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Electrochemical Detection of Single Nucleotide Polymorphism using Gold Electrode Arrays Modified with Thiol Linkers Synthesized by Solid-Phase Strategy

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Abstract

We developed an electrochemical DNA sensor based on gold electrode arrays modified with a thiol linker synthesized by novel solid-phase strategy, and could effectively detect the aging-dependant T414G mutation of mitochondrial DNA. To electrochemically detect the single nucleotide polymorphism (SNP), the gold electrode arrays were patterned on glass wafers, and the thiol linker facilely synthesized on the solid support was assembled on the gold electrode surface. On the SAM electrode surface, three kinds of DNA probes, T414G-T probe, T414G-G probe and control R probe, were covalently immobilized. Using the cyclic voltametry (CV), we could not only discriminate between full-matched DNA and single-mismatched DNA successfully, but also confirm the effectiveness of the synthesized thiol linkers for reducing the non-specific DNA binding.

Keywords: Electrochemical DNA sensor, Gold electrode arrays, Mitochondrial DNA, Single nucleotide polymorphism, Solid-phase synthesis

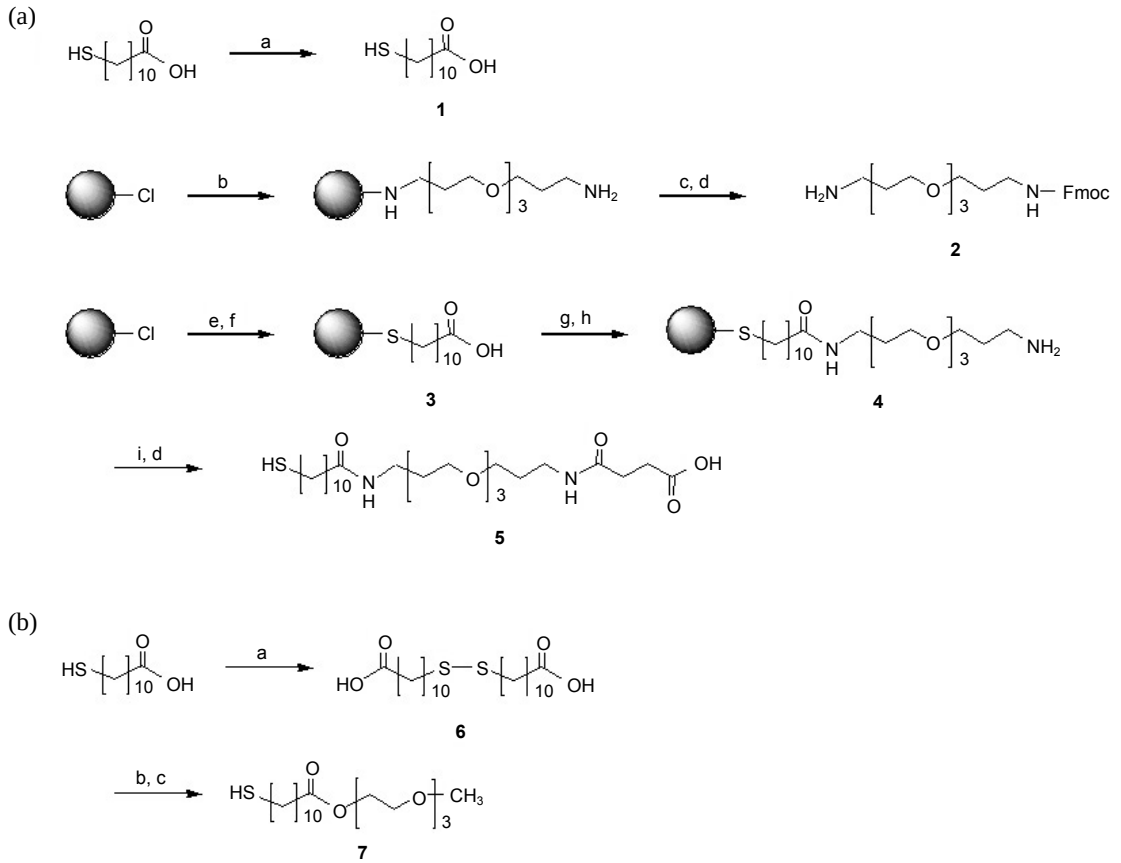
Introduction

Genetic factors contribute to virtually every human disease, conferring susceptibility or resistance, or influencing interactions with environmental factors. Single nucleotide polymorphism (SNP) which is the main cause of genetic variations is the point mutation that is found at a rate of 0.5-10 per 1000 base pairs within the human genome[1]. Since the early 1990's, DNA chips have been used to discover SNPs and a variety of different sensing methods have been developed[2,3].

Among the different DNA chips, fluorescence-based DNA chips are of particular interest, because they are sensitive and suitable for the array-based format. However, these chips require expensive and sophisticated instrumentation[4,5]. In addition, the post-synthetic labeling of the target DNA is also required, which substantially limits their application to real systems. The detection method such as a quartz crystal microbalance (QCM) involves the monitoring of mass changes on the surface of the chip[6,7]. The largest advantage of this method is the real-time detection of the surface changes. However, ensuring the reliable operation of the QCM still remains a serious technical challenge. Recently, several electrochemical-based methods, which are simple and sensitive, have been developed[8,9]. These methods do not require expensive instrumentation or post-synthetic labeling (label-free detection).

Electrochemical sensors have novel metal-plated electrodes, especially gold surface. The conductivity changes between the working electrode and the buffer medium induced by the differences of the electrode surface make the meaningful electrical signals. Therefore, it is essential to make the electrode surface stable and unblemished in order to obtain reliable and reproducible signals. The alkane thiol-based self-assembled monolayer (SAM) could afford one to achieve the goals[10,11]. Several research groups have developed alkane thiol-based SAM compounds with various molecular structures. In general, the alkane thiol molecules were synthesized using a solution-phase strategy, and they were used to immobilize various biomolecules such as proteins and carbohydrates on the sensor surface. There have been a wide range of applications including DNA chips[12,13], peptide/protein chips[14-16] and carbohydrate chips[17,18].

