BIOCHIP JOURNAL, Vol. 1, No. 4, 267-272, December 2007

Improvement of DNA Chip Performance by Using a Silver-patterned Chip Substrate

Mikyung Kim[1] **& Changwoo Park**[2]

[1]Advanced Nano Products Co., Ltd,
244 Buyong Industrial Complex, Kumho-ri, Buyong-myeon,
Cheongwon-kun, Chungcheongbuk-do 363-942, Korea
[2]Division of Applied Chemistry & Biotechnology,
Hanbat National University, san 16-1 Duckmyoung-dong,
Yuseong-gu, Daejeon 305-719, Korea
Correspondence and requests for materials should be addressed to M. Kim (Mikyung-kim@anapro.com)

Accepted 30 October 2007

Abstract

We have applied the inkjet technology to the development of Ag-patterned biochip substrates in order to increase the production capability, the sensitivity, and the detection limit of biochips. Although silver has a potential to be a better substrate for biosensors and biochips than gold because of its higher chemical affinity toward biomaterials, conventional Ag films have a high reactivity and poor adhesion to substrates (especially glass), and the films can be easily destroyed during the process of biochip fabrication. However, these problems in conventional silver films were overcome by using the unique ANP Ag ink and ink jet technology, which produced a highly stable and adhesive silver film on glass substrates. By applying this inkjet technology, we were able to produce a great variety of patterns in chip substrates, increase the immobilization efficiency by more than 2 times when compared with conventional substrate technology, and increase the chip sensitivity to allow a 100 times reduction in the amount of probe needed. We also investigated the chip specificity by applying the perfect match and mismatch samples into a silver-patterned chip. These results show that a Ag-patterned chip substrate with high sensitivity and specificity can be used for the application of biochip and biosensors.

Keywords: Silver nanoparticles, DNA chip substrate, Inkjet printing, Chip Sensitivity

Introduction

Biosensors & biochips are tremendously important in various applications. Although they are primarily used for drug screening and the diagnosis of genetic diseases, they are also useful in other areas, such as environmental analysis and food analysis[10-12,20]. The major areas of biosensor & biochip technology include surface chemistry technology, detection technology, and data analysis technology, with the surface chemistry of the chip substrate being critical for the preparation of a robust DNA chip that is capable of stable and efficient DNA immobilization[1-3,6-7,19]. Surface chemistry plays an important role in the overall performance of the DNA chip by increasing the immobilization efficiency, and a large number of studies have been conducted during the last decade in an attempt to increase the detection sensitivity of biomaterials by increasing the immobilization efficiency. The improved technologies that have been developed have increased the immobilization capacity of biomaterials while ensuring efficient hybridization, low background interference, and spot uniformity.

In addition, various immobilization techniques based on chemical mechanisms, the chemical interaction of the linker system used, and the attachment stability have been developed[14,18-20]. Generally DNA can be immobilized on the glass surface using a covalent or non-covalent method. Non-covalent immobilization methods utilize the electrostatic interaction that occurs between the surface of the chip and DNA through the ionic and hydrogen bonds. In case of covalent methods, the glass surface is usually covered with aldehyde-, thiol-, or epoxy-groups, which then immobilize chemically modified DNA using an appropriate chemical reaction[9,14]. DNA can also be immobilized on a gold-coated surface using the self-assembly monolayer technique, in which of the thiol groups are chemically bonded with the gold[16-18].

