Covalent Immobilization of Oligonucleotides on Modified Glass/Silicon Surfaces for Solid-phase DNA Hybridization and Amplification

Mengsu Yang,* Richard Y.C. Kong, Najam Kazmi, and Andrew K.C. Leung
Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, P. R. China

(Received October 29, 1997; CL-970836)

Glass slides and silicon chips were modified with aminosilane and various linker molecules to provide activated surfaces for covalent immobilization of oligonucleotides under ambient condition, and the surface-attached oligonucleotides remain active for solid-phase DNA hybridization and polymerase chain reaction.

Development of DNA-based biosensors and microchips relies heavily on the effective immobilization of DNA onto solid substrates comprising silicate and silicon dioxide surfaces.[1-5] In this study, we have derivatized the surfaces of glass slides and silicon chips with γ-aminopropyl-triethoxysilane (APTES), and various bifunctional linker molecules were compared for efficient and specific attachment of DNA onto the modified surfaces. The surface-attached oligonucleotides through the formation of covalent bonding between 5'-thiol-labeled oligonucleotides and the maleic anhydride linker were shown to be stable under elaborated temperature and thermal cycling conditions and active for solid-phase hybridization and amplification.

Microscope glass slides (Polyscience) or mirror-polished silicon wafers (Wafernet) cut into small chips (2 x 2 cm) were thoroughly cleaned in chloroform and 0.1 M HCl, treated with 1:1:5 $NH_4OH/H_2O_2/H_2O$ and 1:1:5 $HCl/H_2O_2/H_2O$ to generate surface silanol groups,[6-9] as confirmed by FTIR. The resulting chips were treated with 2% APTES (Aldrich) in toluene for 24 h to generate aminosilane-modified chips, and then immersed in 1% p-nitrophenylchloroformate (NPC) (Fluka) in chloroform, or 1% glutaraldehyde (GA) (Sigma) in water, or 1% maleic anhydride (MA) (Aldrich) in chloroform to give NPC-, GA-, or MA-modified surfaces, respectively (Figure 1).

Two oligonucleotide primers (23-mer), LTI-F: 5'-TTACGGCGTTACTATCCTCTCTA-3' and LTI-R: 5'-GGTCTCGGTCAGATATGTGATTC-3', and the 5'-hexylamine

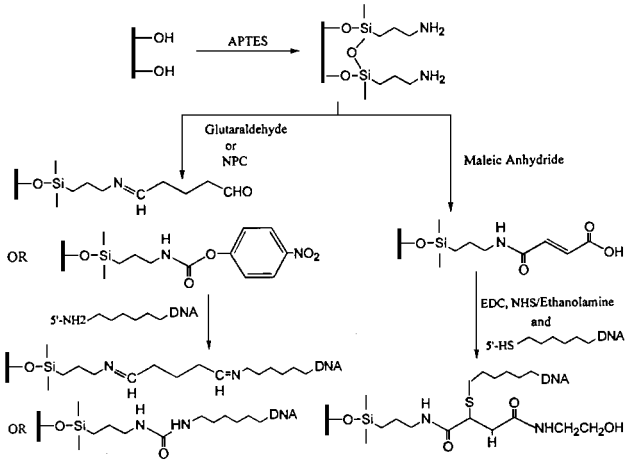

Figure 1. Strategies for covalent immobilization of DNA onto glass/silicon surface. NPC: p-nitrophenylchloroformate. It should be noted that the position of Michael addition of -SH group is not fixed.

(NH_2)-labeled LTI-R were synthesized using an automated DNA synthesizer (Pharmacia). 5'-Mercaptohexyl (SH)-labeled LTI-R was synthesized by DNAgency, PA, USA. The oligonucleotides were purified by reverse-phase HPLC. A 275-bp dsDNA was obtained by amplifying the heat-labile type I toxin gene (LTI) of *Escherichia coli* strains H10407 using polymerase chain reaction (PCR) and the primers LTI-F and LTI-R.[10] For autoradiographic measurements, isotopically-labeled dsDNA was prepared by including ^{35}S-labeled dATP in the PCR solution. Similarly, 5'-NH_2- or 5'-SH-labeled LTI-R primers were used in the PCR to prepare 5'- NH_2- or 5'-SH-labeled dsDNA, respectively.

