Bead-Based Padlock Rolling Circle Amplification under Molecular Crowding Conditions: The Effects of Crowder Charge and Size

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Abstract

Bead-based padlock rolling circle amplification under molecular crowding conditions, which we have developed for ultrasensitive detection of DNA, is examined to improve the detection efficiency and sensitivity of the method as well as to gain insight into the mechanism of the method. Both non-magnetic and magnetic sepharose microbeads were employed. Biotinylated DNA had to be pre-immobilized onto the microbeads in order to obtain the products on the magnetic beads. The optimal concentration of biotinylated DNA was found to be about 5 μM, above which the number of products decreased. The effect of crowder charge was examined, and neutral polymers were found to be effective on ligation and hybridization step, while charged polymers were only effective on hybridization step and inhibited the ligation and primer extension. The effect of the molecular weight of neutral dextran on the number of products was investigated, and the number of products was found to be increased with the increase in the molecular weight of dextran.
Introduction

Ultrasensitive detection of small amounts of DNA is important in various aspects of society, such as criminal investigations and medical diagnoses. Various methods have been proposed to detect small amounts of DNA, such as enzymatic amplification of the DNA \(^1,2\) or amplification of the signal derived in the presence of the DNA.\(^3,4\) In particular, for ultrasensitive detection of DNA in field analysis, a method for amplifying and detecting DNA at room temperature is required, rather than the PCR method, which requires temperature variation.

The padlock/rolling circle amplification (RCA) method is capable of amplifying and detecting DNA at room temperature.\(^5,6\) In this method, both ends of the oligonucleotide, called a padlock probe, are hybridized to the target DNA. If the sequence of interest is present in the target DNA, the padlock probe forms a complementary strand with the target DNA, and the ends of the padlock probe are linked to each other by DNA ligase to form a circular DNA. By adding \(\phi 29\) DNA polymerase to the system, long single-stranded DNA is synthesized at room temperature using the circular DNA as a template. The long single-stranded DNA has a repeated complementary sequence to the circular DNA, so the addition of fluorescently labeled DNA having the same sequence of a part of the circular DNA results in binding of multiple fluorescently labeled DNAs to one molecule of long single-stranded DNA. As a result, a single molecule of long single-stranded DNA can be detected under a standard fluorescent microscope, which is a promising method for high-sensitivity detection of DNA. However, the conventional padlock/RCA method has a problem of low detection efficiency (~0.02 %).\(^5\)

We have made two efforts to improve the detection efficiency and sensitivity of
the padlock/RCA method. The first is the use of microbeads. Primer DNA, which is part of the reaction product, is bound to the agarose-based microbeads and the product is concentrated on the beads.\textsuperscript{7,8} The second is the use of molecular crowding.\textsuperscript{9} Molecular crowding refers to the state in which the activity of the reactant species is increased by the high concentration of polymers in the reaction system. We have reported that the number of products increased when the bead-based padlock/RCA was performed under a molecular crowding conditions.\textsuperscript{10} In addition, using PEG as a crowder, we have investigated the mechanism of the increase in the number of products and evaluated the effect of PEG molecular weight and concentration on the number of products.\textsuperscript{11} However, we believe that further studies are necessary to understand the mechanism of this method and to improve its performance. The first consideration is the timing and amount of the primer DNA to be immobilized to the beads. In previous studies,\textsuperscript{8,10,11} excessive amounts of primer DNA were immobilized onto the beads first, followed by the padlock/RCA. The second was the use of a crowder other than PEG. In addition to PEG, various other crowders have been used in molecular crowding studies.\textsuperscript{9} In this study, we focused on dextran, which is commonly used as a crowder as well as PEG. The effect of molecular weight of the dextran was investigated by experiments because dextran is an electrically neutral polymer like PEG and dextran having various molecular weights are commercially available. In addition, we believe that the influence of the charge of the crowder can be evaluated by using electrically charged analogues, including anionic dextran sulphate and cationic diethylaminoethyl (DEAE)-dextran.

In this study, we examine the effect of the timing and amount of primer DNA immobilization on the bead-based padlock/RCA under molecular crowding conditions. Next, dextran and its analogues were used as crowders to gain insight into the mechanism of bead-based padlock/RCA and to provide guidelines for the use of this
method for ultrasensitive detection of DNA.

