Phosphate Recognition Using Multilayer Charged Thin Films Containing Zirconium (Zr) Ions for Biochip Applications

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ABSTRACT

The surface modification of a solid substrate using organic/inorganic reagent is very important for the immobilization of biomolecules for biochip and biosensor applications. In this work, we fabricated a multilayer charged thin (MLCT) film containing zirconium (Zr) and evaluated its ability to recognize the phosphate group. The MLCT film was composed of alternate, positively (poly(diallyldimethylammonium chloride) (PDDA) and Zr) and negatively (phytic acid) charged layers. Oligonucleotide probes terminated with phosphate and fluorescent dye (Cy5) were spotted directly onto the zirconated surface forming a covalent linkage. The phosphate recognition ability of zirconated surface induced the immobilization of oligonucleotide probes. The fluorescence intensity due to these immobilized oligonucleotide probes was maximized in the conditions of 10 wt% PDDA, 0.1 M phytic acid and 0.05M Zr deposited for 20 min at 25 °C. The recognition ability of the Zr surface decreased with the increasing number of phytic acid/Zr layers and was negatively correlated with the dipping time.

Keywords: Charged polymer; Thin film; Phosphate recognition; Zirconium, Biochip

1 INTRODUCTION

Microarray techniques have emerged as a convenient and powerful tool for high- throughput, highly parallel experimentation in molecular biological research [1]. For example, DNA microarray technology can be used to probe gene expression in panel assays for research and clinical-based diagnostics [2]. In addition, protein microarray technology is rapidly emerging as a promising tool not only for studies of molecular interactions of proteins but also for their application in biomarker analysis and protein expression profile analysis [36]. In these new technologies, the development of diverse chemical linker systems is essential for the immobilization of the target biomolecules such as DNA, protein and carbohydrate. In general, the protocol for anchoring biomolecules onto the substrate surface includes thiol / acrylamide, activated carbohydrate/ amine, amine/aldehyde, epoxide/amine, aldehyde/oxyamine, and biotin/streptavidin [4].

It is well known that the phosphate group plays a role as not only the structural component of DNA and lipid but also the activation signal of protein in the cellular signaling pathways. Therefore, the selective recognition for the phosphate group is very important in the field of biochip and biosensor technologies. Recently, diverse chemical and biochemical applications based on the interaction between zirconium (Zr) and the phosphate group has been conducted [5-6]. Especially, Bruno et al. fabricated a Langmuir-Blodgett membrane using zirconated octadecylphosphonic acid (ODPA-Zr) and selectively immobilized DNA fragment with phosphate [7]. In their report, they suggested the possibility of selective recognition of the phosphate group of biomolecules on a solid phase. The methods for the construction of functional organic thin film include the Langmuir-Blodgett technique, sol-gel coating by dipping method, and self-assembled monolayer. In comparison to the Langmuir-Blodgett method, the other two methods have the advantages of easy and simple processing, with no necessary equipment, and high cost-effectiveness. On the other hand, the application of multilayer charged thin (MLCT) film for the construction of functional organic surface was also reported recently [8]. With this research background, we focused on the advantages held by the two methods, namely dip coating and self-assembly, and the potential of a functional organic surface with MLCT film.

In this work, we investigated the possibility of selective recognition for the phosphate group of biomolecules on the MLCT film with Zr. The MLCT film was constructed on a glass surface using the layer-by-layer self-assembly technique. The recognition ability of the MLCT film for the phosphate group was evaluated by using a Cy5 labeled oligonucleotide probe.

2 MATERIALS AND METHODS

2.1 Chemical and reagents

To fabricate a charged polymer layer, the surface of microscope glass (Matsunami, Japan) pretreated by placing

it in 2 % KOH aqueous solution under sonication for 30 min, in order to build a negative charge. Poly(diallyldimethylammonium chloride) (PDDA, Mw = 200,000 - 350,000), phytic acid and zirconyl chloride octahydrate ($ZrOCl_2 \cdot 8H_2O$) were purchased from Aldrich (Milwaukee, WI). To avoid interruption by the phosphate group in buffer solution, sodium chloride-sodium citrate buffer (SSC) was used as the buffer solution for the oligonucleotide probe. Sodium citrate, NaCl, polyethyleneglycol (PEG), and other reagents were obtained from Sigma Chemicals (St. Louis, MO). Milli-Q grade (>18.2 mΩ/cm) water was used for the preparation of the sample and buffer solutions. In addition, for the probe fabrication, a 20-mer oligonucleotide (3'-GGGGGGGCAAATGTTTAAGC-5') was synthesized and modified with a fluorescence dye located at the 5' terminal (-5'-Cy5) and the phosphate group located at the 3' terminal (-3- phosphate). In this sequence structure, a poly guanine spacer ((G)n, n=7) was designed to enhance the structural stability of the oligonucleotide probe. It was reported that in the presence of potassium or sodium ions, a strand of poly guanine spacer can form stable, four-stranded helices around the potassium or sodium ions [30]. This characteristic of the poly guanine spacer contributes toward increasing the surface coverage of the probe molecules within a spot area. Cy5-conjugated oligonucleotide probe (phosphate-3' GGGGGGGCAAATGTTTAAGC 5'-Cy5) was used as a probe to estimate the recognition ability of the Zr layer for the phosphate group. The structures of PDDA and phytic acid are shown in figure 1.

