International Journal of Oral Bioiogy, Vol. 37, No. 4 December 31 2012, p. 181-188

http://dx.doi.org/10.11620/IJOB.2012.37.4.181

International Journal of Oral Biology

Surface Polarity Dependent Solid-state Molecular Biological Manipulation with Immobilized DNA on a Gold Surface

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(received October 5, 2012 ; revised November 5, 2012 ; accepted December 11, 2012)

As the demand for large-scale analysis of gene expression using DNA arrays increases, the importance of the surface characterization of DNA arrays has emerged. We compared the efficiency of molecular biological applications on solid-phases with different surface polarities to identify the most optimal conditions. We employed thiol-gold reactions for DNA immobilization on solid surfaces. The surface polarity was controlled by creating a self-assembled monolayer (SAM) of mercaptohexanol or hepthanethiol, which create hydrophilic or hydrophobic surface properties, respectively. A hydrophilic environment was found to be much more favorable to solid-phase molecular biological manipulations. A SAM of mercaptoethanol had the highest affinity to DNA molecules in our experimetns and it showed greater efficiency in terms of DNA hybridization and polymerization. The optimal DNA concentration for immobilization was found to be 0.5 μM. The optimal reaction time for both thiolated DNA and matrix molecules was 10 min and for the polymerase reaction time was 150 min. Under these optimized conditions, molecular biology techniques including DNA hybridization, ligation, polymerization, PCR and multiplex PCR were shown to be feasible in solid-state conditions. We demonstrated from our present analysis the importance of surface polarity in solid-phase molecular biological applications. A hydrophilic SAM generated a far more favorable environment than hydrophobic SAM for solid-state molecular techniques. Our findings suggest that the conditions and methods identified here could be used for DNA-DNA hybridization applications such as DNA chips and for the further development of solid-phase genetic engineering applications that involve DNA-enzyme interactions.

Key words: Solid-phase, surface polarity, DNA immobilization, molecular biologic techniques, multiplex PCR.

Introduction

In recent years, biotechnology related to the manipulation of solid-phase DNA has expanded dramatically. These developments have paralleled the increased demands of human genome sequencing and large-scale analysis of gene expression using DNA arrays and RNA array [1-3]. DNA chips contain array of DNA fragments an orderly pattern at which the DNA fragments are available for next step of molecular biological approach [4]. These DNA chips can be used for the gene expression monitoring, detection of polymorphism and mutations, sequencing the human genome and DNA diagnostics [5-7]. Recently, further advanced form of DNA chips including production of protein array from DNA microarray and an aptamer chip have been tried [8-10].

While DNA array based technology is a promising area, not much attention was paid to the surface characterization

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of DNA arrays. The environment of the immobilized DNA fragments at the solid surface depends upon the mode of immobilization and can differ from that experienced in the bulk solution. Thus, in order to be functional after immobilization of DNA fragments on the solid surface, physical characterization of the surface and surface species are important. Since it has been reported that well defined probe orientation and the immobilization nature are critical, various immobilization methods have been attempted [11-19].

In this study, we controlled surface polarity by constructing self-assembled monolayers (SAM) of thiol molecules on the surface of thin gold film with terminal groups of different polarity. Thiolated DNA was immobilized on gold surface by generating mixed monolayers of thiolated oligomer and mercaptohexnol or hepthanethiol. The DNA oligomers in the resulting SAM will be in hydrophilic or hydrophobic environment, respectively. And the feasibility of molecular biological technique manipulation including nucleic acid hybridization, polymerization, restriction enzyme digestion, ligation and polymerase chain reaction (PCR) was compared. We report here that polar surface provide more favorable environment for immobilized DNA to be adaptable to solution phase molecular biological techniques. In addition, multiplex PCR was successfully performed with solid- phase tethered DNA.