**Experimental**

*Optimization of primer DNA immobilization*

Streptavidin-modified sepharose beads (Streptavidin HP SpinTrap, GE Healthcare, mean particle size: 34 μm) and streptavidin-modified magnetic sepharose beads (Streptavidin Mag Sepharose, GE Healthcare, particle size: 37-100 μm) were used. All the oligonucleotides (see Table 1 for sequences) were purchased from Sigma Genosys.

In a control experiment with streptavidin-modified sepharose beads, primer DNA was immobilized on beads prior to the bead-based padlock/RCA as previously described\(^1\) with some modifications. Briefly, suspensions of \(1 \times 10^4\) streptavidin-modified sepharose beads were centrifuged at 200g for 1 min to remove the solution. To couple the primer to the beads, the beads were resuspended in 200 μL of 5 μM biotinylated DNA (biotin-target) dissolved in Tris-buffered saline containing Tween 20 (TBST: 20 mM Tris-HCl, 1.4 M NaCl, 0.05% Tween 20, pH 7.6), followed by incubation at room temperature for 30 min with gentle mixing using a tube rotator. The suspensions were centrifuged at 200g for 1 min to remove the solution. The beads were resuspended in 400 μL of TBST and centrifuged as described above. The same procedures were repeated twice more. The beads were resuspended in 400 μL of TBST to make a stock suspension. Then, 10 μL of ligation solution containing 1 U of T4 DNA ligase (Fermentas) in 0.075 M KCl, 1 mg/mL BSA (New England Biolab), 1× T4 DNA ligase buffer (Fermentas), 0.1 nM padlock probe (gfp-RCA) and \(1.0 \times 10^4\) primer-immobilized beads were added into a polymerase chain reaction (PCR) tube. The mixture was incubated at 30°C for 30 min. Then 10 μL of RCA solution containing 2.5 U of φ29 DNA polymerase (New England Biolabs), 1 mg/mL BSA,
deoxynucleotide triphosphates (dNTPs, TaKaRa Bio Inc., 0.14 mM each), and 1× φ29 DNA polymerase buffer were added and the tube was incubated at 30°C for 60 min. Finally, 20 μL of fluorescent probe solution (0.1 μM detection oligomer DNA (gfp-d) in 0.02 M EDTA–2Na, 20 mM Tris–HCl [pH 7.4], 0.5 M NaCl, and 1 % Tween 20) was added. The temperature of the solution was then gradually decreased from 50°C to 30°C over 30 min. Then, the beads were concentrated to 10 μL by centrifugation and suspended in 90 μL of washing solution (0.02 M EDTA–2Na, 20 mM Tris–HCl [pH 7.4], 0.5 M NaCl, and 1 % Tween 20). Fluorescence images were acquired with a fluorescence microscope (IX71, Olympus) with a 60× objective lens, Chroma 86009 BFP/GFP and DsRed filter set, 555-nm exciter and 620-nm emitter for Cy3. The images were recorded with a charge-coupled device (CCD) camera (Exi Blue, BioImagingSolutions, USA). MetaMorph Software (Molecular Devices, USA) was used to handle the images. We performed z-axis scanning to cover the depth of the beads and summed up the total number of the bright spots from one bead.

In a set of experiments to optimize primer DNA immobilization with streptavidin-modified sepharose beads, the timing of the addition of beads was varied as shown in Fig. 1A. In condition I, 5 μL of ligation solution containing 0.15 M KCl, 2 mg/mL BSA, 2× T4 DNA ligase buffer, 1 U of T4 DNA ligase, and 0.4 nM biotinylated DNA (biotin-target) was added into a PCR tube. Then, 4 μL of 1×10^4 beads suspension was added and vortexed for 2 min. Then 1 μL of 1 nM padlock probe (gfp-RCA) was added. Therefore, the concentration of biotinylated DNA in the mixture was 0.2 nM. The mixture was incubated at 30°C for 30 min. RCA reactions and fluorescent probe hybridization were performed as described above, except that the hybridization was performed at 30°C for 30 min. In condition II, 5 μL of ligation solution and 1 μL of padlock probe were added into a PCR tube, then beads suspension was added. Further
procedures were the same as in condition I. In condition III, 5 μL of ligation solution and 1 μL of padlock probe were added into a PCR tube, and the mixture was incubated at 30°C for 30 min. Then beads suspension was added. Further procedures were the same as in condition I. In condition IV, 5 μL of ligation solution and 1 μL of padlock probe were added into a PCR tube, and the mixture was incubated at 30°C for 30 min. RCA solution was added to the tube and the mixture was incubated at 30°C for 60 min. Then beads suspension was added. Further procedures were the same as in condition I.

