Solid-phase genetic engineering with DNA immobilized on a gold surface

Jeong Hee Kim a,*, Jung-A. Hong a,b, Myungok Yoon c, Moon Young Yoon b, Han-Seung Jeong d, Hyun Jin Hwang c,*

a Department of Oral Biochemistry, Kyung Hee University, Seoul 130-701, South Korea
b Department of Chemistry, Hanyang University Seoul, Seoul 133-791, South Korea
c Department of Chemistry, Kyung Hee University, Seoul 130-701, South Korea
d Biochip Division, Ahram Biosystems Inc., Seoul 130-103, South Korea

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Abstract

A novel method for immobilizing large DNA fragments on a solid surface was developed. A mixed self-assembled monolayer of thiolated single-stranded DNA with inert alkanethiol was generated on a gold (Au) surface through the Au–S reaction. Surface-tethered DNA generated by this method was compatible with various genetic engineering techniques, including hybridization, polymerization, restriction enzyme digestion and ligation. Kinetic control of surface coverage of immobilized DNA was critical for optimizing genetic engineering techniques on solid-phase. Multi-step reaction schemes utilizing various genetic engineering techniques described above were employed for solid-phase gene assembly. We were able to immobilize DNA fragments of up to 1180 bp on a solid surface. Furthermore, we showed that these immobilized genes can be regenerated by PCR. The present work suggests that these types of assembled genes can be used to store and regenerate genes on solid-phase. © 2002 Elsevier Science B.V. All rights reserved.

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1. 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 chips (Bartosiewicz et al., 2000; Hayward et al., 2000; Heiskanen et al., 2000; Xu et al., 2000; Amundson et al., 1999; Mochii et al., 1999; Gerhold et al., 1999; Golub et al., 1999; Yang et al., 1999). The development of efficient methods for immobilizing DNA on a solid sup-
port is essential for generating DNA chip and DNA array-based instruments. Various DNA immobilization methods have been described, including adsorption (Nikiforov and Rogers, 1995), copolymerization (Rehman et al., 1999; Proudnikov et al., 1998), complexation (Niemyer et al., 1999; Nilsson et al., 1995) and covalent attachment (Boncheva et al., 1999; Rogers et al., 1999; Levicky et al., 1998; Herne and Tarlov, 1997). Methods used to covalently attach oligonucleotides generally use silane (Pirrung et al., 2000; Rogers et al., 1999) and self-assembled monolayers of thiols (Boncheva et al., 1999; Higashi et al., 1999; Herne and Tarlov, 1997).

Two DNA chip formats currently in wide use are the cDNA microarray format and the photolithographically synthesized oligonucleotide array (Gerhold et al., 1999). However, neither format is effective for covalent immobilization of a large DNA fragment onto a solid surface. Therefore, we attempted to immobilize a relatively large DNA fragment, i.e. one that could be genetically manipulated, on a solid surface. In order to do this, various laboratory procedures must be verified and systematically optimized for use on the solid phase, including the immobilization process itself and application of various genetic engineering techniques. In principle, the manipulation of various genetic engineering techniques in the solid state is possible. However, in order to use successfully these methods in solid-phase, systematic analysis of the surface coverage of surface-tethered DNA, the kinetic dynamics of DNA immobilization, and the resolution of steric hindrances on surface are very important. Thiolated DNA was immobilized on the surface of a thin gold (Au) film by generating a mixed monolayer of thiolated oligomer and mercaptohexanol. Oligonucleotides attached by this method provided a highly efficient and specific substrate for solid-phase genetic engineering methods, including nucleic acid hybridization, polymerization, restriction enzyme digestion, ligation and polymerase chain reaction (PCR). By optimizing these parameters, we were able to immobilize a large unmodified DNA segment of 1180 bp on the surface of thin Au film.

2. Materials and methods

2.1. Materials

Thiolated single stranded DNA (HS-DNA) was purchased from Research Genetics (Huntsville, AL). The structure of the 5’ thiolated oligomeric DNA is 5’ OH(CH2)6-S-S-(CH2)6-CGAGGTC-GACGATCGATA-3’. Unmodified oligonucleotides were obtained from Bioneer, Korea. Au-coated slides were purchased from EMF, USA. Mercaptohexanol (6-mercapto-1-hexanol, MCH) was synthesized from the reaction of sodium thioacetate with 6-bromo-1-hexanol, as described elsewhere (Throughton et al., 1988). Other chemicals were purchased from Sigma (St. Louis, MO) or from other common sources.