Materials and Methods

Materials

Thiolated single stranded DNA (HS-ssDNA) was purchased from Research Genetics (Huntsville, AL, USA). The 5' thiolated oligomeric DNAs and unmodified oligonucleotides were obtained from Research Genetics (USA) and Bioneer, Korea, respectively. Au-coated slides were purchased from EMF, USA. Mercaptohexanol (6-mercapto-1-hexanol, MCH) hepthanethiol was purchased from Sigma-Aldrich (USA). Klenow fragment and *Taq* DNA polymerase were obtained from Takara (Japan) and Perkin Elmer (USA), respectively. Restriction enzymes were purchased from either Promega (USA) or New England Biolabs (USA). [^{35}S]α-dATP (1,000 Ci/mmol) was from Amersham (UK) and β-emission scintillation cocktail was from Pakard (USA). Other chemicals were purchased from Sigma (USA) or from other common sources.

Preparation of self-assembled monolayers (SAM) on Au surface

The Au substrate used was a glass plate of 3.0 mm×5.0 mm size on which Au was vacuum-deposited to about 1000 Å thickness. The Au surface was cleaned with piranha solution [concH_2SO_4: 30% H_2O_2 = 2:1 (v/v)], rinsed with absolute ethanol and dried under nitrogen gas. Solutions of 1 mM mercaptoethanol or 1 mM hepthanthiol were prepared in ethanol. Au-coated glass slides were incubated with the thiol solutions for 2 hrs at room temperature and rinsed with ethanol. The thiol groups were chemically adsorbed to the Au surface, thereby creating a SAM of either mercaptoethanol or heptanthiol.

In order to test the effect of surface polarity to physical adsorption of DNA molecule, we prepared radio-actively labeled small DNA fragment. A 65 base single stranded DNA were amplified with the KS primer and the SK primer (10 pmol each). The temperature cycle was set as follows: Hot start step: 94°C, 10 min, PCR cycle (20-45 cycles): 94°C, 30 sec; 50°C, 60 sec; 72°C, 30 sec. A part of the PCR solution was sampled and analyzed by agarose gel electrophoresis. Radioactively labeled DNA (200 μL) was added to each Au glass with SAM and incubated at RT for 1 hr. After the incubation, Au glasses were rinsed in 1 x Tris-buffered saline with Tween 20 (TBST) at RT and the resulting radioactivity was counted in a scintillation counter (Beckman LS6500, USA).

Preparation of DNA functionalized monolayers on Au surface

The cleaned bare Au surface was soaked in an aqueous solution of a thiolated single-stranded DNA (HS-ssDNA; thiolated DNA 20mer-1; 5'OH$(CH_2)_6$-S-S-$(CH_2)_6$-CGA GGT CGA CGG TAT CGA TA-3', 1 μM) in a buffer of 1.0 M potassium phosphate, pH 6.7, for 10 min. The Au surface bearing pre-adsorbed HS-ssDNA was then immersed in a solution of 1 mM mercaptoethanol or 1 mM heptanthiol (100 μl) in ethanol for 5 min and then thoroughly rinsed with 0.5% SDS solution. Thiol groups on the mercaptoethanol or heptanthiol was chemically adsorbed to the Au surface, thereby creating a mixed monolayer of HS-ssDNA and mercaptoethanol or heptanthiol.

Hybridization and polymerization of surface tethered ssDNA

For hybridization of surface-immobilized thiolated DNA 20mer-1 to an ssDNA template, gold slides with an ss DNA-functionalized monolayer on the surface were soaked in a solution of 65mer (5'-TAT AGA ACT AGT GGA TCC

TTT TCT TTT CTT GAA TTC TTT CTT TTC TTT TAT CGA TAC CGT CGA CC-3') and the mixture was heated to 65°C for 5 min, then slowly cooled down to room temperature. The surface immobilized DNA and the annealed 65-mer DNA were then polymerized with 2 U of Klenow fragment (Takara, Japan) for 1.5 hr in a reaction containing 4 μM dNTP, 0.2 μCi [α^{35}S] dATP, 10 mM Tris-Cl, 7 mM $MgCl_2$, and 0.1 mM dithiotreitol, pH 7.5. After the reaction, gold films were washed with 0.5% SDS at room temperature and the radioactivity remaining fixed to the slides were counted in a scintillation counter (Beckman LS6500, USA). All experiments performed at least in triplicate. Data was shown as mean± standard deviation (SD).

