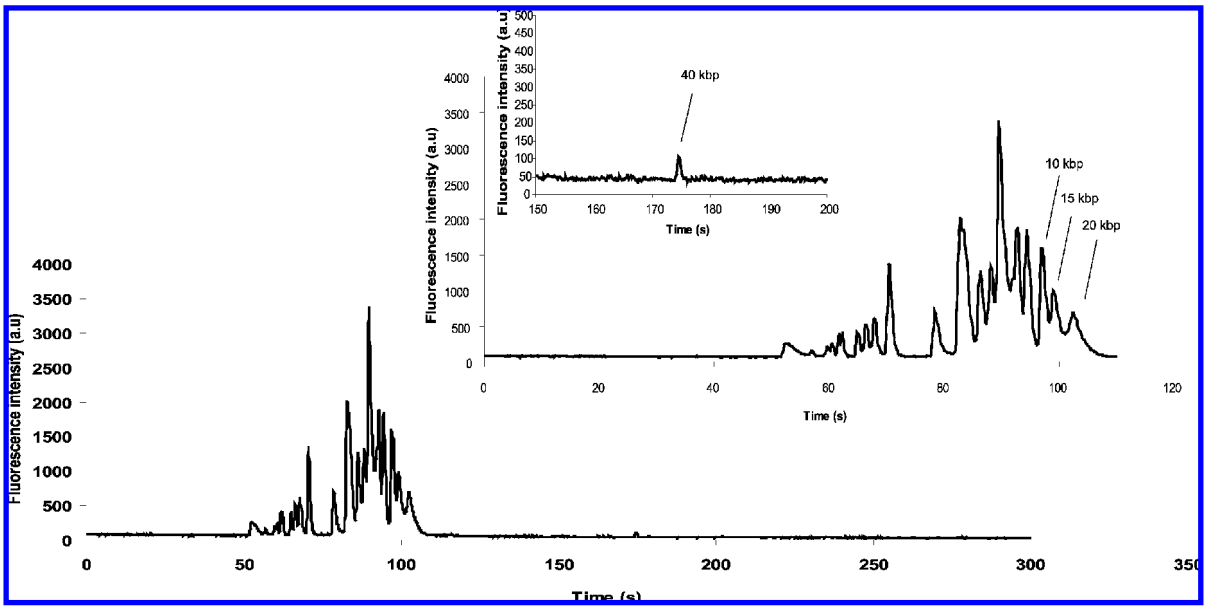

Figure 3. Separation of 1 kbp extension ladder using PEO mixed polymer solution and SYBR Gold. The ladder consisted of the following DNA bands: 1, 1.6, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, and 40 kbp. All the fragments could be detected in less than 200 s. The inset shows the separation from 0 to 120 s, and the internal inset shows the separation from 150 to 200 s.

range of fragment sizes, we chose this mixed polymers solution for analysis of RCA product. Figure 3 shows the separation of a 1-kbp extension ladder with the mixed PEO solution. As shown there, the mixed PEO solution had a high resolving power, separating DNA fragments from 1 to 40 kbp.

For C2CA, 1.5% HPMC-4000 polymer in TBE 1× buffer was chosen to separate the RO+ from the dsDNA C2CA product and be able to be suitable for injection into the channels of the PMMA microchip.

On-Chip RCA and Electrophoretic Detection. RCA was successfully carried out in the sample well space of the microchip, and the product was subsequently detected by μ-CE. RCA product of a synthetic target was detected with high intensity after 60 min and the reaction was monitored for 0, 15, 30, 45, and 60 min amplification time courses too. Figure 4 shows the detection of RCA product of a synthetic target (using a starting concentration of 10 nM DNA circle) over time. RCA product could be detected using either the SYBR Gold dye premixed with polymer solution or after adding it directly to the sample well prior to analysis. RCA product is repeating end-to-end multimeric copies of circular DNA. Observation of several peaks is a normal feature of RCA product in electrophoresis, as the reaction is characterized by smearlike amplicons.[23] In addition, the product was checked with SYBR Green II dye, which is specific for ssDNA (data not shown). The similar general pattern of peaks showed that both dyes could be used for the detection of RCA product. Since SYBR Gold dye was more fitted to the excitation and emission range of our instrument, and had a higher sensitivity, we decided to use this dye.