2.2. Preparation of DNA functionalized monolayers on Au surface

The Au surface was cleaned with piranha solution [concH2SO4; 30% H2O2 = 2:1 (v/v)], rinsed with absolute ethanol and dried under nitrogen gas. The cleaned bare Au surface was soaked in an aqueous solution of HS-ssDNA (1 μM) in a buffer of 1.0 M potassium phosphate, pH 6.7, for 90 min. The Au surface bearing pre-adsorbed HS-ssDNA was then immersed in a solution of 1 mM MCH in ethanol for 5 min and then thoroughly rinsed with ethanol. The thiol group on the MCH was chemically adsorbed to the Au surface, thereby creating a mixed monolayer of HS-ssDNA and MCH.

2.3. Hybridization and polymerization of surface tethered ssDNA

For hybridization of surface-immobilized HS-ssDNA to an ssDNA template, Au slides with an ssDNA-functionalized monolayer on the surface were soaked in a solution of 65-mer DNA (5’-TC-TAGAACTAGTGGATCCTTTTCTTTTCTTG-AATTC TTTTTTTTTATCGATAC-CGTCGACC-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 poly-
merized with 2 U of Klenow fragment (Takara, Japan) for 3 h in a reaction containing 4 μM dNTP, 0.2 μCi [$^{35}$S] dATP, 10 mM Tris–Cl, 7 mM MgCl$_2$, and 0.1 mM dithiotreitol, pH 7.5. After the reaction, Au 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).

2.4. Restriction digestion of immobilized DNA

After immobilization of HS-ssDNA, annealing to the ssDNA template and polymerization with immobilized DNA was performed as described above. Au slides containing surface-tethered double-stranded DNA (dsDNA) were incubated with 12 U of EcoRI (Amersham, USA) in a reaction volume of 50 μl at 37 °C for 90 min. The surface of the Au films was then washed once with a solution containing 5% SDS in 40 mM sodium phosphate, pH 7.2, at 65 °C and four 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 (5’-CGAGGTCGACGTTATCGATA-3’) and P primer (5’-TGGCGAAGGGGGATGTG-3’) for immobilized DNA that was ligated to the 179 bp EcoRI–PvuII fragment, or 20-mer and N primer (5’-GGCGAACGTTGGCGAGA-3’) for immobilized DNA that was ligated to the 1143 bp EcoRI–ScaI fragment, respectively. The amplified PCR products were separated by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining.

3. Results

3.1. Preparation of mixed self-assembled monolayer on the surface of Au thin film

The aims of the present work were to develop a new method for immobilizing relatively large DNA fragments on a solid surface and to determine whether this immobilized DNA can be used as a tool for solid-phase gene storage. In order achieve these goals, we needed to confirm that solution-phase genetic engineering techniques are also applicable to DNA immobilized on a surface. To efficiently attach the DNA segment to the Au surface, the well-known sulfur–Au interaction was employed (Prime and Whitesides, 1991). The DNA molecule to be immobilized was functionalized at the 5’ terminal with a thiol group. An Au-coated glass slide was then treated with thiolated single-stranded DNA (Hs-ssDNA). HS-ssDNA molecules were then chemically adsorbed to the Au surface through the thiol end group, as well as physically adsorbed to the Au surface through backbone contacts. Next, the surface was
treated with mercaptohexanol (MCH) to generate a mixed self-assembled monolayer on the Au surface. MCH is a short alkanethiol with a terminal hydroxyl group that is used as an inert matrix thiol to cover non-reacted Au surface for several reasons. First, MCH is small enough to fill in gaps in the monolayer that might form around the bulky HS-ssDNA. Second, the six-carbon length is the same size of the methylene spacer in HS-ssDNA. Third, the hydroxyl terminal group of MCH generates hydrophilic characteristics of self-assembled monolayer (SAM), thus it might facilitate enzymatic access to the surface immobilized DNA. Fourth, MCH is known to be resistant to nonspecific binding of biomolecules (Herne and Tarlov, 1997). Finally, the formation of the MCH monolayer prevents contacts between the DNA backbone and the Au surface. Thus, after MCH treatment, the DNA is attached to Au only through the thiol end.