Ligation and PCR with immobilized DNA

To prepare DNA fragments to be ligated to the immobilized DNA, pBluescriptII KS(+) was first enzymatically digested with *Hind* III, followed digestion with *Sca* I to generate 1,154 bp digestion products. This DNA fragment was separated by electrophoresis on 1.2% agarose gels and purified by squeezing, subsequent phenol extraction and ethanol precipitation. Concentration of the DNA fragments were adjusted to 5 μM and then used in ligation reactions, as described below.

A HS-ssDNA, thiolated DNA 20mer-1 was immobilized on the gold surface, annealed to 24-mer DNA (CH-24 mer) to generate *Hind* III sticky end, and ligated to a 1,154 bp *Hind* III-*Sca* I digested DNA fragment with T4 DNA ligase (Promega, USA) in a reaction buffer containing 30 mM Tris-Cl, 10 mM $MgCl_2$, 10 mM dithiotreitol, 1 mM ATP and 15% polyethyleneglycol 8000, pH 7.8. Ligation reactions were carried out at 16°C for 2 hr or overnight at 4°C. The gold films were then washed once with a solution containing 5% SDS in 40 mM sodium phosphate, pH 7.2, at 65°C and 4 times with a solution of 1% SDS in 40 mM sodium phosphate, pH 7.2 at 65°C. The DNA immobilized on the slides was then subjected to 35 cycles of PCR, as described above. Primers used for the PCR reactions included 20-mer-1 (5'-CGA GGT CGA CGG TAT CGA TA-3') and *Nae* I primer (5'-GGC GAA CGT GGC GAG AA-3'). The amplified PCR products of 431 bp were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining. All experiments performed at least in triplicate. Data was shown as mean± SD.

Solid- phase mutiplex PCR

DNA fragments for ligation to the immobilized DNA, pBluescriptII KS(+) was digested with *Bam* HI, *Eco* RI or *Hind* III, followed digestion with *Nae* I to generate 359 bp, 377 bp and 389 bp digestion products. These DNA fragments were separated as described above.

HS-ssDNAs (Thiolated DNA 20mer-1, Thio-T3-E and Thio-R-B) were immobilized on a gold slide, annealed with mixture of CH-24-mer, cT3-E and c-R-B and ligated to either the 359 bp *Bam* HI-*Nae* I, 377 bp *Eco* RI-*Nae* I or 389 bp *Hind* III-*Nae* I DNA fragments as described above. The sequence of Thio-T3-E and Thio-R-B are 5'OH-$(CH_2)_6$-S-S-$(CH_2)_6$-ATT AAC CCT CAC TAA AGC CG-3' and 5'OH-$(CH_2)_6$-S-S-$(CH_2)_6$-AAC AGC TAT GAC CAT GCA TG-3', respectively. The gold films were then washed as described above. The DNA immobilized on the slides was then subjected to 35 cycles of PCR with 3 different pairs of primers. Forward primers used were either 20-mer (5'-CGA GGT CGA CGG TAT CGA TA-3'), T3 primer (5'-ATT AAC CCT CAC TAA AG-3') or reverse primer (5'-AAC AGC TAT GAC CAT G-3'). The reverse primer was *Pvu* II primer (5'- TGG CGA AAG GGG GAT GT-3') for all 3 PCR reactions. The amplified PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining. All experiments performed at least in triplicate.

Results

Comparison of hybridization and enzyme accessibility to the immobuilized DNA on gold surface with hydrophilic or hydrophobic matrix.