Similar experiments were performed with streptavidin-modified magnetic sepharose beads. The magnetic beads were employed to easily change the solution surrounding the beads, which was important to evaluate the effect of crowders on each step of the bead-based RCA. The conditions were the same as condition II, except that 5 times amount of biotinylated DNA and padlock probe was used.

In a set of experiments to optimize primer DNA concentrations, suspensions of 1×10^4 streptavidin-modified sepharose beads were centrifuged and resuspended in 200 μL of 1, 5, 10, and 50 μM biotinylated DNA (biotin-target) dissolved in TBST. The concentration of the padlock probe was 10 times higher than the concentration in the control experiment. The other steps were performed as in the control experiment.

The effects of polymers on the bead-based RCA

Polymers with an average molecular weight (MW) of about 40,000 were used, including neutral PEG (Sigma-Aldrich Japan Ltd., MW: 35,000) and dextran (Wako Pure Chemical Industries Ltd., MW: 32,000-45,000), anionic dextran sulfate (Wako Pure Chemical Industries, Ltd., MW: 36,000-50,000), and cationic DEAE-dextran (Sigma-Aldrich Japan Ltd., MW: 40,000).

Streptavidin-modified magnetic sepharose beads were used to fix the beads in the
PCR tube using a magnet and to remove the supernatant in each step of the bead-based RCA. Biotinylated DNA (biotin-target) was immobilized to streptavidin-modified magnetic sepharose beads in a similar way to the control experiment. Then, 10 μL of ligation solution containing 1 U of T4 DNA ligase in 0.075 M KCl, 1 mg/mL BSA, 1× T4 DNA ligase buffer, 1 nM padlock probe (gfp-RCA), 2.0×10³ primer-immobilized beads, and 10 % polymers were added into a PCR tube. The mixture was incubated at 30°C for 30 min. The beads were then fixed in the PCR tube using a magnet, the ligation solution was removed, and 15 μL of washing solution (1 mg/mL BSA and 1× T4 DNA ligase buffer) was added, and the beads were washed. After this procedure was repeated twice, the RCA solution (2.5 U of ϕ29 DNA polymerase, 1 mg/mL BSA, dNTPs (0.14 mM each), 1× ϕ29 DNA polymerase buffer, and 10 % polymer) was added into a PCR tube. The mixture was incubated at 30°C for 60 min. The beads were then fixed in the PCR tube using a magnet, the RCA solution was removed, and 15 μL of washing solution (1 mg/mL BSA and 1× ϕ29 DNA polymerase buffer) was added, and the beads were washed. After this procedure was repeated twice, 10 μL of fluorescent probe solution (0.1 μM detection oligomer DNA (gfp-d) in 0.02 M EDTA–2Na, 20 mM Tris–HCl [pH 7.4], 0.5 M NaCl, 1 % Tween 20, and 10 % polymer) was added. The temperature of the solution was then gradually decreased from 50°C to 30°C over 30 min. The beads were then fixed in the PCR tube using a magnet, the fluorescent probe solution was removed, and 15 μL of washing solution (0.02 M EDTA–2Na, 20 mM Tris–HCl [pH 7.4], 0.5 M NaCl, and 1 % Tween 20) was added, and the beads were washed. After this procedure was repeated twice, further steps were performed as in the control experiment.

