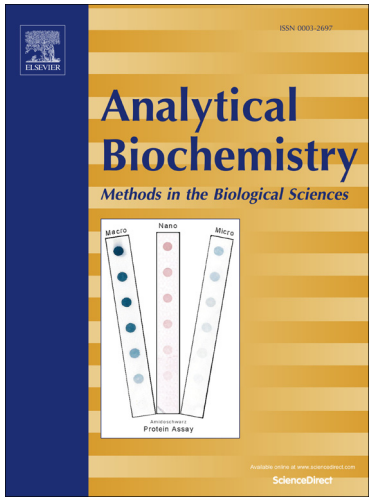

Notes & Tips

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A lipid-based method for the preparation of a piezoelectric DNA biosensor

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ABSTRACT

A piezoelectric DNA biosensor was prepared by immobilizing DNA probes on a quartz crystal microbalance (QCM) using a lipid-based method. A QCM electrode was coated with a hybrid bilayer membrane composed of an octadecanethiol monolayer and a lipid monolayer containing biotinylated lipids to establish biotin groups on the electrode surface. A DNA biosensor was prepared by sequentially immobilizing avidin and the biotinylated probe. The DNA biosensor was stable throughout repeated surface regeneration and showed higher sensitivity than that prepared by the conventional chemical method using diimide. We also optimized the surface regeneration conditions and flow rate for flow injection analysis.

Keywords: DNA biosensor, quartz crystal microbalance, lipid, biotin, avidin

Abbreviations used: QCM, quartz crystal microbalance; HBM, hybrid bilayer membrane; BTPE, biotinyl-phosphatidylethanolamine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, SAM, self-assembled monolayer

A DNA biosensor is an analytical device commonly used for sequence-specific DNA detection, and is an important tool for diagnostics as well as life sciences research. Label-free DNA biosensors involve a single-stranded DNA probe immobilized on a signal transducer, which produces a signal as the target DNA hybridizes to the probe. Immobilization of the DNA probe on the signal transducer surface is a critical step for the preparation of an efficient DNA biosensor. The quartz crystal microbalance (QCM) is a sensitive signal transducer based on the principle that the resonance frequency shifts of the piezoelectric crystal are proportional to the adsorbed molecular mass. Immobilization of a DNA probe on the QCM is commonly achieved through chemisorption of the thiolated probe on the gold electrode surface [1–6]. Alternatively, the biotinylated probe can be

conjugated to streptavidin that is immobilized on the QCM by chemical reactions [1, 2, 6, 7].

Recently, a lipid-based method was reported for immobilizing avidin on the QCM [8]. This method involves the addition of liposome over a hydrophobic self-assembled monolayer (SAM) of alkanethiol to prepare a hybrid bilayer membrane (HBM) composed of a phospholipid layer and an alkanethiol layer. Introduction of biotin groups to the QCM surface was easily achieved by including biotinyl-phosphatidylethanolamine (BTPE) in the liposome. This relatively simple method does not require any chemical reactions. Moreover, the sensitivity and stability of the resulting sensor chip was identical to that prepared by immobilizing avidin using the conventional chemical method with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS).

In this study, the lipid-based method was applied to the immobilization of biotinylated DNA probes to prepare a DNA biosensor. Avidin was immobilized on QCMs (8 × 8 mm AT-cut 10 MHz-QCM with a 5-mm diameter electrode; Crystal Sun Life, Tokyo, Japan) based on the method of Mun and Choi with slight modifications [8]. Dried lipid film was prepared with 0.54 μmol 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 0.27 μmol BTPE (Avanti Polar Lipids, Alabaster, USA) dissolved in 0.2 ml chloroform. The film was hydrated with 0.1 ml HEPES, pH 7.0, for 1 h at 50°C. The lipid suspension was converted to small unilamella vesicles by sonication. The hydrophobic SAM was prepared by immersing a cleaned QCM in 2 mM 1-octadecanethiol for 12–15 h with gentle stirring. The QCM was thoroughly rinsed with ethanol and dried with nitrogen gas. The QCM was installed in a well and washed with 40 mM n-octyl-β-D-glucopyranoside. Immediately following the wash, 0.1 ml liposome suspension was added to the well and incubated for 1.5 h at 50°C for formation of the HBM. Unstructured lipids were removed by washing with 0.1 M NaOH for 30 s.