The aim of this study is to optimize the surface conditions for solid-state molecular biological manipulation. First of all, the surface polarity of the gold surface was modified by the well-known sulfur-gold interaction [20] to observe the relative DNA molecule adsorption to a solid surface with different surface polarity. Bare gold surface was treated with thiol molecules with terminal group with hydroxyl group (mercaptohexanol) or methyl group (hepthanethiol) to form self- assembled monolayers (SAM), thus generating hydrophilic and hydrophobic surfaces, respectively. These were hybridized with radioactively labeled DNA and the remaining radioactivity was measured. As expected polar, hydrophilic merca

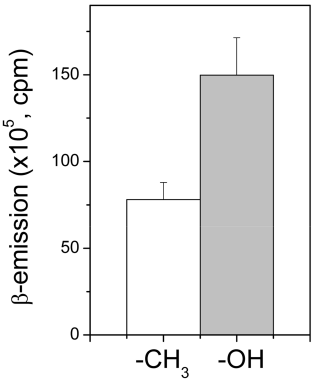

Fig. 1. DNA molecule adsorption to hydrophilic or hydrophobic surfaces created on gold slide. Radioactively labeled DNA was incubated with gold slides which were functionalized with thiol molecules to generate hydrophilic or hydrophobic characteristics of surface with either mercaptoethaol or hepthanethiol. Hydrophilic surface showed higher affinity to DNA molecules.

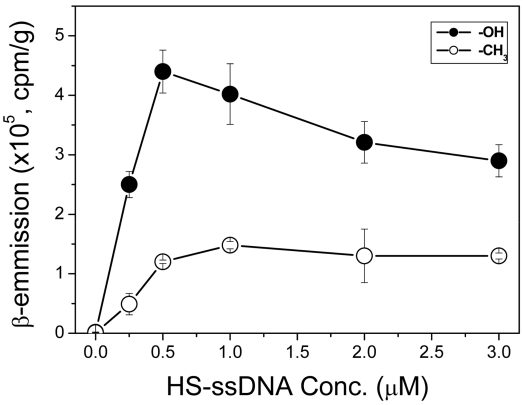

Fig. 2. DNA hybridization and polymerization on solid surface as a function of concentration of DNA immobilized. Thiolated single stranded DNA (HS-ssDNA) was immobilized on the gold surface derivatized with hydrophilic (●) or hydrophobic (○) molecules. partially complimentary ssDNA was hybridized to the immobilized DNA and polymerization was performed in the presence of radioisotope-labeled nucleotides.

ptohexanol-treated surface revealed higher radioactivity than non-polar hydrophobic hepthanethiol-treated surface (Fig. 1). Almost twice more DNA molecule was adsorbed to the polar surface than the non-polar surface that suggests that polar hydrophilic surface has higher affinity to DNA molecules than non-polar hydrophobic surface.

Regulating the surface coverage of DNA is a critical factor in manipulating molecular biological techniques in solid-phase [21]. Therefore, we tried to directly visualize the amount of DNA immobilized on the gold surface. The DNA molecule to be immobilized was functionalized at the 5' terminal with a thiol group. A gold-coated glass slide was then treated with Hs-ssDNA and subsequently treated with mercaptohexanol to generate a mixed SAM on the gold surface. After immobilization, immobilized DNA was hybridization with complimentary fragment of DNA, stained with a fluorescent dye and the result was observed under fluorescent microscope. We observed an increase in the optical density of microscopically observed fluorescent image of stained hybridized immobilized DNA as the immobilized DNA concentration increased (data not shown). However, the detection of fluorescent intensity of immobilized DNA and quantitative analysis were rather limited, thus we tried to monitor surface concentration of immobilized DNA indirectly. Various concentration of thiolated DNA on gold surface was immobilized and the surface was treated with either mercaptohexanol or hapthanethiol to construct hydrophilic or hydrophobic matrix of the surface. The immobilized DNA washybridized with 65-mer ssDNA. The resulting hybrid was used as a template for the Klenow fragment reaction, as described in Materials and Methods. The polymerization reaction was carried out in the presence of [α^{35}S] dATP. The gold slides were washed thoroughly and β-emission of the slides was quantified (Fig. 2). As expected the hybridization and polymerization efficiency was higher in hydrophilic surfaces than hydrophobic surface in the range of 0 to 3 μM of thiolated DNA immobilized on gold surface. The highest efficiency was observed at 0.5 μM of thiolated DNA was immobilized. And at the same concentration of thiolated DNA, approximately 3.7 times higher efficiency was observed in hydrophilic SAM than hydrophobic SAM.