To examine the effect of MW of dextran on the bead-based RCA, dextran was used at a final concentration of 10 % for three different molecular weights of ~6,000
Biotinylated DNA (WtGGTtarget) was immobilized to streptavidin-modified sepharose beads in a similar way to the control experiment. Another padlock probe (PLP-KRASwtGGT) and fluorescent probe (KRAS-d) was used. The concentration of the padlock probe was 10 times higher than the concentration in the control experiment. The other steps were performed as in the control experiment.

Results and Discussion

Optimization of primer DNA immobilization

The effect of addition timing of microbeads on the number of RCA products is shown in Fig. 1B. The number of RCA products was 5.2±2.0 in control experiments in which microbeads were modified with 5 µM biotinylated DNA prior to the RCA experiments. The number increased to 14.0±2.7 by decreasing the concentration of biotinylated DNA to 0.2 nM (Condition I). Conditions II and III, in which microbeads were added prior to the ligation (Condition II) and RCA (Condition III) gave almost the same results as in Condition I. The reason for the increased number of RCA products seems to be the decrease of the density of biotinylated DNA on the microbeads: that is, hybridization of padlock probe is hindered if the density of biotinylated DNA is too high, whereas hybridization is efficiently occurred at a lower density. A decrease in hybridization efficiency at a higher probe density has been reported. On the other hand, no products were observed in Condition IV, where the microbeads were added after the RCA. This is because the biotin moiety, which binds to the microbead, of the biotinylated DNA was screened by the RCA products and could not bind to streptavidin moiety on the surface of the microbead.

Figures 1C and 1D show typical bright field images and fluorescence images of
magnetic sepharose microbeads after RCA experiments. The use of DNA-conjugated microbeads (i.e. primer DNA was immobilized on beads prior to the RCA experiments) yielded products (Fig. 1C), but the use of unconjugated microbeads (i.e. beads suspension was added to the mixture of ligation solution and padlock probe, denoted as Condition II in Fig. 1A and 1B) yielded almost no product (Fig. 1D). In the latter case, the products remained in the supernatant (see SI). Unlike the case when non-magnetic beads were used, conjugation of DNA to the microbeads was essential to obtain RCA products on the magnetic microbeads. In the following experiments, we used DNA-conjugated microbeads.

Figure 2 shows the effect of biotinylated DNA concentration on the number of RCA products. The number of RCA products decreased as the (initial) concentration of biotinylated DNA in the modification step increased. When streptavidin-modified sepharose microbeads were used (Fig. 2A), the number of RCA products was 51.8±9.0 (5 μM) and decreased to 26.0±6.6 (10 μM) and 14.5±4.0 (50 μM). When streptavidin-modified magnetic sepharose microbeads were used (Fig. 2B), the number of RCA products was 92.1±19.5 (5 μM) and decreased to 72.6±15.0 (10 μM) and 32.3±6.5 (50 μM). The reason for the decrease can be considered as follows. From the product specifications, the matrix of streptavidin sepharose beads is highly cross-linked 6% agarose, which is a porous gel, and its molecular weight cutoff is 4,000,000. The molecular weight of biomacromolecules used in this study are 11,259 (biotinylated DNA), 26,610 (padlock probe), 96,000 (phi29 DNA polymerase), and 62,000 (T4 DNA ligase), so these biomacromolecules are expected to penetrate sufficiently into the beads, and molecular weight cutoff is not the reason for the decrease. Next, we estimated how much DNA is conjugated to the microbeads. The binding capacity of biotin to the microbeads is more than 300 nmol per mL of gel. Considering the mean diameter of the
microbeads (34 μm), more than 6 fmol of biotin can be captured to a single microbead. In this study, 200 μL of biotinylated DNA solution was added to 5 × 10^5 microbeads, so the ratio of streptavidin moieties coupled with biotinylated DNA to the whole streptavidin moieties are calculated to be 7 % (1 μM biotinylated DNA solution), 70 % (10 μM biotinylated DNA solution), and ~100 % (50 μM biotinylated DNA solution). In principle, higher densities of biotinylated DNA should increase the probability of biotinylated DNA interacting with padlock probes and enzymes and increase the efficiency of the reaction. However, under the present experimental conditions, the concentration of biotinylated DNA (5 μM) is considered to be sufficient for the dense modification of biotinylated DNA on the microbeads. Higher concentrations would result in a more crowded state than the optimal density, making it difficult for the padlock probes and enzymes to react. In addition, Fig. 2A and 2B show approximately the same trend. Therefore, it was found that even if the types of beads are different, the reaction can be considered to occur in the same way if the amount of biotinylated DNA modified is the same.