In this study, an Ag-patterned biochip substrate developed by using the ANP Ag ink and inkjet technology was employed to increase the production capability of chip substrates, the chip sensitivity, and the detection limit of biochips. Although silver has the potential to be a better substrate for biosensors and biochips than gold because of its higher chemical affinity toward biomaterials, conventional Ag films have a high reactivity and poor adhesion to substrates (especially glass), and the films can easily be destroyed during the process of biochip fabrication. However, these problems in the use of conventional silver

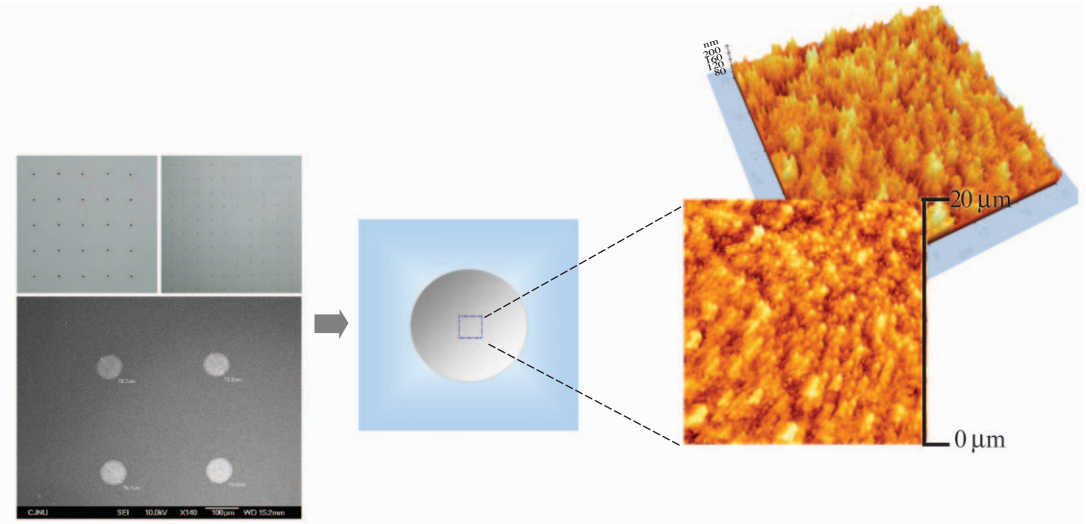

Figure 1. Characterization of the Ag-patterned chip substrate by microscopy and AFM.

films were overcome by using the unique ANP Ag ink in conjunction with inkjet technology, which produced a highly stable and adhesive silver film on glass.

By applying this inkjet technology, we were able to produce a great variety of patterns in the chip substrate, as well as to increase the spot intensity by more than 2 times while increasing the chip sensitivity by up to 100 times when compared with conventional substrate technology.

Results and Discussion

Preparation of Ag-patterned Substrate

The ANP Ag ink was prepared from mixture of metal inks including palladium, platinum, and silver in order to produce Ag-patterned chip substrate by using the inkjet printing technology. The different ratio of metal composition affected the chemical and physical properties of the Ag-patterned substrate such as the adhesiveness, the chip intensities, and the spot uniformity (Data not shown). The metal pattern was produced by using an inkjet printer to apply the chip substrate, which was then thermally treated at different temperatures in order to improve the adhesiveness of the metal film to the substrate. The thin metal pattern was easily formed using the LSL program of the Litrex 70 inkjet printer, which allowed production on a mass scale at low cost. Furthermore, the thin metal pattern could be patterned to a size as small as micrometers so that biomaterials could be integrated at a high density. Pattern diameters ranging from 30 μm to 200 μm were then regulated by the solid content of the Ag metal ink and the number of patterning repeats[8] (*Manuscript in preparation*). Figure 1 shows an example of Ag-patterned chip substrate, as well as the surface characteristics, which were determined by AFM analysis. The Ag-patterned surface showed a roughness that ranged from 15 nm to 30 nm and a height that ranged from 1 μm to 7 μm, depending on the patterning condition. These results suggest that the Ag-patterned substrate had more flexibility in controlling the pattern thickness and roughness, and it could therefore be applied into various areas of biosensors and biochips[5-7].