The sizes of RCA product could be estimated, based on the fact that generally φ29DNA polymerase replicates short DNA circles at an average rate of ~1500 nt/min.[7] Therefore, the size for RCA product after 15, 30, 45, and 60 min of amplification was estimated as 22, 45, 68, and 91 kb. Considering the nature of RCA, which produces a range of sizes of ssDNA fragments, not a single size, we would expect a variety of size ranges to be present in the solution.

The electropherograms for the time-course RCA in Figure 4 could be explained as follows: At 0 min after starting the reaction, only a baseline intensity is observable. At 15 and 30 min after initiation of the reaction, the fluorescent intensity was increasing slightly and some low-intensity peaks were appearing, but as described above, large DNA fragments should be produced after 15- or 30-min reactions, and more peaks with higher intensities would have been expected. The data achieved from in-tube reaction (using 5-fold higher reaction volume) in our recent study confirmed this expectation (data not shown). We reasoned that the high concentration of reagents and buffer components in the reaction solution may interfere with the dye−DNA reaction, explaining the lower peak numbers with lower intensities that appeared than expected. As the reaction time increases to 45 and 60 min, the consumption of buffer components allows more peaks with higher intensities to be observed, which shows the progress of the reaction. Generally, the peak intensities were increasing over time, which could be explained by the reduced effect of buffer components, generation of more amounts of the product in all size ranges, and intercalation of higher amounts of the dye molecules to the longer generated DNA fragments for longer reaction times.

Another point in the electropherograms is the existence of discrete peaks instead of continuous ones as is observable in conventional gel electrophoresis analyses in which low percentages of agarose gel have been used in denaturing or nondenaturing conditions.[8,9] We speculate that this difference may be due to the higher resolution of the PEO mixed solution we used here (each discrete peak did not belong to a single size but a variety of close sizes of DNA fragments). As we could see in Figure 4, the general patterns of the electropherograms for the 45- and-60 min reactions were somewhat different, which could be explained

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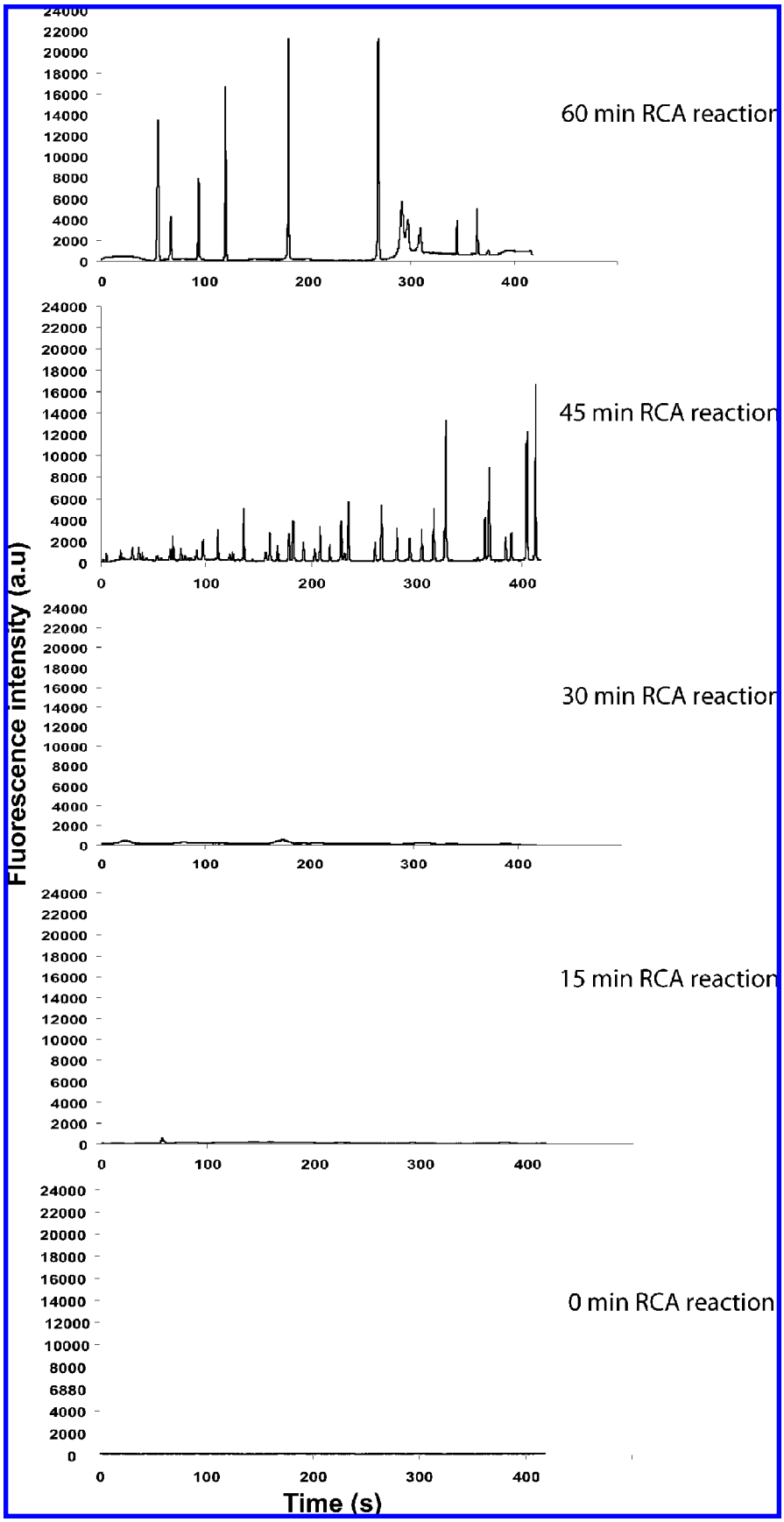