In this study, we facilely synthesized a thiol linker using a novel solid-phase strategy, which was applied to a gold electrode array based electrochemical DNA



Scheme 1. (a) Synthesis of carboxylic acid-terminated thiol linker: a) H_2SO_4, MeOH; b) 4,7,10-trioxa-1,13-tridecanediamine, TEA, DCM; c) Fmoc-Cl, TEA; d) TFA, TES, DCM; e) compound 1, DIPEA, DCM; f) NaOH, TFA; g) compound 2, DIC, HOBT, DIPEA, DCM; h) 20% (v/v) piperidine in DMF; i) succinic anhydride, DIPEA, DMF, and (b) synthesis of methoxy-terminated thiol linker: a) I_2, DMSO, THF; b) tri(ethylene glycol) monomethyl ether, DIC, DMAP, DCM; c) DTT, EtOH.

chip. And we efficiently detected the aging-dependant T414G mutation of mitochondrial DNA, a single nucleotide polymorphism (SNP). The thiol linker was designed to have the triethylene glycol part and hydrocarbon part as the molecular backbone, and the thiol group at one terminus and a carboxylic group at the opposite terminus. The solid phase-synthesis offered us many advantages in the purification and characterization of the linker molecules, compared wiht the solution-phase synthesis method[19-21] or the on-chip synthesis method.[22-24]. For the SNP assay, three kinds of DNA probes (T414G-T probe, T414G-G probe and control probe) were covalently immobilized on the SAM-treated electrode surface. The target DNA which is fully complementary to T414G-T probe was hybridized with the DNA probes on the gold electrode surfaces, and the signals were detected by the cyclic voltametry (CV) system.

Results and Discussion

Synthesis of the Carboxylic Acid-terminated Thiol Linker, 5 and Methoxy-terminated Thiol Spacer, 7

To modify the surface of the gold electrodes, we synthesized a novel linker molecule with the thiol group at one end and the carboxylic acid group at the other end. The synthesized linkers contained triethylene glycol (TEG), which are known to reduce the nonspecific binding of biomolecules[19] and to increase the water solubility of the linker compounds.

The synthetic strategy was described in Scheme 1. The methyl ester of 11-mercaptoundecanoic acid (MUA), 1 was prepared in 97% yield by the esterification of MUA with methanol. Compound 1 was loaded onto 2-cholrotrityl chloride (CTC) resin, with this step being monitored by elemental analysis of the resin. The

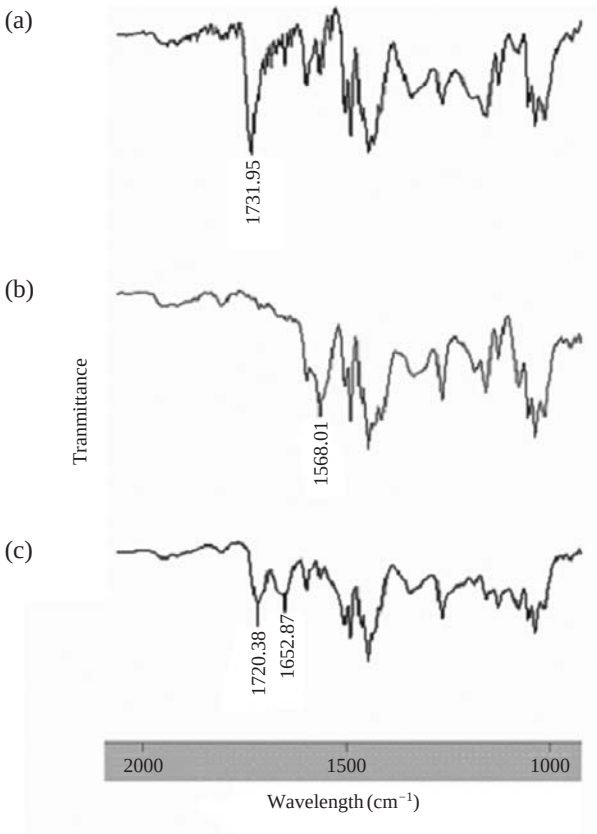


Figure 1. The FT-IR spectra of (a) resin 3 before saponification, (b) resin 3 after saponification, and (c) resin 4.

loading level of compound 1 was determined to be 0.25 mmol/g. After the saponification of methyl ester 1, the carboxyl end group of the MUA on the resin was coupled with Fmoc-NH_2-TEG-NH_2, 2 to introduce the TEG group onto the linker (by DIC coupling). The progress of the hydrolysis and the coupling reaction was monitored by FT-IR. The FT-IR spectra are shown in Figure 1.

As shown from Figure 1a, the thiol linker 1 loaded on CTC resin showed a typical ester bond adsorption at 1,736 cm^{-1}. After saponification, a new peak appeared at 1,567 cm^{-1} (Figure 1b), which confirmed that the saponification reaction proceeded quantitatively. After Fmoc-NH_2-TEG-NH_2 coupling, a new peak appeared at 1,652 cm^{-1} (Figure 1c). The result of the elemental analysis of the resin demonstrated that the TEG coupling reaction proceeded quantitatively. The N-content was determined to be 0.5 mmol/g and the S-content was 0.25 mmol/g, as determined by elemental analysis. The loading level of the TEG molecules was found to be 0.25 mmol/g by Fmoc group titration. These results were consistent with each other. After the removal of the Fmoc-group, the resulting amino group was succinylated by succinic anhydride to introduce the carboxylic acid end group for the purpose of facilitating the coupling with the NH_2-modified DNA. The completion of the succinylation reaction was monitored by the Kaiser test. After removing the final linker compound from the resin by means of trifluoroacetic acid/triethylsilane (TFA/TES) treatment, the by-products were separated by flash chromatography to give the pure compound 5 in 75% yield, which is based on the initial loading of thiol compound 1. In terms of the linker synthesis, our solid-phase based synthetic method proved to be simple and efficient compared to other solution-phase synthesis methods.