Comparison of Ag-patterned Substrate with Conventional Substrates on Immobilization Efficiency

To estimate the immobilization efficiency of the conventional substrates, various concentrations of TAMRA-labeled HPV 16 DNA was spotted onto the aldehyde-treated substrate using a Gentix Q array. The control substrate was then analyzed for chip intensity without washing in order to determine the intensity of the known DNA concentration, however, the chip intensity of the experimental substrate was analyzed after the washing and drying steps. The results showed that only 4-6% of the total applied DNA was immobilized on the conventional aldehyde-treated substrate (Data not shown), therefore, improvement of the immobilization efficiency is a major obstacle that needs to be overcome to allow enhanced chip performance[4,9].

To compare the immobilization efficiency with that

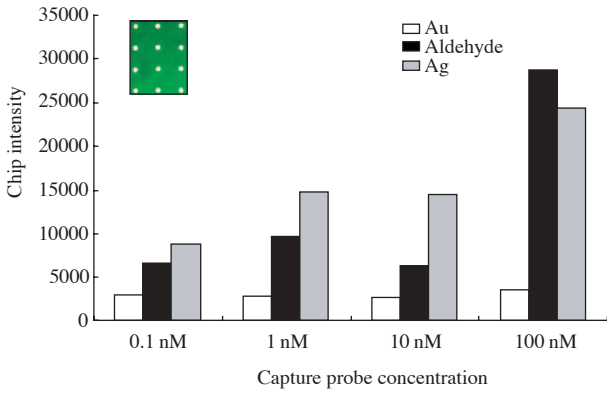

Figure 2. DNA immobilization efficiencies on different types of substrates by the TAMRA-labeled capture probe.

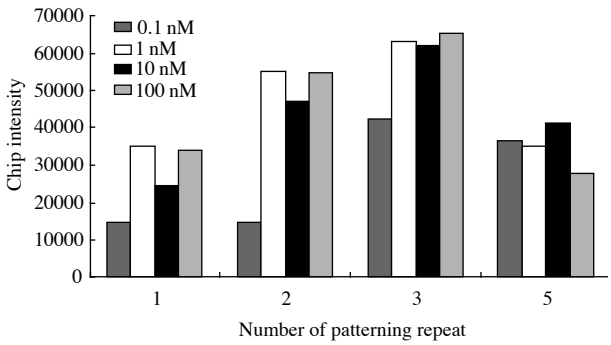

Figure 3. Comparison of chip intensity depending on the number of patterning repeats conducted during the process of inkjet printing.

of the gold substrate, aldehyde-treated, and Ag-patterned substrates were tested (Figure 2). The Ag-patterned substrate (called "ANP-DC1") showed higher immobilization efficiency at a lower probe DNA concentration when compared to conventional Au- or Aldehyde-coated substrates, however, there was no difference observed when higher probe DNA concentrations were used because the substrate became saturated with the DNA probe. A higher DNA immobilization efficiency occurred as a result of the characteristics of the surface, which included a higher surface area of Ag-patterned substrate produced by the dispersion of the Ag nanopowder. This higher surface area and roughness of the Ag-patterned substrate provided more space for the immobilization of capture probes[16-18]. The lower immobilization efficiency observed on the gold substrate resulted from the production process of gold substrate. Generally, gold substrate was prepared by vapor deposition or a sputtering method, therefore the roughness of the coated surface, as well as the surface area, could be less than that of the Ag-patterned substrate[2,15,16].

Comparison of the Ag-patterned Substrate with Conventional Substrates on Hybridization Efficiency

To assess the hybridization efficiency of the different surface treatment methods, 10 μM HPV-16 DNA was immobilized onto a silver-patterned slide and then hybridized with Cy5-labeled single-stranded target DNA at a concentration ranging from 0.1 nM to 100 nM. The chip intensity gradually increased as the number of patterning repeats increased up to three, and then decreased when up to five additional repeats of patterning were used, indicating that the maximum chip intensity was given by three repeats of patterning (Figure 3).