Figure 4. Time-course analysis of RCA product from a synthetic target. RCA was monitored for 0, 15, 30, 45, and 60 min after the amplification was initiated. The generation of RCA product was clearly observable after 45 and 60 min. Separation matrix and conditions are as in Figure 3.

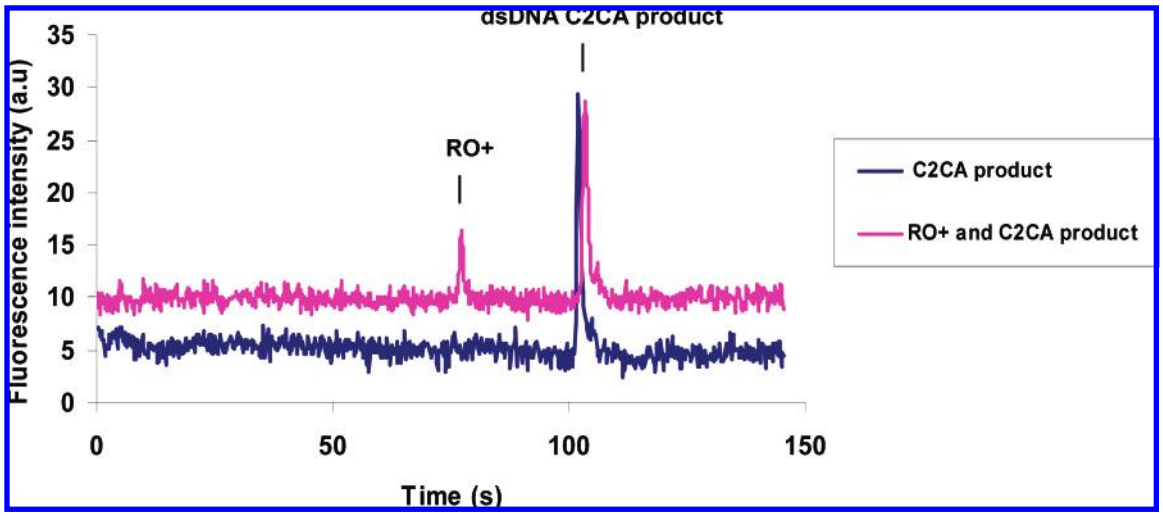

Figure 5. Detection of dsDNA C2CA product using the integrated platfrom. 1.5% HPMC-4000 in TBE 1× buffer mixed with TO-PRO-3 was used for separation. The lower electropherogram shows the detection of dsDNA C2CA product and the upper electropherogram shows separation of the RO+ and dsDNA C2CA product.

by the anomalous migration of RCA product so that we would not expect a well-defined peak.