Moreover, we chose a methoxy-terminated thiol spacer to control the density of the carboxylic acid-terminated thiol linker, 5. As described in Scheme 1b, the compounds 7 was simply synthesized in the solution phase. After the purification, the final yield was 72%.

Preparation and Characterization of Spacer Immobilized Gold Electrodes

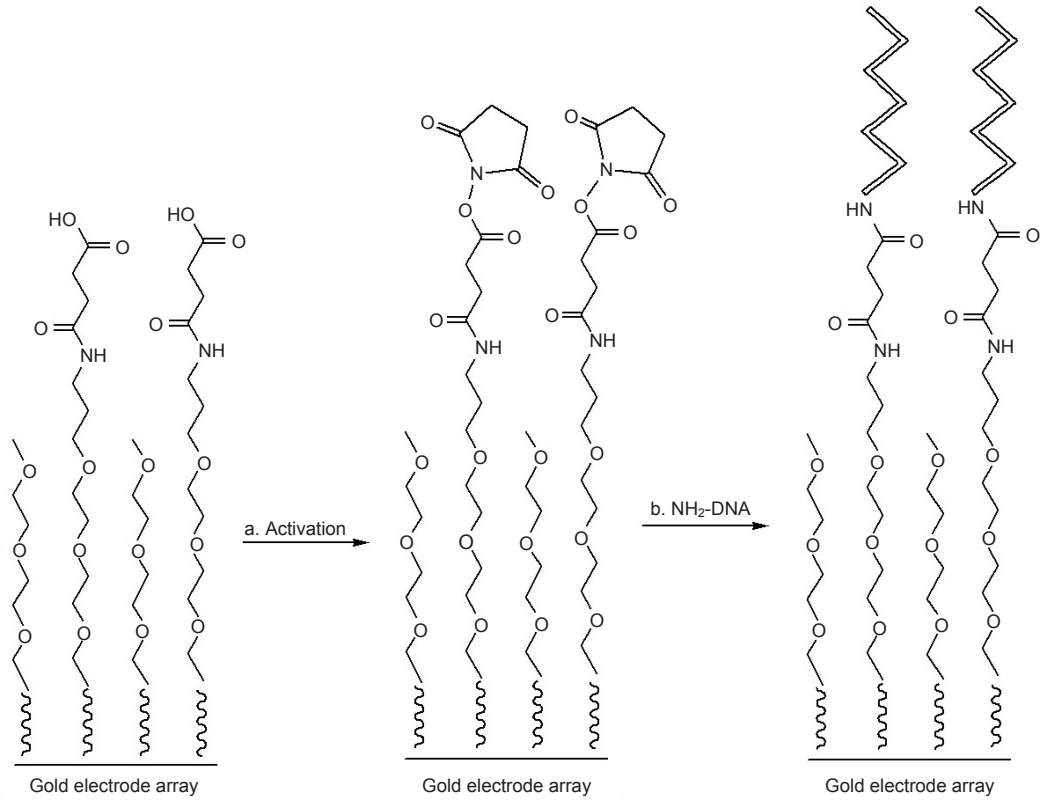
The self-assembled monolayer (SAM) was achieved on the surface of gold electrodes with a 1 : 1 mixture of thiol linker compounds 5 and 7, as depicted in Scheme 2. The resulting surface was characterized by the contact angle measurement. The water contact angle data provided direct evidence of any change in the surface hydrophilicity when the linkers were immobilized on the gold surface. The bare gold surface was hydrophobic and the contact angle was as high as 72°, whereas, after the immobilization of the linkers, the gold surface became more hydrophilic, and the contact angle decreased to 58°. These results prove that the surface became hydrophilic, due to the terminal carboxyl group and the TEG moiety of linkers 5 and 7.

Immobilization of Probe DNA on Gold Electrodes

Before the immobilization of the probe DNA on the gold electrodes, the carboxylic acid group was activated with *N*-hydroxysuccinimide (Scheme 2), in order to facilitate the conjugation of the NH_2-modified DNA. The immobilization of the DNA on the gold electrodes was confirmed by X-ray photoelectron spectroscopy (XPS). As shown in Figure 2, the S and P contents were increased in the probe DNA immobilized gold electrode.

Electrochemical Measurements

Three different types of probe DNA were immobilized on the surface of the gold electrodes of the DNA chip. The probe DNA-T differs from the probe DNA-G at one base, and this generates a single base mismatched dsDNA after hybridization with the target DNA-A, as summarized in Table 1. The probe DNA-



Scheme 2. Preparation of the electrochemical DNA chip based on gold electrode arrays modified with a thiol linker synthesized by novel solid-phase strategy: a) EDC, NHS; b) aminated probe DNAs.

Table 1. Sequences and mismatch locations of probe DNAs.

Probe and Target DNAs	Sequences
NH_2-probe DNA-T	5′-NH_2-TCTTTTGGCGGTATGCACTT-3′
NH_2-probe DNA-G[a]	5′-NH_2-TCTTTTGGCGGGATGCACTT-3′
NH_2-probe DNA-R[b]	5′-NH_2-AAACCCCCTCCCCATGCTTA-3′
Target DNA-A[c]	5′-AAGTGCATACCGCCAAAAGA-3′

[a]Underline represent the mismatched bases of the probe or target DNA

[b]Non-complementary DNA as a control

[c]Target A is complementary to the probe T

R contains random sequences that are not hybridized with the target DNA and, consequently, it remains as a single strand oligonucleotide, even after the hybridization process. Figure 3 shows the cyclic voltammograms (CVs) obtained in the presence of $[Fe(CN)_6]^{3+}$ and methylene blue (MB). We chose MB as an intercalator, since it binds readily to the dsDNA and its association constant is $3.8 \times 10^6\ M^{-1}$ [25]. As expected, the probe DNA-T-coupled electrode demonstrated the highest current peak after hybridization, and its CV was clearly distinguished from those of the probe DNA-A and the probe DNA-R (Figure 3). This result demonstrated that the DNA sensors prepared with the new synthetic linkers successfully generated CVs in the presence of MB and $[Fe(CN)_6]^{3+}$ and were able to discriminate the single G mutation from the wild type (T) of the target DNA.