*The effects of polymers on the bead-based RCA*

Figure 3 shows the effect of polymers on the number of RCA products. RCA products were observed in the absence of polymers (Fig. 3A), and the number of the products increased in the presence of PEG (Fig. 3B) and dextran (Fig. 3C). In contrast, no products were observed in the presence of dextran sulphate (Fig. 3D) and DEAE-dextran (Fig. 3E). These results clearly indicate the difference between electrically neutral polymers (PEG and dextran) and charged polymers (dextran sulphate and DEAE-dextran). For the better understanding of the difference, the effect of polymers to each step of bead-based RCA (*i.e.* ligation, primer extension, and
hybridization of detection probe) was studied (Fig. 3F). All the polymers tested increased the number of RCA products if the polymers were added to the hybridization step (H in Fig. 3F). Since hybridization is caused by the formation of hydrogen bonds between nucleobases, the hybridization was promoted independently of the crowder's charge. The addition of dextran in the ligation step (L in Fig. 3F) increased the number of products in a similar manner to PEG. As described in our previous report,\textsuperscript{10} stabilization of hybridized DNA or DNA-enzyme complexes in a crowded environment led to an increased number of RCA products. The addition of dextran as well as PEG in the primer extension step (E in Fig. 3F) gave almost no effect in the number of RCA products. As described in our previous report,\textsuperscript{10}, de-hybridization of the primer-template complex, which should occur for continuous extension of the primer, is suppressed in a crowded environment, and therefore primer extension is not facilitated. When dextran sulfate and DEAE-dextran were added in the ligation or primer extension steps, no products were obtained. The reason for this is that (i) the charged dextran sulfate and DEAE-dextran are electrostatically attracted to the enzyme and form a complex, and (ii) the negatively charged dextran sulfate deprives the enzyme of magnesium ions and inhibits the enzymatic reaction. Therefore, it was found that electrically neutral polymers were advantageous to increase the number of RCA products.

Figure 4 shows the effect of molecular weight of dextrans on the number of RCA products. The number of RCA products increased in the presence of Dex40k and Dex500k. The highest number of RCA products was obtained in the presence of Dex500k, and the number was approximately 5.5-fold higher than that of the control experiment. The padlock probe with a single-nucleotide mismatch produced no products in the absence and presence of dextran (data not shown). Therefore, the selectivity of bead-based padlock RCA was retained in the presence of dextran, and the applicability
of the present technique to SNP typing has been demonstrated.

**Conclusions**

We have examined the effect of (i) the timing and amount of primer DNA immobilization and (ii) the charge and size of the crowders on the bead-based padlock/RCA under molecular crowding conditions. In the case of sepharose microbeads, it was found that adding biotinylated DNA before the primer extension step was more productive than prefixing the microbeads with biotinylated DNA. On the other hand, when magnetic sepharose microbeads were used, the biotinylated DNA had to be pre-immobilized onto the microbeads in order to obtain the products. The concentration of biotinylated DNA at about 5 μM was optimal, and the number of products decreased at higher concentrations. The effect of crowder charge was examined and it was found that neutral polymers were effective on ligation and hybridization step, while cationic and anionic polymers were only effective on hybridization step and inhibited the ligation and primer extension. Finally, the effect of the molecular weight of dextran on the number of products was investigated and it was found that the number of products increased with the increase in the molecular weight of dextran, which was up to 5.5 times higher than that without the addition of the polymer. These results provide insight into the mechanism of bead-based padlock/RCA and provide guidelines for the application of this method to the ultrasensitive detection of DNA.