We could not determine the exact sizes of RCA product, because of the lack of a large DNA marker over 1 kb and also the mobility of ssDNA in nondenaturing conditions, which strongly depends on its sequence-dependent conformation.[24,25] Further experiments are necessary to observe the injection and behavior of such long ssDNA fragments' migration during the electrophoresis using fluorescence microscopy.

Since the analysis of RCA product using a linear polymer was performed for the first time in this study and in an on-chip format, carrying out the time-course analysis of this product in a microtube reaction (using higher reaction volumes), followed by a purification step before the microchip electrophoretic analysis, will be helpful for better understanding of the peak distribution in the electropherograms.

On-Chip C2CA and Electrophoretic Detection. Duplicated product of C2CA, which is more reliable for the detection of C2CA product using μ-CE, was detected successfully using the integrated platform. Figure 5 shows detection of the dsDNA C2CA product of a synthetic target after the first cycle of C2CA. The lower electropherogram shows the detection of dsDNA C2CA product using 1.5% HPMC-4000 in TBE 1× buffer mixed with TO-PRO-3 dye. The remaining RO+ should have a low concentration and would not be detected. In another analysis (upper electropherogram), the same sample plus extra RO+ (0.6 μL of 5 μM RO+) was added to the sample prior to the electrophoresis. Analysis of this sample showed a good separation of the RO+ (20 b) and the dsDNA C2CA product (98 bp), confirming that the peak of the lower electropherogram was for the dsDNA C2CA product. For further confirmation, product size was compared with a 100-bp ladder using agarose gel electrophoresis (data not shown). The relative standard deviation of peak migration times was calculated to 1.5% ($n = 6$) indicating the reproducibility of the method. We observed that using a high-viscosity mixed PEO solution, with MWs and percentages of 8×10^6, 0.3%; 1×10^6, 0.6%; 4×10^5, 0.9%; and 1×10^5, 1.2%, in TBE buffer 1× was not suitable for our system, since the polymer dried rapidly; so after a 60-min incubation, the solution inside the channels had nearly all evaporated. Due to the problems with the size detection of ssDNA fragments, we avoided analyzing the monomerized C2CA product, and the reaction proceeded to produce dsDNA C2CA product.

Detection of Clinical Sample. The padlock probe ligated to species-specific sequence in the 16S rRNA gene of a bacterial pathogen *V. cholerae* (an acute human diarrhea agent) was amplified and successfully detected using the introduced platform. Figure 6A shows the detection of the clinical sample using a starting 25-ng amount of genomic DNA. This result proved that even 1 h RCA, with a linear amplification mode, was sufficient to detect a bacterial genomic DNA. This meant that our integrated platform was sensitive enough for the detection of clinical samples.

A clinical sample was then detected using the on-chip C2CA (Figure 6B). In our previous report, two cycles of C2CA plus a purification step were shown necessary for the detection of C2CA products of a genomic DNA sample using μ-CE.[20] In our present microchip, the maximum space in the well was 12 μL, while for performing the second cycle using the established method, at least a 15-μL volume would be needed. So we first tried to detect a variety of concentrations of the genomic DNA sample after one cycle of C2CA using 1.5% HPMC and TO-PRO-3 for which no peak was gained. Then, we used the same polymer (1.5% HPMC) but changed the dye to SYBR Gold, which provides higher intensity. As a result, we could successfully detect a clinical sample using 700 ng of genomic DNA after one cycle of C2CA. This amount of genomic DNA could not be detected when TO-PRO-3 dye was used (data not shown). Figure 6B (top) shows the detection of a 700 ng of the genomic DNA after one cycle of C2CA. The size of the product was confirmed using a 20-bp DNA ladder (Figure 6B, bottom). The reason for requiring such a high concentration of genomic DNA was attributed to the presence of buffer components in the reaction solution. It meant that, for analysis of a clinical sample, the microchip is needed to be equipped with a purification