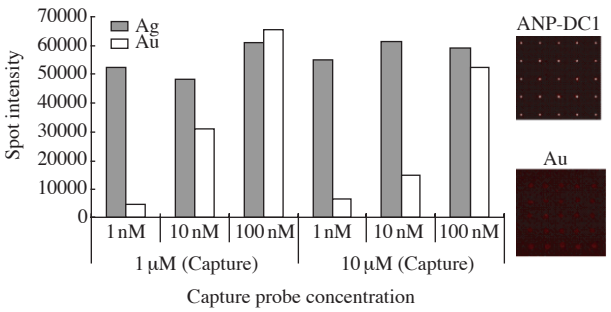

Figure 4. Chip intensities at different capture and target probe concentrations for Ag-patterned (ANP-DC1) and Au substrates. 1 nM, 10 nM, and 100 nM of target probe were applied to the immobilized capture probe (1 μM and 10 μM).

To compare the chip performance among the different types of substrates, 1 μM, 10 μM, and 100 μM of capture probe (HPV-16 DNA) were immobilized onto substrates and then hybridized with Cy5-labeled single-stranded target DNA at a concentration ranging from 0.001 pM to 100 nM. The Ag-patterned substrates showed a higher chip intensity at a lower target probe concentration than other types of substrate such as gold- or aldehyde-treated substrates (Figure 4), which may have occurred as a result of the higher immobilization efficiency at the lower range of the capture probe concentration (Figure 2). The lower detection limits of the different types of substrates were 0.001 pM for the Ag-patterned chip substrate, 10 pM for the aldehyde-treated chip substrate, and 1 nM for the gold-coated substrate (Figure 4 & 5), which was consistent with the results of the immobilization efficiency experiments. Taken together, these results indicate that the higher chip sensitivity occurred as a result of the higher immobilization efficiency. Therefore, the immobilization efficiency of the capture probe may

be the most important factor in determining the chip performance in that the chemical stability between DNA and the substrate may inhibit the nonspecific binding to the substrate[7,9].

We investigated the chip intensities and signal-to-noise ratios (S/N ratio) by regulating the different types of linker and the linker or spacer length of the capture probe (Figure 6). Although the chip intensity increased as a result of increasing the linker length, it was not significantly different between the C_{12} and C_{18} linker lengths. However the S/N ratio of the DNA chip was higher in the C_{12} linker system than in the C_{18} linker system, which indicates that the capture probe should be maintained at a certain distance from the surface for optimal chip performance.

We also tested the chip specificity by applying different types of target probe, such as HPV 16 alone, a mixture of HPV16 & 18, and HPV 18 alone to the immobilized HPV 16 capture probe[13]. As shown in Figure 7, the Ag-patterned chip substrates could clearly distinguish the perfect match probe from the mismatch probe.

