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Single nucleotide mismatch analysis using oligonucleotide probes synthesized on bacterial magnetic particle

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Abstract

An approach to analyze mismatches using short and specific oligonucleotide probes directly synthesized on bacterial magnetic particles (BMPs) by phosphoramidite methods was exploited. Approximately 126 molecules of 4-mer oligonucleotides/particle were synthesized on BMPs with high reaction efficiencies. Hybridization between FITC-labeled oligonucleotides and chemically synthesized oligonucleotides on BMPs was performed. Perfect matched and mismatched hybridizations were successfully discriminated by using the oligonucleotide probes on BMPs.
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Keywords: Bacterial magnetic particles; Mismatch discrimination; Oligonucleotide synthesis; Hybridization

1. Introduction

Currently, there is great interest in solid phase assays for nucleic acid analysis. DNA chip, microarrays [1,2] and particle-based assays [3,4] are all gaining prominence for nucleic acid applications. These techniques offer multiplex capabilities required for high throughput analysis since a large number of different assays can be conducted simultaneously on a single sample.

Solid phase methods for synthesizing oligonucleotides offer the opportunity for developing hybridization assay, which obviate the need of detaching them from the matrix. Based on these properties, simultaneous detection of several polynucleotides from a single biological sample has been developed on these bases [5,6]. A number of methods have been reported for immobilizing oligonucleotides on micrometer-sized beads [3,4,7].

Magnetic bacteria produce intracellularly nano-sized magnetic particles. Bacterial magnetic particles (BMPs) are 50–100 nm in diameter surrounded by a phospholipid bilayer membranes which promote high surface area, superior dispersion in fluid suspensions and beneficial for automated processes due to magnetic susceptibility [8]. Many organic molecules have been successfully integrated on BMP surfaces by chemical coupling [9–15] and gene cloning [16,17]. The constructed functional BMPs are beneficial for highly sensitive immunoassays [9–11], substance recoveries [12–14] and microarrays [15]. For the analysis of DNA genotypes and SNPs, single-stranded DNA or oligonucleotides have been integrated on BMPs. Biotin-labeled single-stranded DNA or oligonucleotides were introduced on the surface of streptavidin coated BMPs [12–15]. These modified BMPs were successfully used for the discrimination of tuna species [12], identification of cyanobacterial DNA [13,14] and SNP analysis for aldehyde dehydrogenase 2 gene (*ALDH2*) in human blood [15].

In this study, oligonucleotides were directly synthesized on BMP surface. The detection of single nucleotide

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mismatches was investigated using the 4-mer oligonucleotides synthesized on BMPs.

2. Materials and methods

2.1. Chemicals

3-[2-(2-aminoethyl)-ethylamino]-Propyltrimethoxysilane (AEEA) was purchased from Fluka Chemical (Buchs, SG, Switzerland). Sulfo-NHS-LC-LC-biotin was purchased from Pierce Chemical Co. (Rockford, IL, USA). Streptavidin was obtained from New England Biolabs Inc. (Beverly, MA, USA). Oligonucleotides with biotin (4-mer) or FITC (11-mer) for mismatch analysis were obtained from Sawady Technology Co., Ltd. (Tokyo, Japan).

2.2. Amino silane modification

BMPs covered with lipid membrane were extracted from *Magnetospirillum magneticum* AMB-1 as described previously [9]. The purified BMPs were washed with chloroform, methanol and hexane 1:1:1 (v/v/v) solution and dispersed with sonication using an ultrasonic bath to remove the lipid membranes. Naked BMPs were dried under reduced pressure for 15 min to remove residual solvent. The BMPs were suspended in AEEA (1% (v/v) in 99% ethanol, pH was adjusted to 4.0 by adding acetic acid) solution. After washing with 20 mM Tris–HCl (pH 4.0), BMPs were resuspended in 1% (v/v) of AEEA in Tris–HCl.