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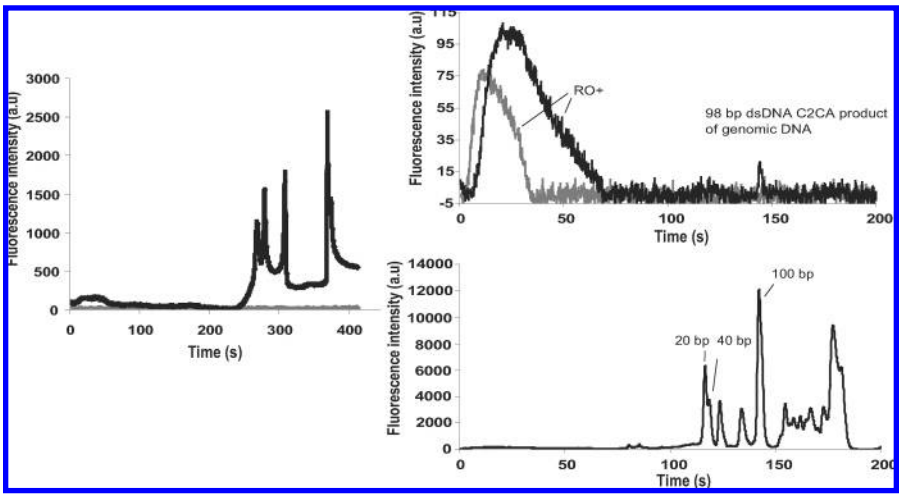

Figure 6. Detection of *V. cholerae* with on-chip RCA or C2CA integrated platform. The black and gray colors correspond to the amplified genomic DNAs and the negative controls, respectively. (A) Analysis of RCA product. The separation conditions are as in Figure 3. (B) Top: The 98-bp dsDNA product. 1.5% HPMC-4000 mixed with SYBR Gold in TBE 1× buffer was used for the separation. Bottom: separation of a 20-bp ladder using the same matrix.

chamber and a higher volume reaction chamber to be able to perform two or three cycles of C2CA. These modifications are necessary for quantification of genomic DNA samples, which are usually available in low concentrations.

In addition to the requirement of elevated temperatures for normal PCR techniques, RCA and C2CA have the following advantages over PCR. RCA shows a good reproducibility, with the amplification errors being lower compared to PCR. Furthermore, the simplicity, and more importantly, the specificity for detecting single base mutations is much superior compared to PCR. Although the linear mode reaction of RCA limits generation of product in high quantities, the use of highly processive enzymes assists having more product. Furthermore, C2CA is also superior to PCR, having higher accuracy for detection of single nucleotide polymorphisms (SNPs), a more highly controllable reaction and yielding ~100-fold higher concentrations of product. It would be significantly advantageous to design an integrated platform that connects RCA and C2CA to the high-throughput μ-CE.

CONCLUSIONS

We have shown that two very specific and precise methods to amplify DNA, RCA and C2CA, could be carried out in a PMMA chamber with good compatibility. RCA product was detectable even a few minutes after the reaction was initiated. One of the major advantages of using the integrated platform was that it combined the simplicity of RCA, which requires only one reaction temperature for amplification (37 °C), with a simple and fast μ-CE, providing a reliable and fast technique for SNPs analyses. To our knowledge, this is the first time that a platform for RCA and C2CA with subsequent μ-CE has been reported. There are no reports describing detection of RCA product using μ-CE, in either off-chip or on-chip formats. The cost for RCA and C2CA was reduced remarkably because of the 5-fold reduction in reagent consumption. A bacterial genomic DNA was successfully detected using the introduced method. Due to clinical importance of C2CA-based detections in which single stable dsDNA fragment can be produced, modification of chip design to include a purification chamber and a reaction chamber with larger space is necessary for the quantification of low copy number genes in genomic DNA samples. Because of the obvious ability of RCA to detect antibodies, with a suitable coating, this kind of microchip could be used for immuno-RCA and detection of proteins. Finally, this integrated platform shows promise for fast and reliable detection of specific genes.

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