To demonstrate the TEG effect of the linker system, we prepared another system without the TEG group. MUA and thiol linker 1 were used for the construction of the SAM on the gold electrodes. The conditions used for the probe DNA immobilization and hybridization were the same as those used for the synthetic linker system. With this linker system which did not contain the TEG group, we could not discriminate the dsDNA from either the ssDNA or the single-base mismatched DNA. Moreover, the peak currents generated by the ssDNA and single-base mismatched DNA had low reproducibility (Figure 4). This result indicates that a significant amount of non-specific binding occurred on the surface of the gold electrode when the linker did not contain the TEG group.

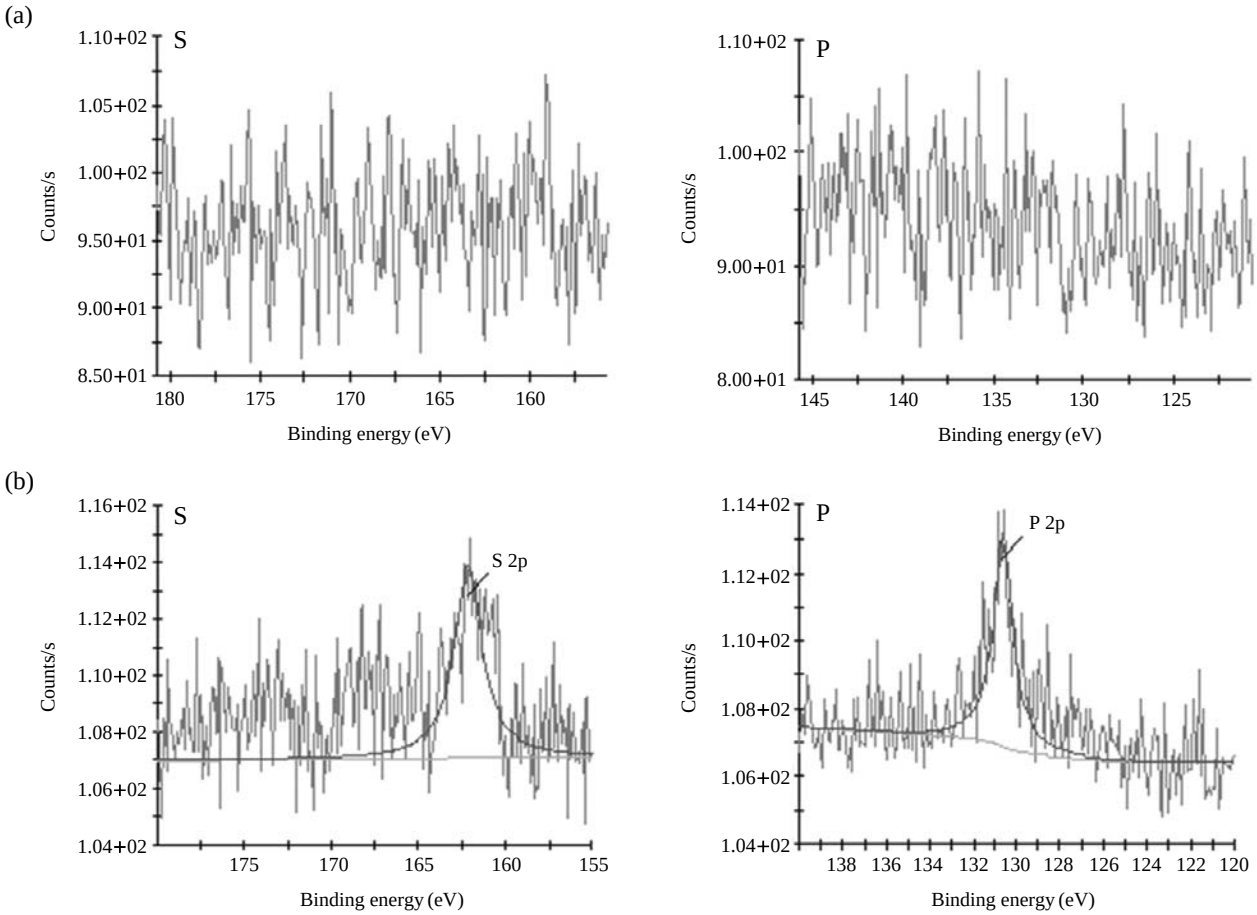


Figure 2. Characterization of the oligonucleotide immobilized gold surface by X-ray photoelectron spectroscopy (XPS) for sulfur and phosphorus; (a) bare gold surface, and (b) DNA immobilized gold surface.