The QCM was placed in a flow cell that was installed in an AMO-QC4 QCM biosensor system (Amogreentech, Gimpo, Korea). All solutions were injected into the QCM through an injection valve with a 100-μl sample loop. The resonant frequency was stabilized in a flow of phosphate-buffered

saline, which was used as a carrier buffer throughout the experiment. Avidin and the biotin probe (5′-TGGGCGGCATGAACCGGAGGCCCATC-3′) were sequentially immobilized by injecting 10 μg/ml avidin and 5 μM biotin probe. Nonspecifically adsorbed avidin and biotin probes were removed with 0.2 M R buffer (0.2 M glycine-HCl, pH 2.0, 0.1% dimethyl sulfoxide (DMSO)) after each injection. Injection of avidin and the biotin probe caused frequency shifts of approximately 90 Hz and 40 Hz, respectively. The frequency shifts were not reversed by surface regeneration with 0.2 M R buffer, revealing high-affinity binding of biotin to avidin. When 1 μM cDNA (5′-GATGGGCCTCCGGTTCATGCCGCCCA-3′) was injected into the resulting DNA sensor and the surface was regenerated with 0.2 M R buffer, recovery to the frequency baseline level was not complete due to incomplete dissociation of the hybridized cDNA (upper panel of Fig 1a). As a result, the frequency shift caused by injection of the same cDNA sample decreased after the first injection.

The application of two consecutive treatments of 1 mM HCl for 30–60 s in a well-type cell has been suggested for the regeneration of piezoelectric DNA biosensors [1, 2, 6, 7]. However, in our experiment, surface regeneration by injection of 1–4 mM HCl was not sufficient for complete removal of the hybridized DNA. Complete dissociation of the hybridized DNA was previously achieved by a flow of 0.5 M NaOH, 3 M NaCl for 20 min [4]; however, this method is time consuming, and the lipids can become hydrolyzed as a consequence of extended exposure to high NaOH concentration. After testing several different conditions, including variation of glycine-HCl concentration, we found that high glycine-HCl concentration resulted in efficient regeneration of the DNA biosensor. As shown in the lower panel of Fig. 1a, effective restoration to the baseline level was achieved by using 0.8 M glycine-HCl, pH 2.0, 0.1% DMSO (0.8 M R buffer), and the same frequency shift was observed with repeated injection of 1 μM cDNA. Fig. 1b shows the frequency shifts of avidin immobilization, biotin probe immobilization, and cDNA hybridization in experiments using R buffers containing different concentrations of glycine-HCl. The amount of immobilized avidin and biotin probe was similar with all of the R buffers tested, but the response to 0.2 μM and 1 μM cDNA differed significantly. As an optimal response was obtained at concentrations >0.8 M glycine-HCl, 0.8

M R buffer was chosen as the regeneration buffer for subsequent experiments.

In flow injection analysis experiments, the analyte should diffuse from the bulk sample solution into the receptor immobilized at the sensor surface as the sample passes over the surface. Therefore, the flow rate of the sample solution as well as the diffusion rate of the analyte can affect the sensitivity of the DNA biosensor. Although low flow rate might be advantageous for sensitivity, it requires a longer time for each analysis. In order to optimize the flow rate, we conducted the experiments at flow rates of 10 μl/min, 25 μl/min, and 50 μl/min. Immobilization of the biotin probe was not affected by the flow rate, but the frequency shift induced by cDNA hybridization was significantly lower at 50 μl/min than at 25 μl/min (Supplementary Figure 1). The binding rate of biotin to streptavidin was reported to be 3.0×10^6–4.5×10^7 M^{-1} s^{-1}, which is considered to be an extremely fast reaction [9]. Moreover, the biotin-binding site of avidin is exposed at the surface and is thus accessible to the biotin probe. By contrast, hybridization of cDNA to the surface-immobilized probe DNA is much more complex and occurs at a slow rate [10, 11]; therefore, the hybridization efficiency is expected to be more sensitive to the flow rate. However, decreasing the flow rate to 10 μl/min did not show improvements in sensitivity. Therefore, a flow rate of 25 μl/min was used in subsequent experiments.