2.3. Oligonucleotide synthesis on BMPs

Oligonucleotides were synthesized on BMPs based on the conventional phosphoramidite method [18]. The 3′ terminal nucleotide in the probe sequence was introduced onto AEEA coated BMPs using β-cyanoethyl (CE) phosphoramidite. AEEA coated BMPs (5 mg) were mixed with 20 mM CE-phosphramidite in acetonitrile anhydride with 0.5 M tetrazol (5 ml) and dispersed for 4 h using an ultrasonic bath. The phosphate groups in 3′ terminus nucleotide with BMPs were oxidized with 50 mM iodine in 5 ml of tetrahydrofuran/pyridine/water (7:2:1) solution. After washing with acetonitrile six times, BMPs were suspended in 5 ml of 3% dichloroacetic acid/trichloroacetic acid to release the dimethoxytrityl group blocking the 5′ terminus hydroxyl group and dispersed for 30 min using an ultrasonic bath. After the introduction of the next nucleotide, the sample was washed with acetonitrile three times and the unreacted hydroxy groups were capped by acetic anhydride solution (acetic anhydride/methylimidazol/acetonitrile/pyridine 6:6:83:5). The above stepwise synthesis was repeated four times. BMPs bearing oligonucleotide were then washed three times with the buffer used in the hybridization assays (1 M NaCl, 5 mM EDTA, 1% Tween, 10 mM NaH_2PO_4 pH 6.8). To evaluate the quality of oligonucleotide synthesis on BMPs, the amount of dimethoxytrityl (DMTr) cation in each step was determined by monitoring the absorbance at 498 nm ($\varepsilon = 70\,000$). The reaction efficiency was calculated from the increasing percentages of DMTr amounts for each step.

2.4. Immobilization of oligonucleotide on BMPs

Synthesized oligonucleotide with biotin (4-mer) in 5′ terminus was immobilized on streptavidin chemically conjugated to BMPs (SA-BMPs). Streptavidin was conjugated onto BMPs using Sulfo-NHS-LC-LC-biotin as described previously [15]. Biotin-labeled perfect match oligonucleotide or the derivatives (250 pmol) containing 1, 2 or 3 base mismatches were mixed with 1 mg of SA-BMPs. The solution was incubated for 30 min with sonication. The oligonucleotide-immobilized on SA-BMPs were washed three times with 1 ml PBS buffer.

2.5. One base mismatch discrimination using 4-mer oligonucleotide probes synthesized on BMPs

FITC-labeled oligonucleotide (11-mer) was used as a target probe. The designed 4-mer probes have 50% GC contents (Table 1). FITC-labeled target probes (100 pmol) were dissociated at 95 °C and hybridized to 100 μg of complementary and mismatched oligonucleotides immobilized on BMPs in 100 μl of hybridization buffer (1 M NaCl, 5 mM EDTA, 1% Tween, 10 mM NaH_2PO_4, pH 6.8) at 6, 8, 10 and 12 °C for 10 min using PTC-2000 Peltier Thermal Cycler (MJ Research, Waltham, MA). The hybrids were washed three times in hybridization buffer and resuspended in 200 μl of buffer and the fluorescence intensity was measured using a microtiterplate reader (FLUOstar galaxy, BMG Labtechnologies, Offenburg, Germany). AEEA coated

Table 1
Sequence of oligonucleotides synthesized on BMPs and target oligonucleotide with FITC

Probes	Sequence[a]
Target	FITC-5′-ATGCTGATTCC-3′
Perfect match	5′-**TCAG**-3′
1 base mismatch	5′-**AGC**T-3′
2 base mismatch	5′-GT**TC**-3′
3 base mismatch	5′-CTTG-3′
1 base mismatch (1)	5′-**TCA**C-3′
(2)	5′-**TC**T**G**-3′
(3)	5′-A**CAG**-3′
(4)	5′-**T**G**AG**-3′

[a] Complementary part to target oligonucleotide was in bold.

BMPs (BMPs without oligonucleotide) were used as a control in the same procedure.

2.6. Four-mer oligonucleotide probes synthesized on BMPs hybridized with different mismatch positions

Four-mer probes with 50% GC contents were designed containing different mismatch positions (Table 1). The FITC-labeled oligonucleotide (11-mer) was used as target probe. Each 4-mer oligonucleotide containing different mismatch position on BMPs was hybridized with the target probe for 10 min. The BMPs were washed three times with hybridization buffer and resuspended in 200 μl of buffer and the fluorescence intensity was measured using a microtiterplate reader. AEEA coated BMPs (BMPs without oligonucleotide) were used as a control in the same procedure.