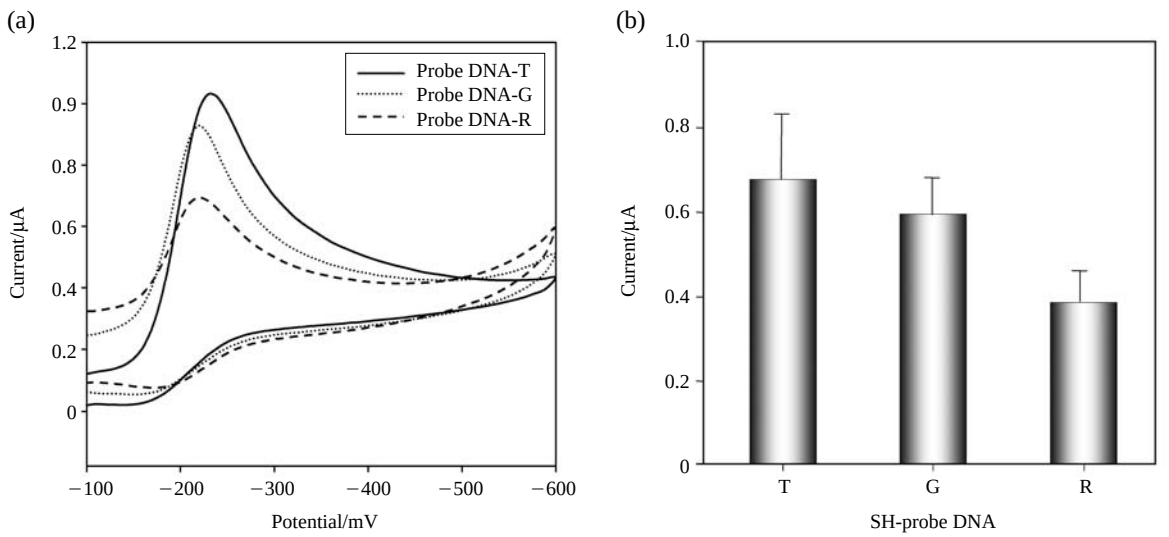


Figure 3. Detection of T414G mutation of mtDNA using DNA-electrode sensor functionalized with novel thiol linker; (a) representative CV, and (b) the peak currents of the CVs which were quantified by the symmetric curve method (Data represent mean (four data)+standard deviation).

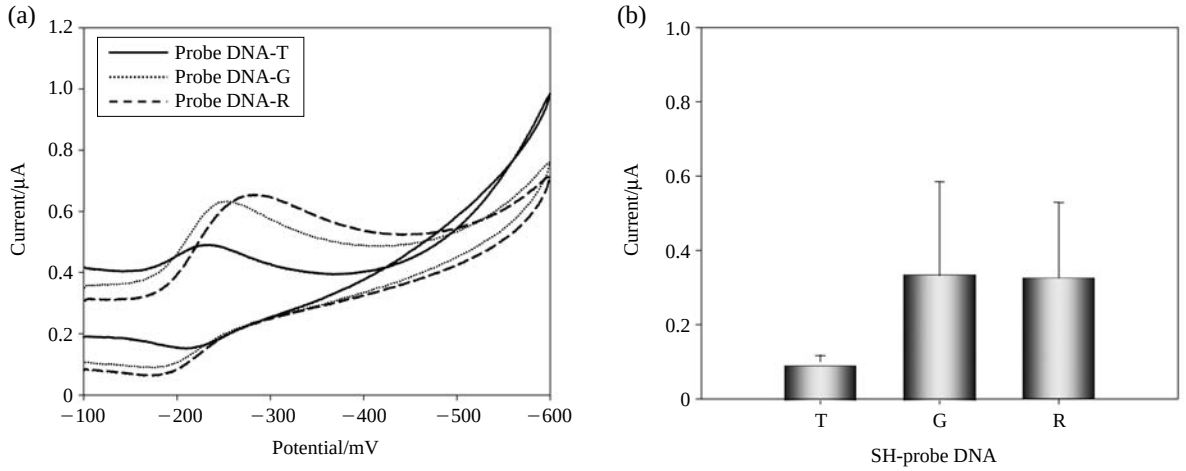


Figure 4. CV graph from DNA-electrode sensor functionalized with MUA; (a) representative CV, and (b) the peak currents of the CVs which were quantified by the symmetric curve method (Data represent mean (four data)+standard deviation).

Conclusions

In conclusion, we developed an electrochemical DNA sensor based on the gold electrode arrays modified with a thiol linker, which could effectively detect the aging-dependant T414G mutation, single nucleotide polymorphism (SNP). To detect SNP electrochemically, the gold electrode arrays were fabricated on the glass wafers, and the thiol linker which was facilely synthesized on the solid support was assembled on the gold electrode surface. On the SAM electrode surface, three kinds of DNA probes, T414G-T probe, T414G-G probe and control probe, were covalently immobilized. Using the cyclic voltametry (CV) system, we could not only discriminate between fully-matched DNA and single-mismatched DNA successfully, but also confirm the effectiveness of the synthesized thiol linkers on reducing the non-specific DNA binding. Moreover, we believe that the thiol linker, synthesized by solid-phase strategy, for gold surface will have broad applications in the field of diagnostic biosensors.

Materials and Methods

Chemicals

2-Chlorotrityl chloride (CTC) resin was purchased from BeadTech Inc. (Korea). *N*-hydroxysuccinimide (NHS) was purchased from Fluka (Japan). Methylene blue, 2-[*N*-morpholino]ethane sulfonic acid (MES), and sodium nitrate, were purchased from Sigma-Aldrich (USA). The photosensitive polyimide (PI2771) was purchased from HD Microsystems (USA). All other reagents were purchased from Sigma-Aldrich (USA). The solvents used for the substrate synthesis were of analytical grade and were used without purification.

Design of Oligonucleotides

The probe DNAs, used for the immobilization on the gold electrode, were designed to have 20 nucleotides. The probe DNAs contained either T_{414} (probe DNA-T) or G_{414} (probe DNA-G) in a 20 nucleotide stretch of the mtDNA D-loop region. The control probe DNA (probe DNA-R), which had a random DNA sequence, and other probe DNAs, which were modified with the amino group at the 5′-end, were purchased from Bioneer (Korea); the probe sequences are summarized in Table 1. The target DNA oligonucleotide is complementary to the probe DNA-T.

Instrumentation

For the resin characterization and analysis, FT-IR (Bomem, FTLA2000) and elemental analysis (Leco, CHNS-932) were used. For the fabrication of the gold electrode, an e-beam evaporator (Anelva, VN-43) was used. In order to analyze the compound's structure, NMR (Japan Electronic Optics Laboratory, JNM-LA300) was used. For the surface characterization and analysis, atomic force microscopy (AFM) (Digital Instrument, NanoScope IIIa), X-ray photoelectron spectroscopy (XPS) (SIGMA PROBE, ThermoVG) and static water contact angle measurement (SEO, Phoenix 300) were used. For the electrochemical analysis, the electrochemical analyzer (Bioanalytical Systems, CV-50W) was used.