A 100 μL solution of the 5'-NH_2-labeled dsDNA (1.0 μg/mL) was placed on each of the NPC-, GA-, or MA-modified surfaces (glass or silicon wafer) at room temperature for 2 h, followed by washing in 1.0 M NaCl solution for 3 h. Autoradiographes and phosphor images of the surfaces showed that the ^{35}S-labeled dsDNA was attached to the surfaces treated with APTES and the bifunctional linkers. No radioactivity was detected on untreated surfaces or surfaces treated with APTES or linkers only. However, similar amount of radioactivity was detected when the linker-modified surfaces were treated with the same dsDNA (^{35}S-labeled) without the 5'-NH_2 group. This indicates that DNA may be non-specifically linked to the surfaces through the amino groups on the bases. After the NPC- and GA-modified surfaces were treated with ethanolamine, and the MA-linked surface was treated with N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 0.2 M) and 0.05 M N-hydroxysuccinimide (NHS), followed by ethanolamine hydrochloride (1 M, pH 9), no attachment of dsDNA (with or without 5'-NH_2) was observed

For preliminary hybridization experiments, 5'-NH_2-labeled or unlabeled LTI-R primers were attached to all three linker-modified surfaces and the remaining linker groups were deactivated with ethanolamine. The resulting surfaces were then incubated with a complementary dsDNA (275-bp, 1 μg/ml) or a non-complementary dsDNA (225-bp) (both ^{35}S-labeled) at 90 °C for 20 min and cooled down to room temperature. Positive signals were observed only in the presence of the complementary dsDNA, suggesting that the immobilized ssDNA primer, whether with 5'-end attachment or not, may still undergo speficfic hybridization.

After the ethanolamine treatment, only the MA-modified glass or silicon surfaces were found to be active for immobilization of 5'-SH-labeled DNA. This indicates that the DNA is exclusively linked to the surface via the 5'-end, probably through the formation of a thiol ether bond. Positive radioactivity signals were observed after the MA-modified surface was treated with 5'-SH-labeled LTI-R primer, EDC/NHS and ethanolamine, and incubated with the ^{35}S-labeled complementary dsDNA under hybridization condition. The silicon surface following each step of treatment described above was analyzed by x-ray photoelectron spectroscopy (XPS) to confirm the elemental composition of the modified surfaces, and the results (data not shown) correlate well with the radioactive measurements. These results suggest that the MA-modified surface may be better controlled for the

immobilization of DNA than the NPC- or GA-treated surfaces.

Hybridization experiments on the MA-modified surfaces after various treatments were carried out by incubating with a PBS solution of the ^{35}S-labeled, 5'-SH-labeled, complementary dsDNA (275-bp, 1 μg/ml) under hybridization condition (Table 1). The phosphor imaging results listed in Table 1 confirm that the immobilized LTI-R primer was required for sequence-specific hybridization, and that non-specific adsorption/attachment of dsDNA was effectively blocked by ethanolamine and octanethiol treatments, which are believed to react with the COOH and C=C groups of the maleic acid moiety, respectively. A series of diluted dsDNA solutions were tested and no signal above the background was detected at about 0.2 μg/ml (total dsDNA about 2 ng).

Table 1. Radioactivity measurements of maleic-anhydride-modified glass slides under various chemical treatments and DNA hybridization conditions

*Surface	$HOC_2H_4NH_2$	SH-Primer	Octane-thiol	dsDNA	Radio-activity
1	+	+	+	+	+
2	+	-	+	+	-
3	+	-	-	+	+
4	-	-	+	+	+

* Surfaces were treated by APTES, maleic anhydride, and 5'-SH-labeled LTI-R according to Figure 1. Standard protocol includes COOH blocking by ethanolamine, 5'-SH-labeled LTI-R immobilization, C=C blocking by octanethiol, and hybridization with complementary 5'-SH-labeled dsDNA (surface-1). No hybridization was observed without the immobilized LTI-R (surface-2). Non-specific attachment of dsDNA occurred without octanethiol blocking (surface-3) or ethanolamine blocking (surface-4).