We compared immobilization efficiency of thiolated oligomeric ssDNA, one containing a disulfide bond and the other containing a sulfhydryl group. A chemical reductant, dithiothreitol, was used to reduce the disulfide bond present in thiolated ssDNA prior to immobilization to the Au surface. However, no significant differences in immobilization efficiency were observed between reduced and non-reduced thiolated DNA (data not shown). Thus, non-reduced thiolated oligomeric DNA was immobilized on the Au surface throughout this study.

3.2. Hybridization and enzymatic extension of immobilized DNA

To evaluate the hybridization of free DNA to DNA immobilized on a solid surface and enzymatic extension of hybridized DNA, we incubated the immobilized DNA with 65-mer ssDNA, and used the resulting hybrid as a template for the Klenow fragment reaction, as described in Section 2. We used the Klenow fragment because it has strong 5’ to 3’ polymerase activity and weak 3’ to 5’ exonuclease activity. Since the 5’-primer was immobilized on Au in our system, we expected the Klenow fragment to exhibit only polymerase activity. As a control, non-thiolated ssDNA with the same sequence as HS-ssDNA was included in some reactions, and in other reactions, DNA was omitted. The polymerization reaction was performed in the presence of [\(^{35}\)S]dATP. The Au slides were washed thoroughly and \(\beta\)-emission was quantified (Fig. 1). As shown in Fig. 1, Au slides immobilized with HS-ssDNA exhibited about 15-fold higher levels of radioactivity, as compared to slides treated with non-thiolated ssDNA or slides incubated in the absence of DNA, which exhibited background levels of radioactivity.

3.3. Kinetic control of DNA immobilization on solid surface

Regulating the surface coverage of DNA is a critical factor in manipulating genetic engineering techniques in solid-phase. Therefore, we deter-
Kinetics of immobilization of thiolated ssDNA on the Au surface. Thiolated DNA (HS-ssDNA) was covalently attached to the Au surface and then hybridized to a 65-mer oligonucleotide and polymerized with Klenow fragment in the presence of $^{35}$S-labeled dATP, as described in Section 2. $^{35}$S levels were determined by liquid scintillation counting. (A) Immobilization efficiency was determined in the presence of various concentrations of HS-ssDNA. (B) Immobilization efficiency was determined at various reaction times.

3.4. Restriction digestion of immobilized DNA

Next, we observed the enzymatic cleavage of immobilized DNA on Au. As described above, HS-ssDNA was immobilized on Au and dsDNA was generated by polymerization with the Klenow fragment in the presence of $[^{35}\text{S}]dATP$. The 65-mer DNA that hybridizes to immobilized DNA is designed to contain the contribution from one strand to the recognition sequence for the endonuclease EcoRI. Thus, dsDNA obtained by extension with Klenow polymerase should include the complete recognition site for EcoRI. To evaluate if an endonuclease is active on solid-phase immobilized DNA, dsDNA tethered to the Au surface was digested with EcoRI and washed, as described in Materials and Methods. Digestion with EcoRI should remove a 31 bp DNA fragment from the surface, while the rest of the DNA would remain tethered to Au. The level of radioactivity on the Au slides was measured both before and after restriction digestion. As shown in Fig. 3, we found that radioactivity levels were significantly reduced after restriction digestion. Taking into account that the DNA fragment re-
remaining on the surface after restriction digestion contains 37% of the radioactivity, the efficiency of restriction digestion of surface-tethered DNA in our experimental conditions was calculated to be 51.6%.

3.5. Gene assembly on Au surface by ligation reaction and confirmation by PCR

As outlined in Fig. 4, a series of experiments was performed to monitor the assembly of DNA fragments in a stepwise manner using the Au surface as a solid-phase anchor. HS-ssDNA was immobilized on Au, hybridized to single-stranded template DNA, extended with Klenow fragment, and digested with EcoRI. The surface-tethered DNA containing a 5' overhang was then annealed with either the 179 bp DNA fragment I [EcoRI–PvuII fragment of pBluescriptII KS(+)]) or the 1143 bp DNA fragment II [EcoRI–ScaI fragment of pBluescriptII KS(+)] with compatible ends, as described in Section 2. The ligation of surface-tethered DNA and free DNA fragments in solution was confirmed by PCR amplification. Primers used for PCR amplifications were designed such that the hybridization sites of the forward primers are localized on surface-tethered DNA and those of the reverse primers are localized on the free DNA fragment to be ligated. Therefore, PCR products are generated only when free DNA fragments are ligated to surface teth-