Synthesis of Thiol Linkers

Synthesis of Methyl 11-mercaptoundecanoate, 1

11-Mercaptoundecanoic acid (MUA, 1.0 g, 4.58

mmol) was dissolved in dried MeOH (15 mL) under nitrogen. Sulfuric acid (44.9 mg, 0.458 mmol) was added to the mixture, which was stirred for 4 h. Then, the solvent was evaporated, replaced by dichloromethane (DCM, 50 mL), and the solution was washed with water. The organic phase was neutralized with 5% $NaHCO_3$ in water, and dried over $MgSO_4$. The solution was filtered and concentrated. The residue was purified by flash-chromatography (silica gel; $CHCl_3$/MeOH, 20 : 1). The yield of the isolated products was 97% (1.06 g), and was in the form of a white solid. ^{1}H NMR (300 MHz, $CDCl_3$, 25°C, TMS): δ=1.3 (broads, 12H), 1.6 (m, 4H), 2.3 (t, 2H), 2.6 (q, 2H), 3.6 (s, 3H).

Preparation of Fmoc-NH-TEG-NH_2, 2

A 4, 7, 10-trioxa-1, 13-tridecanediamine (NH_2-TEG-NH_2, 2.9 g, 13 mmol) and 667 mg triethylamine (667 mg, 6.5 mmol) were added to 2-chlorotrityl chloride (CTC) resin (1.0 g, 1.3 mmol) in 10 mL of DCM. After 18 h, the resin was filtered and washed with MeOH and DCM (3 × 10 mL, each). Then, the resin was treated with Fmoc-chloride (672 mg, 2.6 mmol) and triethylamine (263 mg, 2.6 mmol) in 10 mL of DCM. The termination of the coupling reaction was monitored by the Kaiser's ninhydrin test[26]. After 24 h, the beads were washed with MeOH and DCM (3 × 10 mL, each). After drying in vacuo, the product, Fmoc-NH-TEG-NH_2, 2 was cleaved from the resin by treating it with 5% trifluoroacetic acid (TFA)/triethylsilane (TES) (2 : 1) in DCM (10 mL) 3 times for 10 min each. The cleavage solvents were collected and evaporated, yielding a single product. The final product 2 was purified by flash-chromatography (silica gel; $CHCl_3$/MeOH, 5 : 1), yielding 490 mg of yellow oil (85% yield). ^{1}H NMR (300 MHz, $CDCl_3$, 25°C, TMS): δ=1.63 (t, 2H), 1.91 (t, 2H), 3.15-3.25 (broads, 4H), 3.36-3.67 (m, 14H), 4.19 (t, 2H), 4.36 (d, 2H), 7.30 (d, 2H), 7.41 (t, 2H), 7.65 (d, 2H), 7.73 (d, 2H).

Anchoring of MUA to 2-chlorotrityl Chloride (CTC) Resin

Compound 1 (906 mg, 3.9 mmol) and *N*,*N*-diisopopylethylamine (DIPEA, 505 mg, 3.9 mmol) were added to the CTC resin (1.0 g, 1.3 mmol) in 10 mL of DCM; the mixture was shaken at 25°C. After 24 h, DIPEA/MeOH (10% v/v) was added to quench the residual chloride groups on the CTC resin. After shaking the mixture for 1 h, the resin was filtered and washed with MeOH and DCM (3 × 10 mL, each). The methoxy groups were removed from the protected MUA on the CTC resin by hydrolysis in 1 M NaOH/THF, (50%, v/v) at 55°C for 24 h[27]. The resulting resin 3 was filtered and washed with water, MeOH and DCM (3 × 10 mL, each).

Coupling of TEG Group to MUA Coupled Resin 3

The carboxylic group of the MUA on resin 3 was activated with *N*,*N*′-diisopropylcarbodiimide (DIC, 5eq), 1-hydroxybenzotriazole hydrate (HOBT, 5eq) and DIPEA (5eq) in DCM (15 mL) for 3 h. Then, Fmoc-NH-TEG-NH_2, 2 (3eq) was added, and the resin mixture was shaken for 18 h. The resin was filtered and washed with MeOH and DCM (3 × 10 mL, each). The resulting Fmoc group of the resin was removed by treating it with 20% (v/v) piperidine in DMF (twice, for 3 min and 17 min). Then, the resin was filtered and washed with DMF, MeOH and DCM. The TEG group coupling and the Fmoc deprotection step were monitored by elemental analysis of the resin and the Fmoc group titration method respectively.

Introduction of Carboxylic Acid Group to Resin 4 by Succinylation

Succinic anhydride (3 eq) and DIPEA (3 eq) in DMF were added to the amino group of the TEG on resin 4, then the resin mixture was shaken at room temperature. After 3 h, the resin beads were filtered and washed with DMF, MeOH and DCM (3 × 10 mL, each). The termination of the succinylation reaction was monitored by the Kaiser test. After drying in vacuo, the loading of the linker was found to be 0.25 mmol/g resin by elemental analysis of the resin.

Recovery of Thiol Linker 5 from the Resin

The thiol linker 5 was cleaved from the resin by treating it with 3% TFA in DCM containing 1% TES (10 mL) 3 times for 10 min each. The solvent was concentrated and then the product was purified by flash-chromatography, yielding 98 mg (75%) of a white solid (silica gel; $CHCl_3$/MeOH, 7 : 1). ^{1}H NMR (300 MHz, $CDCl_3$, 25°C, TMS): δ=1.31 (m, 12H), 1.60 (q, 4H), 1.77 (q, 4H), 2.18 (t, 2H), 2.53 (m, 4H), 2.65 (m, 2H), 3.37 (q, 4H), 3.56-3.67 (m, 12H).

