SINGLE DNA MOLECULE DETECTION BY ON-BEAD ROLLING CIRCLE AMPLIFICATION USING MICROCHIP FOR EFFICIENT DETECTION

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ABSTRACT
We realized efficient DNA detection by microchip-based on-bead rolling circle amplification (RCA). Detection efficiency was improved by enrichment onto agarose beads.

KEYWORDS: Rolling circle amplification, DNA, Beads

INTRODUCTION
Ultra sensitive DNA detection from complex matrixes is of great importance for a variety of diagnostics. One powerful analytical reagent is a padlock probe, which utilizes rolling circle amplification (RCA) for generation of 1-µm-sized fluorescent dots from single DNA templates in a solution [1]. However, counting the fluorescent dots in bulk scale is difficult. To improve the detection efficiency, we developed on-bead rolling circle amplification using a microchip [2], and succeeded in detection of 35 amol of Salmonella genomic DNA [3]. Although property of the bead material may affect the reaction and detection efficiency of the method, detailed study have not been reported. In this study, we investigated bead materials for efficient detection.

THEORY
Molecular recognition of DNA targets through padlock probe ligation results in the formation of a single circular DNA molecule. The circular molecule acts as an endless template for a RCA reaction. This produces a long single-stranded concatemer DNA molecule complementary in sequence to the DNA circle, repeated approximately 1,000 times, which spontaneously collapses into a random coil of DNA. Fluorescently tagged probes are hybridized to the repeated DNA molecule. The circular molecule acts as an endless template for a RCA reaction. This produces a long single-stranded concatemer DNA molecule complementary in sequence to the DNA circle, repeated approximately 1,000 times, which spontaneously collapses into a random coil of DNA. Fluorescently tagged probes are hybridized to the repeated sequence, resulting in a confined cluster of up to 1,000 fluorophores, visible in a fluorescence microscope as a bright dot with a diameter of approximately 1 µm.

EXPERIMENTAL
Polystyrene or agarose beads were used for on-beads RCA. A 5'-amino-modified primer was immobilized on NHS-activated Sepharose beads (φ = 34 µm, agarose beads). A 5'-biotin-modified primer was immobilized on streptavidin modified polystyrene beads (φ = 19 µm) (Fig.1). First, 5 µL of a ligation solution (0.1 U/µL T4 ligase, 0.08 M KCl, 0.1 mg/mL BSA in 1 × T4 ligase buffer) with padlock probe (0–10 nM padlock probe, gfp-RCA in Table 1) was added to beads solution (5 µL) and incubated for 30 min at 30˚C. Next, 10 µL of a RCA solution (5 U/µL of φ29 polymerase, 0.1 mg/mL BSA, 0.25 mM dNTP in 1 × φ29 buffer) was added and incubated for 60 min at 30˚C. Finally, 20 µL of a fluorescence probe solution (1 µM detection oligomer DNA, gfp-d in Table 1) was added. Fluorescence and DIC images were acquired with a fluorescence microscope (IX 71, Olympus) using a 60× objective lens. Forty z-slices were collected with 1 µm step. The reaction products were counted and analyzed with Image J software.

Table 1. Oligonucleotide sequences.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>amino-linker (primer)</td>
<td>5'-NH₂-GAC GGG AAC TAC AAG ACG CGT GCT GAA GTC AAG TT -3' (35 bases)</td>
</tr>
<tr>
<td>gfp-RCA</td>
<td>5'-P-ggc tct tgt cgt c-CT GCT CCA CGA TGG TGT A-CT GCT CCA CGA TGG TGT A-CT GCT CCA CGA TGG TGT A-aa ctt gac ttc agc ac-3' (89 bases)</td>
</tr>
<tr>
<td>gfp-d</td>
<td>5'-TexasRed-CTG CTC CAC GAT GGT GTA-3' (18 bases)</td>
</tr>
<tr>
<td>SE_pp (Padlock probe)</td>
<td>5'-P-cgt caa tgg cgg tta AGA GCG CAT GAA TCC GTA GTA ACT TGA CTT CAG CAC GCG TGA GGT CGG TAC ACT Ctg ctt ctt ceg gta a-3' (91 bases)</td>
</tr>
<tr>
<td>SE_tar (Sample DNA)</td>
<td>5'-AAT AAC CGC AGC AAT TGA CGT TAC CCG CAG AAG AAG CAC C -FITC-3' (40 bases)</td>
</tr>
<tr>
<td>SE_do_488 (Detection Probe)</td>
<td>5'-Alexa 488- AGA GCG CAT GAA TCC GTA GT-3' (20 bases)</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

The enzymatic reactions were realized on both beads successfully. Individual RCA products on the beads were visible with a fluorescence microscope as bright objects (Fig. 2). The detection ratio of counting objects per theoretical value (number of including padlock probe/number of beads) is calculated by the following equation (1).

\[
\text{Detection ratio (\%)} = \frac{\text{Counting objects/Bead}}{\text{Number of including padlock probe/Number of Beads}} \times 100 \quad (1)
\]

Using 2.5 fmol DNA in 40 µL reaction buffer with 10,000 agarose beads in a PCR tube, 1,800 countable RCA products per one bead could be detected (n=3), corresponding to a detection ratio of ~1% (Table 2). With 3,000 polystyrene beads in a PCR tube, 250 countable RCA products per one bead could be detected (n=3), corresponding to a detection rate of ~0.04%. Polystyrene bead showed lower detection rate because amplification rate was low at polystyrene surface and the bead was not suitable for optical detection because of its lower transparency than agarose bead. These are superior to the previously reported value of 0.02% (7 × 10^8 padlock probes, approximately 10^5 counted objects) [1]. Moreover, the detection rate was improved to 8% when all processes were performed with the agarose beads packed in a microchip. Fewer beads were suitable to detect smaller amounts of sample DNA. To improve the detection efficiency, we designed bead array in a microchip and investigate the effect of dead distance.
CONCLUSION
We concluded that agarose is suitable material for on-bead RCA and enrichment to the beads in a microspace is effective for efficient detection.

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REFERENCES

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**Table 2. Analysis results of on-bead Padlock/RCA, sequence 1**

<table>
<thead>
<tr>
<th></th>
<th>Counting objects (dot/bead)</th>
<th>Theoretical value (molecules/bead)</th>
<th>Detection ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-modified Agarose Beads</td>
<td>1813</td>
<td>1.5×10^7</td>
<td>1.2</td>
</tr>
<tr>
<td>Unmodified Agarose Beads</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DNA-modified Polystyrene Beads</td>
<td>254</td>
<td>6.6×10^3</td>
<td>0.04</td>
</tr>
<tr>
<td>Unmodified Polystyrene Beads</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Liquid phase [1]</td>
<td>5×10^3</td>
<td>7×10^7</td>
<td>0.02</td>
</tr>
</tbody>
</table>

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**Figure 2: Detection of single RCA on-bead.**