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**Fig. 4.** Schematic diagram of gene assembly on Au surface. Duplex DNA was digested with EcoRI and ligated with DNA fragment I or II containing compatible ends. Ligation of immobilized DNA and free DNA fragments was confirmed by PCR with primers that anneal specifically to either immobilized DNA or to free DNA fragments. →, forward primer; ←, reverse primer.
ered DNA. First, we ligated the 179 bp DNA fragment I to the surface tethered DNA. After ligation and PCR amplification with appropriate primers, a 216 bp band of the expected size was detected on agarose gels (Fig. 5A). A DNA product of the same size was detected in solution-phase ligation and in PCR, whereas no band was detected when T4 ligase was not added to the ligation reaction. Even when DNA fragment I was diluted by a factor of 1000 prior to ligation-immobilization, a detectable PCR product was still generated. Next, we attempted to immobilize a larger DNA fragment, 1143 bp DNA fragment II, onto the Au surface. To do this, we immobilized a 1180 bp DNA fragment by ligating DNA fragment II to the surface-tethered DNA, as described above. In order to confirm immobilization, we performed PCR amplification with 20-mer and N primers that can anneal to surface-tethered DNA or to DNA fragment II, respectively. Thus, the PCR product would be generated only when the 1180 bp DNA fragment was immobilized to the Au surface. As shown in Fig. 5B, expected size of PCR product, 415 bp was detected in solid-phase and solution-phase PCR (Fig. 5B, lanes 1 and 3). However, no PCR product was detected when T4 ligase was not added to the ligation reaction (Fig. 5B, lane 2).

### 4. Discussion

We prepared a mixed self-assembled monolayer of thiolated ssDNA and mercaptohexanol on the surface of a thin Au film by using the well-known spontaneous reaction of Au–S bond formation (Prime and Whitesides, 1991). The covalent Au–S bonds formed between oligonucleotides and the Au surface were highly stable, thereby resulting in chemically and thermally stable oligonucleotides attached to the Au surface. In addition, the immobilized DNA presented in this study is specifically oriented; the 5′-termini of oligonucleotides are covalently attached to the Au surface in a highly specific manner, and the 3′-termini of oligonucleotides are therefore located away from the Au surface. This orientated immobilization provides structural flexibility to the immobilized DNA molecules, enabling them to appropriately interact with other biomolecules, such as complementary DNA and various DNA binding proteins.

In this study, we have demonstrated that it is possible to apply solution-phase genetic engineering techniques to solid-phase DNA. The present study showed that oligomeric ssDNA immobilized on the surface of Au through Au-S bond formation can be hybridized to the larger complementary DNA. Furthermore, this DNA hybrid contained a long protruding 5′-terminal that could be extended by Klenow DNA polymerase. The immobilized duplex DNA generated by enzymatic replication can serve as a substrate for restriction endonucleases, as a template of PCR, and also as a substrate for ligation. We
successfully used the immobilized DNA generated in this study multiple times as a template for PCR (data not shown). We also demonstrated that kinetic control of the surface coverage of DNA is an important factor in optimizing hybridization and polymerization efficiency. The surface coverage rate of the immobilized DNA can be controlled by the kinetic control of mixed monolayers. Gene assembly was performed by restriction digestion of immobilized DNA with EcoRI, followed by ligation with a longer DNA fragment that contained a compatible end. The most important aspect of the present work of the finding that long pieces of unmodified DNA can be immobilized. Up to now, immobilization of unmodified dsDNA has been reported only for relatively short fragments of 50 bp (Bamdad, 1998), 69 bp (Nilsson et al., 1995), and 75 bp (Nikura et al., 1998). Here, we show that dsDNA molecules of up to 1180 bp in size can be immobilized on an Au surface and subjected to modifications with standard genetic engineering protocols optimized for the solid phase.

The present results demonstrate that the solid-state genetic engineering techniques developed in this study are possibly applicable for preparation of immobilized genes, i.e. immobilization of large DNA fragments each of which contains a complete information for a specific gene. This along with the fact that copies of the large, immobilized DNAs can be reproduced multiple times by using the PCR method open up the potentially immense possibility for development of artificial gene storage and regeneration devices and related new application methods. For example, an immobilized genetic library of the standard human genome could be generated based on techniques demonstrated in this study. Such a regenerative solid-state genetic library could provide essential material for various functional genomics and proteomics research programs.

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References