When the immobilization reaction time of thiolated time was varied from 0 to 90 min and the hybridization and polymerization with Klenow fragment was performed in the presence of radioisotope as described above. As shown in Fig. 3, higher radioactivity was measured with hydrophilic SAM of mercaptoethanol and than with hydrophobic SAM of hepthanethiol in the range of time varied in this experiment. Highest activity was acquired at 5-10 min of immobilization reaction time of thiolated DNA. At 5 min of immobilization reaction time, hybridization and polymerization of the immobilized DNA on hydrophilic SAM of mercaptoethanol was approximately 5.9 times higher activity than hydrophobic SAM of hepthanethiol.

It was reported that when a thiolated DNA molecules of 166 Å was immobilized on a surface the actual thickness measured was about 33 ± 2 Å [12]. The change of surface thic-

kness was far less than the actual size DNA molecule immobilized. This result suggests that most of immobilized DNA is not fully extended to its full length or oriented perpendicular to the surface. In order to avoid the non-specific binding of DNA molecules to the surface and, matrix molecules such as mercaptohexanol or heptanthiol were used to fill in the surface between DNA molecules. Both of these matrix molecules contained 6 carbon backbones which are the same length of carbon backbone in thiolated DNA. DNA molecules immobilized would protrude out on the matrix molecules, thus could be available for subsequent reactions.

Thiolated DNA was immobilized and subsequently incubated with either mercaptohexanol or heptanethiol for 0-90 min time period. As shown in Fig. 4, DNA hybridization

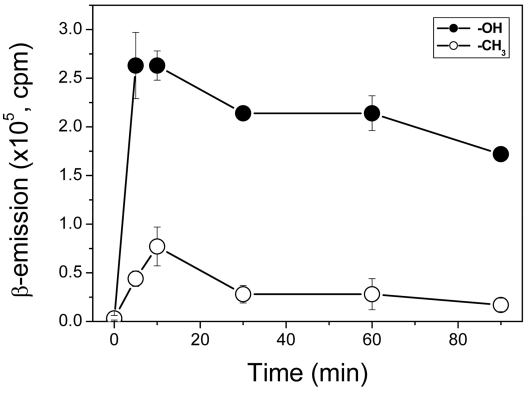

Fig. 3. The DNA immobilization time dependent DNA hybridization and polymerization on solid surface. The reaction time was varied for 0 to 90 min and the radioactivity was measured and plotted as a function of time on the gold surface derivatized with hydrophilic (●) or hydrophobic (○) molecules.

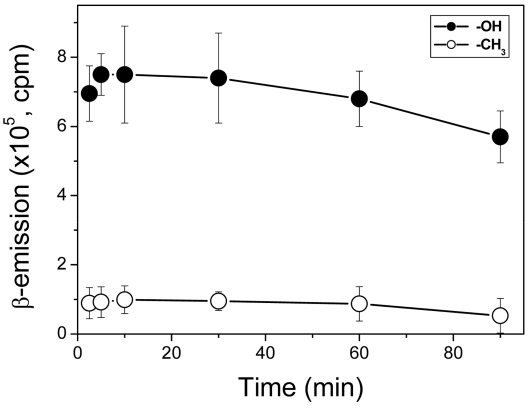

Fig. 4. The matrix molecule immobilization time dependent DNA hybridization and polymerization on solid surface. The reaction time was varied for 0 to 90 min and the radioactivity was measured and plotted as a function of time on the gold surface derivatized with hydrophilic (●) or hydrophobic (○) molecules.

andpolymerization was maximized at 10 min of incubation time with hydrophilic or hydrophobic matrix molecules. It seem that once matrix molecules occupies the space between immobilized thiolated DNA molecules, they prevent direct contact between DNA molecules and gold surface, thus they secure enough room for other complementary DNA or enzymes be functional. When DNA hybridization, polymerization efficiency was compared at 10 min of incubation, it was obvious that hydrophilic surface offered better environment for DNA and enzyme accessibility; approximately 7.5 times higher activity with hydrophilic surface than that of hydrophobic surface.