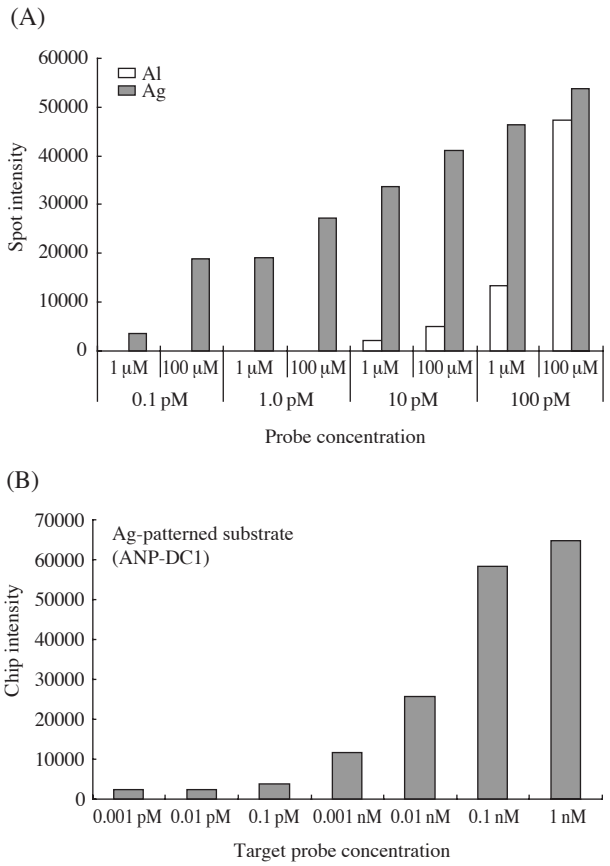

Figure 5. Chip sensitivity based on the range of target probe concentration.

Materials and Methods

Preparation of Nano Silver-patterned Substrate

Glass microscope slides were cleaned by immersion in boiling acetone for 1 minute, followed by immersion in boiling isopropyl alcohol. The slides were then washed twice in dH_2O for 2 min and dried under filtered nitrogen gas. Next, the slides were sonicated for 5 min in a Branson 1510 ultrasonicator in isopropanol, washed twice in dH_2O for 2 min, and then dried using filtered nitrogen.

The dried glass slides were kept in a slide carrier and treated with the different concentrations of the surface coating chemical, XC95-C1658 (GE Toshiba Silicones, Japan). The surface properties of the substrate were then measured using contact angle measuring equipment[8] (Surface Tech Co., Korea).

The silver ink patterning of the substrate was conducted using a Spectra SE-128 head with a Litrex 70 Inkjet System (Litrex Corporation, Pleasanton, CA). The Litrex Substrate Layout Tool (LSL) was used to produce various types of substrate patterns. In order to compare the substrate efficiency of the chips, an Au-coated substrate (BioGold™, Erie Scientific Company) and an aldehyde-treated substrate (CEL Associates, Inc.) were tested. The surface analysis of the

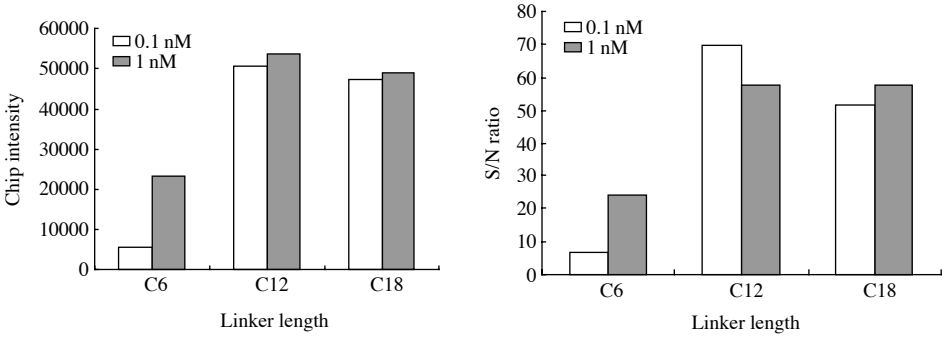

Figure 6. Chip sensitivity depending on the linker and spacer types.

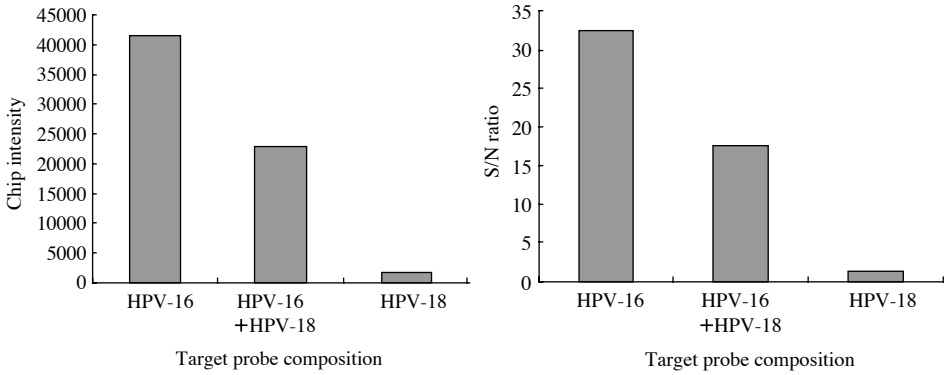

Figure 7. Chip specificity of PCR products of HPV-16 & 18 DNA.