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Supporting information: Detection of RCA products in the supernatant of the reaction solution. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.
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| Name         | Oligonucleotide sequence                                                                 | Length | Modification 5’ |
|--------------|-----------------------------------------------------------------------------------------|--------|-----------------|
| Biotin-target| GACGGGAACCTACAAGACCGCTGCTGAAGTCAAGTT                                                  | 35     | biotin          |
| gfp-RCA      | GCGTCTTGTAGTTCCCGTCCCTGCTCCAGATGGTACTGCTCCACGATGGTACTGCTCCACGATGGTGAAACTTTGACCTTCAGCAC | 89     | phosphate       |
| gfp-d        | CTGCTCCACGATGGTGTAACTCTTGCCTACGCCACCCTCCAACTACC                                         | 18     | Cy3             |
| WtGGTtarget  | ACTCTTGCTACTACGCCACACCCTCCAACTACC                                                       | 31     | biotin          |
| PLP-KRASwtGGT| GCGTAGGCAAGAGTTCTCTGCTAGTAAAGTACCCGACTATCGATCGAATCTAAGGTAAGCTGGTGTAGCTGGTAGTGAGCTGGTG | 80     | phosphate       |
| KRAS-d       | AGTAGCCGTGACTATCGATC                                                                     | 20     | Cy3             |
Figure Captions

Fig. 1  Optimization of conjugation of biotinylated DNA with microbeads. (A) Schematic illustration of experiments. Conditions I to IV denote the addition timing of streptavidin-modified sepharose microbeads. (B) The effect of addition timing of streptavidin-modified sepharose microbeads on the number of RCA products. One fmol of padlock probe (gfp-RCA) was used. N = 15. Error bars: ±1SD. **: P < 0.01 (one-way ANOVA). (C and D) Typical bright field images (top row) and fluorescence images (bottom row) of streptavidin-modified magnetic sepharose microbeads. (C) DNA-conjugated microbeads (i.e. primer DNA was immobilized on beads prior to the RCA experiments). (D) Unconjugated microbeads (i.e. beads suspension was added to the mixture of ligation solution and padlock probe, denoted as Condition II in Fig. 1A and 1B). Scale bar: 20 μm.

Fig. 2  The effect of biotinylated DNA concentration on the number of RCA products. (A) Results obtained by streptavidin-modified sepharose microbeads. (B) Results obtained by streptavidin-modified magnetic microbeads. Ten fmol of padlock probe (gfp-RCA) was used. N = 15. Error bars: ±1SD. **: P < 0.01 (one-way ANOVA).

Fig. 3  The effect of polymers on the number of RCA products. (A-E) Typical fluorescence images of streptavidin-modified magnetic sepharose microbeads following RCA. The experiments were conducted (A) in the absence of polymer and in the presence of (B) PEG, (C) dextran, (D) dextran sulphate, and (E) DEAE-dextran, respectively. In (B-E), polymers (10%) were present in the ligation, extension and hybridization steps. Scale bar: 10 μm. (F) The effect of polymers on the number of RCA products. The experiments were conducted in the absence of polymer (open) and in the
The presence of PEG (filled), dextran (hatched), dextran sulphate (border), and DEAE-dextran (cross), respectively. Polymers were present in the following steps: Cont., no step; L, ligation; E, primer extension; H, hybridization of detection probe; LEH, ligation, extension and hybridization. Ten fmol of padlock probe (gfp-RCA) was used. \( N = 15 \). Error bars: \( \pm 1SD \). **: \( P < 0.01 \) (one-way ANOVA).

Fig. 4 The effect of molecular weight of dextrans on the number of RCA products. Streptavidin-modified sepharose microbeads were used. Ten fmol of padlock probe (PLP-KRASwtGGT) was used. \( N = 15 \). Error bars: \( \pm 1SD \). **: \( P < 0.01 \) (one-way ANOVA).
**Fig. 1**

**A**

Condition I → Bead → Padlock probe → Ligation → RCA → Fluorescent probes

**B**

| Condition | RCA products / (dots/bead) |
|-----------|-----------------------------|
| Cont.     | 5                          |
| I         | 15                          |
| II        | 15                          |
| III       | 15                          |
| IV        | 15                          |

**C**

Images showing different conditions.

**D**

Images showing fluorescent probes.
Fig. 2
Fig. 3
Fig. 4
Graphical Index

- Small neutral crowder
- Enzyme
- RCA product
- Agarose bead
- Anionic crowder
- Large neutral crowder
- Cationic crowder