3. Results

3.1. Oligonucleotide synthesis on BMPs

DMTr cations were released from the 5′ terminus of the introduced nucleotides in the first detritylation step (Fig. 1). The generated free hydroxyl groups were used as initiators for the next cycle. After the introduction of the next nucleotide, the unreacted hydroxyl groups were capped by acetic acid anhydride. To evaluate the oligonucleotide synthesis on BMPs, the amount of released DMTr cation in each step was monitored by measuring the absorbance at 498 nm ($\varepsilon = 70\,000$). The amounts of DMTr cation derived from each step are shown in Fig. 2. DMTr cation derived from the first nucleotide introduced on BMPs was approximately 3.87 μmol/g BMPs. The reaction efficiency was 78.3–98.7% in each step. After 4-mer oligonucleotide synthesis, the amount of oligonucleotide was approximately 126 molecules/particle.

3.2. Detection of FITC-labeled target oligonucleotide using 4-mer oligonucleotides synthesized on BMPs

Hybridization reaction was performed with FITC-labeled target oligonucleotides (11-mer) and 4-mer oligonucleotides synthesized on BMPs at 6, 8, 10 or 12 °C. Perfect match oligonucleotide and the derivatives (Table 1), which contained 1, 2 or 3 base mismatches, were examined. The obtained fluorescence intensities of the hybrids are shown in Fig. 3. The highest fluorescence intensity was obtained at 6 °C. However, the perfect match oligonucleotide could not be discriminated from the other oligonucleotides with mismatches at 6 °C. No significant difference in fluorescence was observed at 10 and 12 °C. The clear discrimination between perfect match and mismatched oligonucleotides were obtained

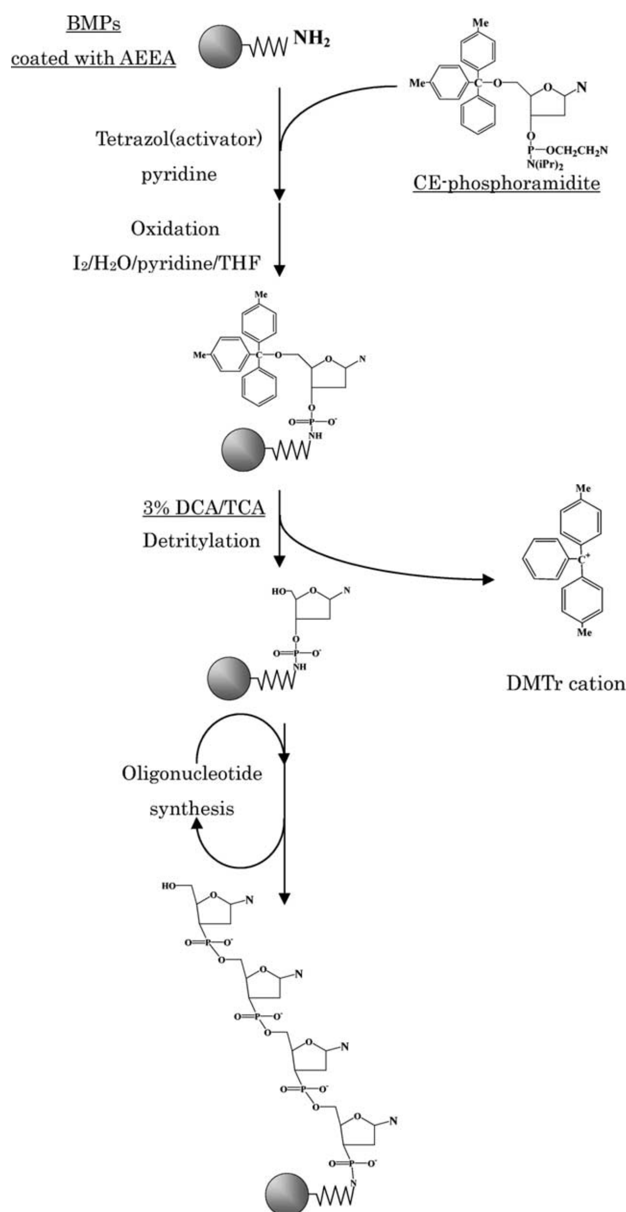

Fig. 1. Schematic diagram for direct oligonucleotide synthesis on BMPs.

in the hybridization at 8 °C. A similar result was obtained when the same examination was investigated using oligonucleotide-immobilized on SA-BMPs (Fig. 4).