Synthesis of 2-(2-(2-methoxyethoxy)ethoxy)ethyl 11-mercaptoundecanoate, 7

11-Mercaptoundecanoic acid (MUA) (218 mg, 1 mmol) was dissolved in THF (5 mL), and iodine (5 mg, 0.04 mmol) was dissolved in dimethyl sulfoxide (DMSO, 1 mL). The resulting iodine solution was added to the MUA solution and the mixture was stirred until the color of the solution turned red. After evaporating the solvent, the residue was dissolved in ether and washed with water 3 times. After evaporating the solvent in vacuo, the disulfide compound 6 was obtained as a white solid in 94% (204 mg) yield. ^{1}H NMR (300 MHz, DMSO, 25°C, TMS): δ=1.25 (b, 24H), 1.48 (q, 4H), 1.61 (q, 4H), 2.18 (t, 4H), 2.68 (t, 2H).

The disulfide compound 6 (200 mg, 0.460 mmol) was dissolved in DCM (20 mL). To this solution, DIC (138 mg, 1.10 mmol) and 4-dimethylaminopyridine (DMAP, 134 mg, 1.10 mmol) were added. After 30 min, triethyleneglycol monomethyl ether (HO-TEG-OCH_3)

(181 mg, 1.10 mmol) was added to the solution. After stirring for 2 h, the solvent was evaporated. Then, the residue was purified by flash-chromatography (silica gel; $CHCl_3$/MeOH 30 : 1). The yield of the product was 86% (287 mg). The resulting disulfide was dissolved in EtOH (20 mL), and then dithiothreitol (DTT) (67 mg, 436 mmol) was added. After 3 h, the solvent was evaporated and the thiol linker diluent 7 was purified by flash-chromatography ($CHCl_3$: MeOH=30 : 1), giving a white solid in 72% (196 mg) yield. ^{1}H NMR (300 MHz, DMSO, 25˚C, TMS): δ=1.28 (broads, 14H), 1.64 (q, 4H), 2.33 (t, 2H), 2.68 (t, 2H), 3.39 (s, 3H), 3.54 (t, 2H), 3.64 (m, 8H), 4.23 (t, 2H).

Fabrication of Gold Electrode Array

A 4×4 gold electrode array was designed for the purpose of detecting various DNA samples simultaneously, and was fabricated as described previously[28]. Firstly, an approximately 500Å thick Ti film (99.9% in purity) was deposited on a 4-inch Pyrex glass wafer by e-beam evaporation, and then a 2000Å thick gold thin film was deposited on top of the Ti layer. The Au/Ti layer was patterned by photolithography and three dies, each consisting of sixteen gold working electrodes with an area of 2 mm^2, were formed on the Pyrex glass wafer. The three dies were diced using a dicing saw, and each die had dimensions of 45 mm × 20 mm. To insulate the multi array DNA sensor, a photosensitive polyimide (Pyralin®PI2771) was spin coated on top of the sensor, baked for 30 min at 130˚C, and patterned so that the gold sensing areas and interconnection pads were exposed for the purpose of immobilizing the probe DNAs and electrical testing, respectively.

Immobilization of Probe DNA on Gold Electrode Surface

The gold electrodes fabricated in the multi-array were electro-polished by cyclic voltammetry in the potential range of 0.1 V-1.5 V versus Ag/AgCl in 0.1 M H_2SO_4 solution at a scan rate of 100 mV/s using an electrochemical analyzer. Once a stable cyclic voltammogram was obtained, the multi-gold electrode array was rinsed with water. The polished gold electrodes were immersed in 1 mM thiol linker solution (1 : 1 mixture of thiol linker 5 and thiol linker 7 in ethanol) for 24 h at room temperature. Thereafter, they were rinsed with ethanol 5 times and dried under N_2. The resulting gold surface was immersed in 50 mM of freshly prepared EDC and 10 mM of *N*-hydroxysuccinimide (NHS) in pH 5.5 MES buffer (4 mL) for 24 h, rinsed with water 5 times and then dried under N_2. The amino-modified probe oligonucleotides (NH_2-probe DNA, 500 pmole/μL) in $NaHCO_3$ pH 8.0 buffer (1 μL) were spotted onto each of the linker-modified electrodes, and incubated for 3 h at 25˚C in a humidity chamber. The electrodes were then rinsed with buffer solution and deionized water (5 times each). The target DNA-A, which is complementary to the probe DNA-T, was added to the probe DNA-modified electrodes and incubated in a humidity chamber for 16 h at 40˚C. 20 μL of 1 M NaCl/TE hybridization solution (10 mM Tris, 1 mM EDTA, pH 7.4) was applied to the probe DNA-gold electrode surface, which was then covered with a cover glass. Then the electrodes were washed sequentially with the following washing solutions; 6× SSPE, 0.1% SDS, 39˚C for 10 min; 3× SSPE, 0.05% SDS, 39˚C for 10 min; SSPE, 0.01% SDS, 50˚C for 5 min; 1 M $NaNO_3$ in PBS at room temperature for 10 min, and deionized water at room temperature for 10 min.

Cyclic Voltammetry Measurement

The hybridized DNA multi-array electrodes were immersed in an electrolyte solution containing 5 mM phosphate buffer (pH 7.0), 0.4 mM potassium ferricyanide, 50 mM NaCl and 0.5 μM methylene blue (MB) at room temperature under dark conditions for 10 min, prior to the cyclic voltammetry (CV) measurement. CV measurement was performed using a CV50W electrode chemical analyzer (BioAnalytical System). The electrochemical detection system consisted of a modified gold working electrode, an Ag/AgCl reference electrode (BAS Model MW-4130) and a platinum wire as the auxiliary counter electrode (BAS Model MF-2052). CV was carried out at room temperature, at a sweep rate of 100 mV/sec between −600 mV and −100 mV. The measured data were stored in a computer connected to the electrochemical analyzer. The peak current of the CV was quantified by the symmetric curve technique, using the method provided by the BAS CV manufacturer. The symmetric curve method calculated the peak height from the base line of the peak, and was more reliable than the tailed curve method.