Solid-phase polymerase chain reaction (PCR) experiments were also carried out on the MA-modified surfaces after treated in succession with ethanolamine, 5'-thiol-labeled LTI-R primer, and n-octanethiol. A typical PCR mixture (800 μL) was prepared containing the following reagents: 80 μL 10 x PCR buffer, 200 mM deoxyribonucleotide triphosphate (dNTPs), 50 mM ^{35}S-labeled dATP, 10 μL 275-bp dsDNA fragment (1 μg/ml) as the template, 2 μL LTI-F (upstream primer, 1 pmol/μL), 0-2 μL LTI-R (downstream, 1 pmol/μL), and 1 unit of *Taq* polymerase. Controlled experiments were carried out in the absence of the template or the *Taq* polymerase. Various concentrations of the LTI-R primer were used to study the effect of primer ratio on the amplification reaction. About 50 μL of the mixture was placed on a reaction chamber constructed on the glass slides using Gene-Frame™ in situ PCR accessories (Bio-Rad). The template dsDNA concentration is about 0.01 μg/ml and the total amount of the template dsDNA in the mixture is about 0.6 ng. The PCR thermal cycle (MTC-100, MJ Research) was as follows: step 1, 94 °C 5 min; step 2, (94 °C 1 min, 54 °C 1 min, 72 °C 1 min) 35 cycles; step 3, 72 °C 10 min; step 4, 4 °C 30 min. After the PCR, the surfaces were washed thoroughly prior to phosphor imaging.

Positive radioactivity signals were observed on the LTI-R-linked surfaces with all the necessary PCR ingredients but not on the controls (Figure 2). No signal was observed when the template dsDNA was replaced with a non-complementary dsDNA. The radioactivity increased as the primer ratio LTI-R/LTI-F in the PCR solution increased, indicating that the DNA template was amplified in the solution and then hybridized with the surface-linked LTI-R primer. However, a small quantity of radioactivity was detectable (after the background attenuation) on the primer-modified surface without the solution LTI-R primer. Two possibilities, not mutually exclusive, may account for the observation: (1) the surface-linked LTI-R were elongated during the PCR cycles; (2) the LTI-R were partially detached during the thermal cycling and amplified in the solution while the remaining immobilized LTI-R captured the PCR products by hybridization. Investigation is underway in our laboratories to optimize the reaction conditions, which may offer a possibility to integrate DNA amplification, hybridization, and detection on one chip.

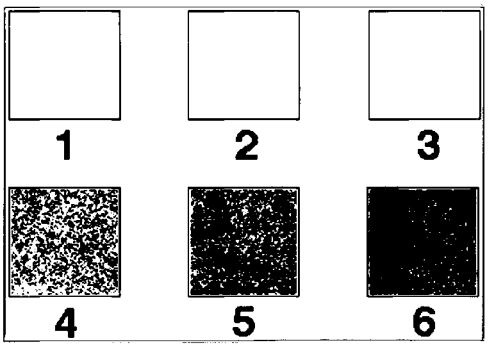

Figure 2. Phosphor images of the LTI-R-linked glass slides after solid-phase PCR experiments. Various PCR solutions were applied on different slides: (1) without the dsDNA template; (2) without the *Taq* polymerase; (3) without the LTI-R primer; (4) LTI-R/LTI-F = 0.2/1; (5) LTI-R/LTI-F = 0.5/1; (6) LTI-R/LTI-F = 1/1. All the slides were subject to same degree of attenuation to minimize the signals on the controlled slides.

This work is supported by an Industry Support Fund (AF/102/96) from the Industrial Technology Development Council, Hong Kong Government Industry Department.

References

1 M. Yang, M. E. McGovern and M. Thompson, *Anal. Chim. Acta*, **346**, 259 (1997).
2 A. Caviani-Pease, D. Solas, E. J. Sullivan, M. T. Cronin, C. P. Holmes, and S. P. A. Fodor, *Proc. Natl. Acad. Sci. USA*, **91**, 5022 (1994).
3 S. S. Ghosh and G. F. Musso, *Nucleic Acids Res.*, **15**, 5354 (1987).
4 R. J. Lipshutz, D. Morris, M. Chee, E. Hubbell, M. J. Kozal, N. Shah, N. Shen, R. Yang, and S. P. A. Fodor, *Biotechniques*, **19**, 442 (1995).
5 U. Maskos and E. Southern, *Nucleic Acids Res.*, **20**, 1679 (1992).
6 U. Jonsson, B. Ivarsson, I. Lundstrom and L. Berghem, *J. Colloid Interface Sci.*, **90**, 148 (1982).
7 L. A. Chrisey, G. U. Lee, and C. E. O'Ferrall, *Nucleic Acids Res.*, **24**, 3031 (1996).
8 M. A. Shoffner, J. Cheng, G. E. Hvichia, L. J. Kricka and P. Wilding, *Nucleic Acids Res.*, **24**, 375 (1996).
9 L. Henke, P. A. E: Piunno, A. C. McClure and U. J. Krull, *Anal. Chim. Acta*, , **344**, 210 (1997).
10 R. Y. C. Kong, W. F. Dung, L. L. P. Vrijmoed, and R. S. S. Wu, *Mar. Pollut. Bull.*, **31**, 317 (1995).