Ag-patterned substrate by AFM was kindly conducted by Dr. Lim at Chungju National University.

Preparation of Oligonucleotides and PCR Products

Oligonucleotides were synthesized and purified by HPLC by Genotech (Daejeon, Korea). Synthetic target probes were 30 bases long and were conjugated with Cy5 dye at their 5′ ends. The synthetic capture probes were 30 bases long with 5′-thiol modification and were conjugated with TAMRA dye at their 3′ ends.

To optimize the linker length, we used C_6, C_{12}, and C_{18} linkers with thiol-modification at their 5′ ends. In order to test the performance of the Ag-patterned substrate, HPV capture and target probe sequences (HPV 16, 18, & 31) were used for the chip test.

Preparation of Printing Solutions and Spotting

Printing solutions containing DNA in saline sodium citrate (Sigma) buffer and Array-It solution (TeleChem International Inc.) were prepared for the capture probe immobilization. SSC printing solutions were prepared by combining appropriate amounts of 20X SSC buffer stock (Sigma, St. Louis, MO), dH_2O (GIBCO) and DNA solutions to achieve a final concentration of 3X SSC with different concentrations of DNA. The array -It solution was diluted to 1X for the final concentration of spotting DNA solution from the 2X stock solution.

The Genetix Q array mini was used for robotic printing of the DNA solution onto the substrate. Before robotic printing, pins were ultrasonically cleaned in dH_2O, vacuum cleaned, and then dried under a N_2 stream. Several patterns were then used to print the DNA onto the substrate. For DNA immobilization and the chip efficiency tests, an Axon 4000B microarray scanner (Axon Instruments Inc., Union City, CA) was used. The fluorescence intensities were analyzed using a GenePix 4000B fluorescence scanner (Axon Instruments, Union City, CA, USA) and software provided by manufacturer. All spot intensities were corrected for background intensity and then used as the median intensity value. In addition, the signal-to-background ratio was calculated.

Chip Performance on Target Probe Hybridization

For target hybridization, a hybridization chamber (Grace-Bio) and cover glasses were used. All hybridization steps in this study were conducted at 4˚C, 25˚C, or 40˚C in a humidified chamber. Fluorescently labeled targets were hybridized to the arrays at different temperatures for 2-15 hours. Fluorescently-labeled PCR products were mixed with 12XSSPE hybridization buffer solution, denatured for 5 min at 95˚C, and then incubated on ice immediately. Next, the denatured mixtures were applied onto the microarray slides under a cover glass or in a hybridization chamber and then placed in 40˚C humidified incubators for 15 hours. After hybridization, the slides were washed sequentially in 3XSSPE for 2 min and twice in 1XSSPE for 2 min at room temperature. Residual fluid was then removed from the slide surface using a micro-slide dryer centrifuge (Fisher Scientific Co.).

Data Analysis

In order to analyze the fluorescence intensities of the DNA immobilization and chip efficiency tests, a GenePix 4000B fluorescence scanner (Axon Instruments, Union City, CA, USA) and software was used. All spot intensities were corrected for background intensity and then used as the median intensity value. The signal-to-background ratio was also calculated.

Acknowledgements

This study was supported by a grant from the Ministry of Commerce, Industry, and Energy (MOCIE).

The project number is 10021489.