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References

1. Kirk, B.W., Feinsod, M., Favis, R., Kliman, R.M. &

Barany, F. Single nucleotide polymorphism seeking long term association with complex disease. *Nucleic Acids Research* **30**, 3295-3311 (2002).
2. Li, Y.A., Wark, A.W., Lee, H.J. & Corn, R.M. Single-nucleotide polymorphism genotyping by nanoparticle-enhanced surface plasmon resonance imaging measurements of surface ligation reactions. *Analytical Chemistry* **78**, 3158-3164 (2006).
3. Zhong, X.B. *et al.* Single-nucleotide polymorphism genotyping on optical thin-film biosensor chips. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 11559-11564 (2003).
4. Epstein, J.R., Biran, I. & Walt, D.R. Fluorescence-based nucleic acid detection and microarrays. *Analytica Chimica Acta* **469**, 3-36 (2002).
5. Sassolas, A., Leca-Bouvier, B.D. & Blum, L.J. DNA biosensors and microarrays. *Chemical Reviews* **108**, 109-139 (2008).
6. Lazerges, M., Perrot, H., Zeghib, N., Antoine, E. & Compere, C. In situ QCM DNA-biosensor probe modification. *Sensors and Actuators B-Chemical* **120**, 329-337 (2006).
7. Pang, L.L., Li, J.S., Jiang, J.H., Shen, G.L. & Yu, R.Q. DNA-point mutation detection based on DNA ligase reaction and nano-Au amplification: A piezoelectric approach. *Analytical Biochemistry* **358**, 99-103 (2006).
8. Kim, Y.S., Lee, S.J. & Gu, M.B. Electrochemical aptamer-based biosensors. *BioChip J.* **2**, 175-182 (2008).
9. Pumera, M., Sanchez, S., Ichinose, I. & Tang, J. Electrochemical nanobiosensors. *Sensors and Actuators B-Chemical* **123**, 1195-1205 (2007).
10. Qiu, F., Jiang, D.W., Ding, Y.B., Zhu, J. & Huang, L.L. Monolayer-barcoded nanoparticles for on-chip DNA hybridization assay. *Angewandte Chemie-International Edition* **47**, 5009-5012 (2008).
11. Mrksich, M. Mass spectrometry of self-assembled monolayers: A new tool for molecular surface science. *Acs. Nano*. **2**, 7-18 (2008).
12. Riepl, M. *et al.* Functionalized surfaces of mixed alkanethiols on gold as a platform for oligonucleotide microarrays. *Langmuir* **18**, 7016-7023 (2002).
13. Umek, R.M. *et al.* Electronic detection of nucleic acids-A versatile platform for molecular diagnostics. *Journal of Molecular Diagnostics* **3**, 74-84 (2001).
14. Houseman, B.T., Gawalt, E.S. & Mrksich, M. Maleimide-functionalized self-assembled monolayers for the preparation of peptide and carbohydrate biochips. *Langmuir* **19**, 1522-1531 (2003).
15. Koehler, A.N., Shamji, A.F. & Schreiber, S.L. Discovery of an inhibitor of a transcription factor using small molecule microarrays and diversity-oriented synthesis. *Journal of the American Chemical Society* **125**, 8420-8421 (2003).
16. Xiao, S.J., Textor, M., Spencer, N.D. & Sigrist, H. Covalent attachment of cell-adhesive, (Arg-Gly-Asp)-containing peptides to titanium surfaces. *Langmuir* **14**, 5507-5516 (1998).
17. Fazio, F., Bryan, M.C., Blixt, O., Paulson, J.C. & Wong, C.H. Synthesis of sugar arrays in microtiter plate. *Journal of the American Chemical Society* **124**, 14397-14402 (2002).
18. Adams, E.W. *et al.* Oligosaccharide and glycoprotein Microarrays as tools in HIV glycobiology: Glycan-dependent gp120/protein interactions. *Chemistry & Biology* **11**, 875-881 (2004).
19. Roberts, C. *et al.* Using mixed self-assembled monolayers presenting RGD and (EG)(3)OH groups to characterize long-term attachment of bovine capillary endothelial cells to surfaces. *Journal of the American Chemical Society* **120**, 6548-6555 (1998).
20. Nelson, K.E. *et al.* Surface characterization of mixed self-assembled monolayers designed for streptavidin immobilization. *Langmuir* **17**, 2807-2816 (2001).
21. Houseman, B.T. & Mrksich, M. Efficient solid-phase synthesis of peptide-substituted alkanethiols for the preparation of substrates that support the adhesion of cells. *Journal of Organic Chemistry* **63**, 7552-7555 (1998).
22. Brockman, J.M., Frutos, A.G. & Corn, R.M. A multi-step chemical modification procedure to create DNA arrays on gold surfaces for the study of protein-DNA interactions with surface plasmon resonance imaging. *Journal of the American Chemical Society* **121**, 8044-8051 (1999).
23. Yang, Z.P., Frey, W., Oliver, T. & Chilkoti, A. Light-activated affinity micropatterning of proteins on self-assembled monolayers on gold. *Langmuir* **16**, 1751-1758 (2000).
24. Madoz, J., Kuznetzov, B.A., Medrano, F.J., Garcia, J.L. & Fernandez, V.M. Functionalization of gold surfaces for specific and reversible attachment of a fused beta-galactosidase and choline-receptor protein. *Journal of the American Chemical Society* **119**, 1043-1051 (1997).
25. Kelley, S.O., Barton, J.K., Jackson, N.M. & Hill, M.G. Electrochemistry of methylene blue bound to a DNA-modified electrode. *Bioconjugate Chemistry* **8**, 31-37 (1997).
26. Kaiser, E., Bossinger, C.D., Colescott, R.L. & Olsen, D.B. Color test for terminal prolyl residues in the solid-phase synthesis of peptides. *Analytica Chimica Acta* **118**, 149-151 (1980).
27. Lee, J., Gauthier, D. & Rivero, R.A. Solid-phase synthesis of 3,4,5-substituted 1,5-benzodiazepin-2-ones. *Journal of Organic Chemistry* **64**, 3060-3065 (1999).
28. Cho, S., Pak, J.J., Hong, J. & Pak, Y.K. Fabrication of a multi-electrode array DNA sensor for electrochemical genotyping. *Journal of the Korean Physical Society* **41**, 1054-1057 (2002).