Again, the time period of polymerase reaction was varied from 0 to 3 hrs. Polymerization efficiency was higher with hydrophilic matrix molecules than hydrophobic matrix molecules (Fig. 5). Highest radioactive emission reached at 150 min of polymerase reaction with hydrophilic matrix and became saturated after 150 min of reaction (solid circles in Fig 5). The maximum was reached at 90 min of polymerase reaction hybridization and polymerization was performed with hydrophobic matrix (empty circles in Fig. 5).

Solid-phase ligation and PCR with DNA immobilized on gold surface.

As shown above we optimized the condition of solid-phase hybridization, polymerization with immobilized DNA on gold surface. For the next step, we tried to ligate a DNA fragments with immobilized DNA to gold surface. Thiolated DNA 20mer-1 was immobilized on the gold surface and the matrix was filled with mercaptohexanol. Partially comple

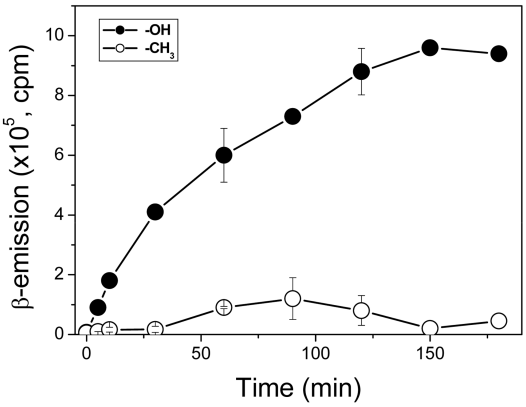

Fig. 5. The polymerase reaction time dependent DNA hybridization and polymerization on solid surface. Polymerase was incubated for 0 to 180 min and the radioactivity was measured and plotted as a function of time on the gold surface derivatized with hydrophilic (●) or hydrophobic (○) molecules.

mentary single stranded DNA (CH-24mer, 5'-AGC TTA TCG ATA CCG TCG ACC TCG-3') to immobilized DNA was hybridized to generate sticky ended which is compatible with *Hind* III restriction digested DNA for subsequent ligation. A DNA fragment of 1154 bp was prepared to contain *Hind* III sticky end and *Sca* I blunt end by digestion of a plasmid with respective restriction enzymes. Then the prepared DNA fragment was ligated with immobilized DNA. The resulting ligation was confirmed by PCR with primers 20mer-1 primer and *Nae* I primer where the annealing sites of each primers reside 5' and 3' sides of the ligation site. The target amplicon size was 413 bps. As shown in Fig. 6, the positive control solution phase ligation and PCR resulted in 413 bps amplicon as expected (lane 1 in Fig 6). Solid-phase ligation and PCR generated the same size amplicon with slightly lower intensity (lane 2 in Fig. 6). However, no PCR product was detected with negative controls of no ligation addition or no thiolated DNA (lanes 3 and 4 in Fig. 6).

Multiplex PCR with DNA immobilized on gold surface

For the next attempt, we tried to ligate three different 20mer-1, thio-T3-E and thio-R-B) were immobilized on the gold surface and the matrix was again filled with SAM of mercaptohexanol. Partially complementary single stranded DNAs (CH-24mer, c-T3-E, and c-R-B) to each immobilized DNA were hybridized all together to generate 3 different sticky ended (*Hind* III, *Eco* RI, and *Bam* HI) DNA for ligation. Three different size of DNA fragments were prepared with either *Hind* III/*Nae* I (389 bp), or *Eco* RI/*Nae* I (377 bp), or *Bam* HI/ *Nae* I (359 bp) double digestions, respectively. Then these DNA fragments were mixed and incubated all together with gold slide which contained 3 different immobilized DNA inthe presence of ligase. The ligation was confirmed by PCR reaction with primers described in Materials and methods to generate amplicons of 212 bp, 200 bp and 182 bp, respectively (Fig. 7). As a control, the same experiments were performed except that non-thiolated single stranded DNA with the same sequence of Thiolated DNA 20mer-1, Thio-T3-E Thio- R-B. And no detectable DNA band was detected in the negative controls of no ligase addition (data not shown).