References

1. Call, D., Chandler, D. & Brockman, F. Fabrication of DNA microarrays using unmodified oligonucleotide probes. *BioTechniques* **30**, 368-379 (2001).
2. Taira, S. & Yokoyama, K. Immobilization of single-stranded DNA by self-assembled polymer on gold substrate for a DNA chip. *Biotechnology and Bioengineering* **89**, 835-838 (2005).
3. Vainrub, A. & Pettitt, B. Surface electrostatic effects in oligonucleotide microarrays: Control and optimization of binding thermodynamics. *Biopolymers* **68**, 265-270 (2003).
4. Mary-Huard, T. *et al*. Spotting effect in microarray experiments. *BMC Bioinformatics* **5**, 63-71 (2004).
5. Wei, C.-W. *et al*. Using a microfluidic device for 1 μL DNA microarray hybridization in 500s. *Nucleic Acid Research* **33**, e78 (2005).
6. Benters, R., Nienmeyer, C.M. & Wohrle, D. Dendrimer-activated solid supports for nucleic acid and protein microarrays. *Chembiochem* **2**, 686-694 (2001).
7. Martinsky, T. Microarrays: Not just for genomics anymore. *Microarray Technology* 12-24 (2004).
8. Krishnan, A. *et al*. An evaluation of methods for contact angle measurement. *Colloids and Surfaces B: Biointerfaces* **43**, 95-98 (2005).
9. Chiu, S.-K. *et al*. Synergistic effects of epoxy- and amine-silanes on microarray DNA immobilization and hybridization. *Biochem J*. **374**, 625-632 (2003).
10. Shi, G., Wen, S.-Y., Chen, S.-H. & Wang, S.-Q. Fabrication and optimization of the multiplex PCR-based oligonucleotide microarray for detection of *Neisseria gonorrhoeae, Chlamydia trachomatis* and *Ureplasma urealyticum*. *J. of Microbiological Methods* **62**, 245-256 (2005).
11. Liu, S. *et al*. Analysis of the factors affecting the accuracy of detection for single base alterations by oligonucleotide microarray. *Experimental and Molecular Medicine* **37**(2), 71-77 (2005).
12. Yu, F., Yao, D. & Knoll, W. Oligonucleotide hybridization studied by a surface plasmon diffraction sensor (SPDS). *Nucleic Acid Research* **32**(9), e75 (2004).
13. Hwang, T.S. *et al*. Detection and typing of HPV genotypes in various cervical lesions by HPV oligonucleotide microarray. *Gynecologic Oncology* **90**, 51-56 (2003).
14. Hackler, L. *et al*. Development of chemically modified glass surfaces for nucleic acid, protein and small molecule microarrays. *Molecular Diversity* **7**, 25-36 (2003).
15. Choi, J.-W., Chun, B., Oh, W., Lee, W. & Lee, W. Fabrication of DNA-protein conjugate layer on gold-substrate and its application to immunosensor. *Colloids and Surfaces B: Biointerfaces* **40**, 173-177 (2005).
16. Herne, T.M. & Tarlov, M.J. Characterization of DNA probes immobilized on gold surfaces. *J. Am. Chem. Soc*. **119**, 8916-8920 (1997).
17. Du, W., Marsac, C., Kruschina, M., Ortigao, F. & Florentz, C. Functionalized self-assembled monolayer on gold for detection of human mitochondrial tRNA gene mutations. *Analytical Biochemistry* **322**, 14-25 (2003).
18. Sakao, Y., Nakamura, F., Ueno, N. & Hara, M. Hybridization of oligonucleotide by using DNA self-assembled monolayer. *Colloids and Surfaces B: Biointerfaces* **40**, 149-152 (2005).
19. Ivanova, E.P. *et al*. Poly(L-lysine)-mediated immobilization of oligonucleotides on carboxy-rich polymer surfaces. *Biosensors and Bioelectronics* **19**, 1363-1370 (2004).
20. Tudorache, M. & Bala, C. Biosensors based on screen-printing technology, and their applications in environmental and food analysis. *Anal Bioanal Chem*. **388**, 565-578 (2007).