The dose response of the DNA biosensor was analyzed with samples containing different concentrations of cDNA. The frequency change rate and shift were proportional to the concentration applied within the range of 1.6–1000 nM (Fig. 2a). For comparison, avidin was immobilized on the QCMs by the chemical method using EDC and NHS, according to the method of Mun and Choi [8]. The avidin-functionalized QCM was used to immobilize the biotin probe for the preparation of a DNA biosensor. Upon injection of cDNA samples, frequency shifts of the lipid-based DNA biosensor were much higher than those of the biosensor prepared by the chemical method, even though the amount of immobilized biotin probe was the same in both sensors (Fig. 2b). Injection of 1000 nM rDNA of a random sequence did not cause a significant frequency shift in either of the DNA biosensors, indicating that the difference in the responses of the two DNA biosensors to cDNA samples were

caused by specific hybridization of the cDNA to the surface-immobilized probe.

The higher sensitivity of the lipid-based DNA biosensor was unexpected since the previous experiment showed that the two biosensors had the same sensitivity when antibody was immobilized by the lipid method and the chemical method [8]. Antibody and oligonucleotide probes have different characteristics as sensing elements. Antigens bind to the antigen-binding sites of antibodies, which are exposed upward. However, cDNA must penetrate deeply into the probe layer because hybridization between cDNA and a probe occurs in a side-by-side manner. Therefore, the sensitivity of DNA biosensors is more likely to be affected by the surface density of the probe, especially in flow injection analysis experiments. A previous study showed that the hybridization rate decreased as the density of a surface-immobilized probe increased [12]. For two DNA biosensors with the same probe density on their surfaces, the sensitivity would be higher in a biosensor with a more homogeneous probe distribution. In the lipid-based functionalization method, the lipids would be maintained in a fluid state at 50°C and are thus expected to form a compact and homogeneous layer through the equilibration process.

We compared the distribution of fluorescein groups prepared on the QCM surfaces using the two methods. In the chemical method, fluorescein cadaverine (Invitrogen, Carlsbad, USA) was conjugated to the QCM surface through NHS groups as described above. In the lipid method, liposome was prepared as described above, except that BTPE was replaced by 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (Avanti Polar Lipids), and was used to prepare the HBM. The two QCMs were observed and photographed with a fluorescence microscope (Olympus BX51) equipped with a camera. The fluorescent points were identified and marked with a Kodak 1D image analysis program (Eastman Kodak, Rochester, USA). We conducted the experiment several times and found that a more homogeneous distribution of the fluorescein group could be obtained by using the lipid method compared to the chemical method. Representative images are shown in Supplementary Figure 2.

This study describes a lipid-based method for the immobilization of DNA probes on QCM surfaces. This method does not involve any chemical reactions and is very simple to carry out. Moreover, the DNA biosensor prepared using this method showed higher sensitivity than that prepared with the chemical method. We also found that the biosensor surface was effectively regenerated by treatment with 0.8 M glycine-HCl, pH 2.0, 0.1% DMSO for 4 min under flow conditions. In conclusion, our findings suggest that the lipid-based DNA immobilization method is a convenient and efficient method for the preparation of label-free DNA biosensors.

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Figure legends

Fig. 1 Optimization of the surface regeneration buffer. (a) Frequency profiles of whole experiments (immobilization of avidin and the biotin probe and cDNA hybridization) when the surfaces were regenerated with 0.2 M R buffer (upper panel) or 0.8 M R buffer (lower panel). The baseline and injection points are indicated by arrows. The gray bars represent the flow of R buffer. (b) Comparison of frequency shifts caused by the immobilization of avidin and the biotin probe and cDNA hybridizations when different concentrations of R buffer were used. Each data point represents the mean from duplicate determinations, and the error bar represents the standard deviation.

Fig. 2 Dose responses of DNA biosensors. (a) Frequency change profiles upon injection of different concentrations of cDNA samples to a DNA biosensor prepared by the lipid method. (b) Comparison of dose response curves of DNA biosensors prepared by the lipid method or chemical method. Frequency shifts of biotin-probe immobilization are shown in the inset. Each data point represents the mean from duplicate determinations and the error bar represents the standard deviation.