3.3. Investigation of mismatch position in oligonucleotides synthesized on BMPs

Effect of the mismatch positions on the hybridization between the target oligonucleotide (11-mer) and 4-mer oligonucleotides synthesized on BMPs was investigated. The 4-mer oligonucleotides containing 1 base mismatches at different position were synthesized on BMPs. The highest fluorescence intensity was obtained

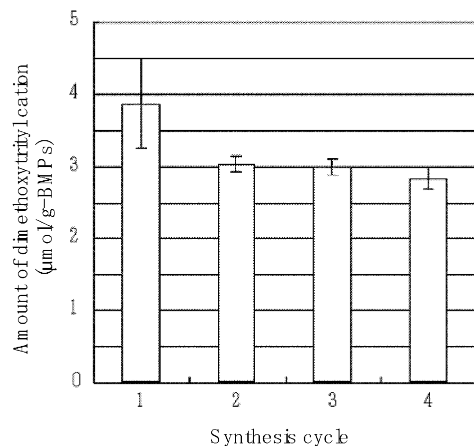

Fig. 2. Amount of dimethoxytrityl cation in each cycle of oligonucleotide synthesis on BMPs.

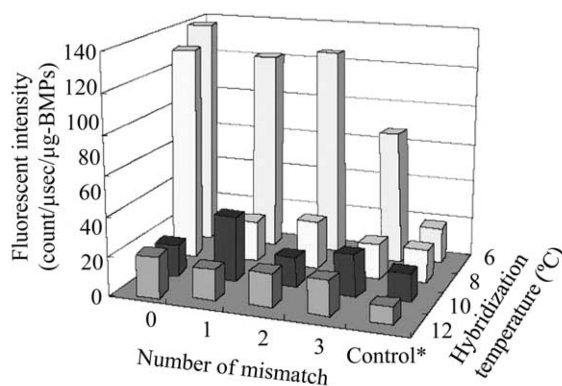

Fig. 3. Fluorescence intensities obtained in the hybridization with FITC-oligonucleotide (11-mer) and perfect matched or mismatched oligonucleotides (4-mer) synthesized on BMPs. 0: 5′-TCAG-3′ (perfect match). 1: 5′-AGCT-3′ (containing 1 base mismatch). 2: 5′-GTTG-3′ (containing 2 base mismatches). 3: 5′-GTTC-3′ (containing 3 base mismatches). *Control: BMPs without 4-mer nucleotide.

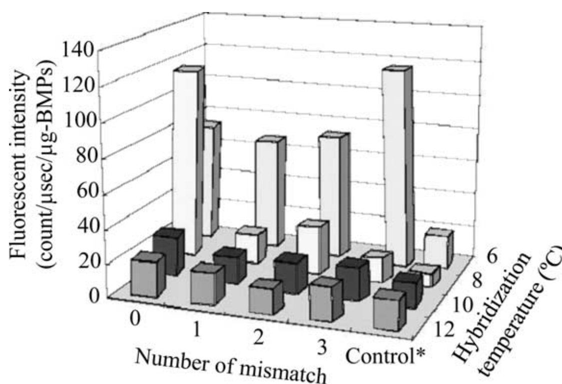

Fig. 4. Fluorescence intensities obtained in the hybridization with FITC-oligonucleotide (11-mer) and perfect matched or mismatched oligonucleotides (4-mer) immobilized on BMPs by streptavidin–biotin interaction. 0: 5′-TCAG-3′ (perfect match). 1: 5′-AGCT-3′ (1 base mismatch). 2: 5′-GTTG-3′ (2 base mismatches). 3: 5′-GTTC-3′ (3 base mismatches). *Control: BMPs without 4-mer nucleotide.