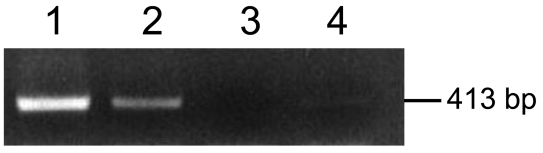

Fig. 6. Solid-state DNA manipulation on the gold surface. Thiolated DNA was immobilized, hybridized to single stranded DNA and hybridized to ssDNA to generate a compatible end for restriction enzyme (Hind III)-digested DNA fragment. After DNA ligation in the presence or absence of ligase, PCR was performed with primers positioning either side of the ligation site. The amplicon generated was 413 bp. Positive solution phase control (lane 1) and solid- phase DNA manipulation (lane2) show DNA amplicons amplified. No amplicon was generated from no ligase negative control (lane 3) or no immobilized DNA control (lane 4).

Discussion

Recently, technological progress in DNA microarrays has been extremely rapid and have been used widely in research areas including gene discovery, detection of mutations and medical diagnostics [5, 22-24]. In order to have good DNA chips, the immobilization of DNA molecules is a critical factor. The immobilized DNA onto a solid surface should permit their accessibility for subsequent steps including binding with the complementary strands. Numerous attempts have been tried to immobilizing DNA molecules on solid surfaces [11-19, 25-27]. A general approach for immobilization methodology is to modify the biomolecules of interest with a functional group that allows covalent attachment to a reactive group on the surface. These include using silanized DNA [25], amine-modified oligo nucleotides [16], poly(dT)-modified DNA by UV irradiation [26] and heterobifunctional DNA [19, 27].

Thiolated DNA was used in this study. It was immobilized on the surface of a thin gold film by generating a mixed monolayer of thiolated oligomer and thiol molecules. It was because shown that mercaptoalkyl (thiol)-modified oligonu

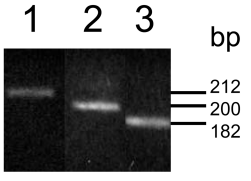

Fig. 7. Solid- phase multiplex PCR. Three different thiolated DNA were immobilized and ligated to a bigger size DNA fragments as described in Materials and methods. PRC was performed each set of primers specific to each DNA fragment was used. Amplicons size of 212 bp (lane 1), 200 bp (lane 2) and 182 bp (lane 3) were detected from thiolated DNAs; Thiolated DNA 20mer-1, Thio-T3-E and Thio-R-B.

cleotide showed the highest thermal stability with comparable accessibility and specificity of the surface-bound probes [28]. Another advantage of using 5' thio-functionalized DNA molecule in immobilization is that the orientation of DNA molecule can be controlled. The orientation of the immobilized DNA is critical for the subsequent steps including hybridization to a complementary DNA which is broadly applied in DNA chip technology and the interaction with proteins as employed in this study, and for the reduction of background noise which is directly related to device sensitivity and selectivity [29,30].

Unlike DNA microarrays in which the subsequent step is just hybridization to complementary DNA, the DNA chip developed in this study is for both interactions with DNA and proteins, specifically enzymes. To find an optimal condition for solid-state DNA-Protein interaction, the surface polarity was controlled by changing matrix molecules which cover the gold thin film. In addition, we revealed that various multiple solid-state molecular biological techniques wereas applicable with reasonable efficiency using the DNA chip developed in this study.

Acknowledgement

This research was supported by a grant from Ministry of Health and Welfare, Korea (02-PJ1-PG11-VN01-SV02-0036).

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