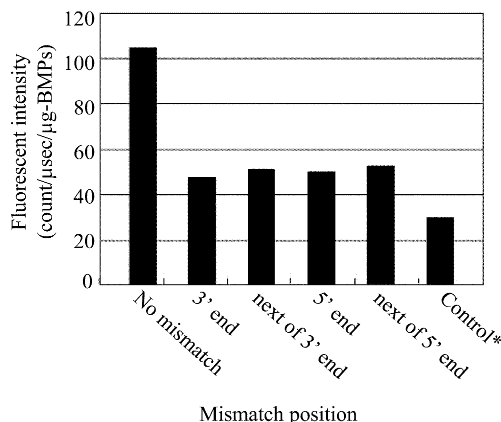

Fig. 5. Fluorescence intensities obtained in the hybridization with FITC-oligonucleotide (11-mer) and 1 base mismatched oligonucleotides (4-mer) at different positions on BMPs. *Control: BMPs without 4-mer nucleotide.

when complementary oligonucleotide was used (Fig. 5). A significant difference in fluorescence was observed between perfect match oligonucleotide and the derivatives containing different 1 base mismatch. Mismatch hybridization showed 44–48% decrease in fluorescence from the perfect match hybridization. It is, therefore, possible to design mismatch in the 4-mer oligonucleotides in any position for this method.

4. Discussion

In this study, the direct synthesis of 4-mer oligonucleotides on BMPs, which can be applied for single nucleotide discrimination by hybridization reaction, were demonstrated. The incidences of mismatched positions were investigated by changing the hybridization temperatures. One-base mismatches in any base position can be discriminated at 8 °C using 4-mer oligonucleotides directly synthesized or immobilized on BMPs. The hybridization efficiency is highly susceptible to the temperature. The melting temperature (Tm) calculated from oligonucleotide sequence is 12 °C for hybridization. However, the difference in fluorescence between perfect match and mismatch sequence was obtained at 8 °C, which is lower than calculated Tm value. The difference could be due to lower flexibility of the synthesized oligonucleotide on solid support (BMPs) than that of free oligonucleotide.

This study extends the capability of an approach to a three-dimensional SNP detection using short and specific oligonucleotide probes directly synthesized on BMPs. Controlled pore glass (CPG) [19], polystyrene [20] and colloidal silica particles [21] have been used as typical solid supports in oligonucleotide synthesis. BMPs offer multiple advantages as magnetic carriers of DNA due to a large surface to volume ratio of its size.

This is useful for faster condensation of DNA onto BMPs and hence, faster completion of assays. Based on these properties, SNP detection has been successfully achieved by BMP-microarray system [15]. For high throughput SNP analysis based on hybridization reactions, a large number of probes are required. This direct method on BMPs can reduce laborious work, time and cost in the preparation of oligonucleotides onto BMPs.

Bacterial identification has become an important procedure in healthcare, environmental monitoring and food and water quality testing. Traditional methods using microscopy and culturing techniques have various limitations and are time consuming. Recently, molecular approaches have been developed for bacterial identification, mostly based on hybridization to specific DNA fragment or sequence determination of conserved regions from a bacterial genome after amplification of the DNA by PCR. The analysis of 16S ribosomal RNA sequences is currently the approach chosen by most workers because of the following benefits: (1) specific for bacteria at the species level, (2) does not need to be subcultured for DNA analysis, (3) much amount can be obtained and stable in all living cells and (4) can be extracted from the originally isolated cell or colony. However, the currently available methods for 16S rDNA analysis are labor-intensive and often not suitable for high throughput isolation and diagnostics. Cyanobacterial genera have been successfully identified by 16S rRNA-targeted oligonucleotide probes immobilized on BMPs [13]. An exploited approach for analysis of DNA sequence using short and specific oligonucleotide probes directly synthesized on BMPs was shown in this study. This system has potential for identification of insertion and deletion of polymorphisms and even for SNP genotyping. The library of 4-mer probe synthesized on BMP containing all possible oligonucleotides is large enough to afford sufficient specificity to the variable region of 16S rDNA, but is small enough for practical fabrication and readout. It provides the sequence-specific hybridization pattern for high throughput bacterial identification.

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