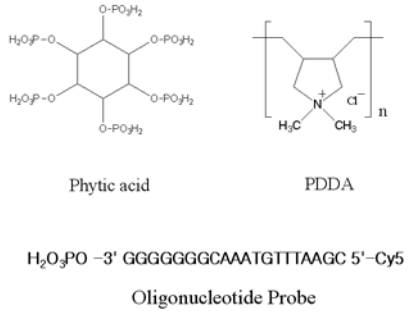

Fig. 1. Chemical structure of reagent for construction of charged thin film and sequence structure of Cy5-labeled oligonucleotide probe terminated with phosphate group.

2.2 Fabrication of multilayer charged thin film

PDDA was used as a polyelectrolyte for the preparation of the positively charged, self-assembled film. The glass substrate with a negative charge on its surface was dipped into the different concentration of PDDA solutions (1 wt% or 10 wt %) and incubated with different incubation time (20, 60, and 90 mins). After the formation of the positively charged, self-assembled, polymer layer, the glass substrate was immersed into a 0.1 M phytic acid solution and incubated for 20, 60, and 90 mins. Phytic acid has many phosphate residues around the benzene ring. Therefore, it can give a strong negative charge on the glass surface and binds to the Zr atoms as a crystal structure. After the formation of the phytic acid layer with the phosphate group, the glass substrate was dipped into a Zr solution with 0.05 M or 0.5 M concentration. A schematic diagram of the constructed MLCT films is shown in figure 2. For optimizing the thin film condition, the repetition number of phytic acid/Zr layers was increased from 1 to 3. In addition, the dipping time of the glass substrate into the reagent solution was modulated for 20, 60, and 90 mins.

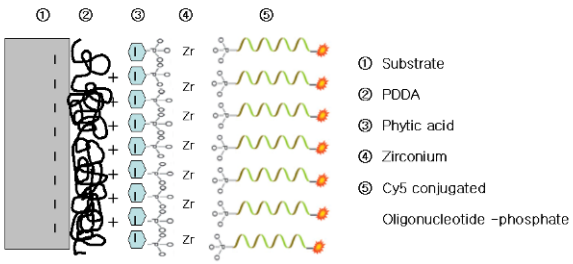

Fig. 2. Schematic diagram of multilayer charged thin film and oligonucleotide probe.

2.3 Microarray analysis

Microarray experiments for immobilization of Cy5-conjugated oligonucleotide probe (20-mer, phosphate-3' GGGGGGGCAAATGTTTAAGC 5'-Cy5) were carried out with a slightly modified version of the method described by MacBeath [9] and Pavlickova et al. [34]. A Cy5-conjugated oligonucleotide was diluted to a working concentration varying between 2μ M and 20 mM in 1X SSC buffer solution with 20% PEG. PEG was added to prevent evaporation of the micro-droplets. This Cy5-conjugated oligonucleotide in 1X SSC solution was then spotted on the surface of the Zr chip with 1 uL volume per spot and incubated at 37°C for 2 h. After incubation for the stable binding event between Zr and phosphate, the chip was then washed twice with current distilled water (DW) for 10 min at room temperature and dried under a stream of nitrogen gas [34]. The immobilized oligonucleotide probes were analyzed by a GenePix 4000B (Axon Instruments, CA, USA) scanner using a 635 nm laser as an excitation source. The signal intensity was quantified with GenePix Pro 6.0 software purchased from Axon Instruments (California, USA).

3 RESULTS AND DISCUSSION

3.1 Effect of layer repetition

To evaluate the effect of repeated layers, we examined films with 1, 2 and 3 repeated phytic acid/Zr layer pairs.

Multilayer charged thin film was constructed in the condition of 1 wt% PDDA, 0.1 M phytic acid, and 0.05 M zirconium with 20 min dipping time. The concentration of oligonucleotide probe ranged from 50 mM to 5 mM. As shown in figure 3a, the fluorescence intensity, corresponding to various concentrations of Cy5-labeled oligonucleotide probe solution, decreased with increasing number of thin film phytic acid/Zr layers. Figure 3b shows the plot of the averaged numerical value transferred from the spots of the scanning images and clearly presents the tendency for the fluorescence intensity to decrease with increasing number of repeated layers. These results indicate that the structural stability of the thin film is negatively correlated with the number of repeated phytic acid/Zr layers. The structural stability of the thin film is essential for the establishment of a crystal structure between the Zr atoms and phosphate group in the oligonucleotide probe. However, based on these results, we postulate that the increased number of repeated phytic acid/Zr layers excessively lengthens the dipping time, which may explain the unstable structure of positively (PDDA) and negatively (phytic acid) charged layers.

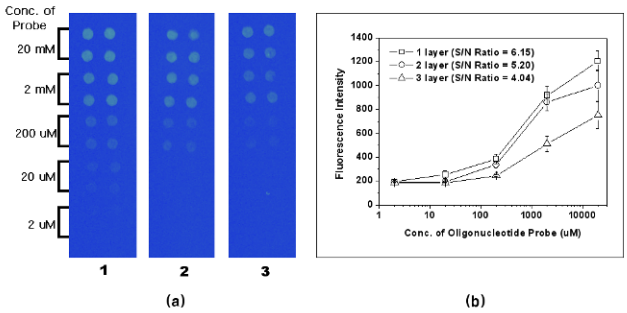

Fig. 3. Microarray images and plot of fluorescence intensity according to the number of layer repetitions. (a) Microarray image. The number under each image indicates the repetition number of phytic acid/zirconium layers. (b) Plot of fluorescence intensity

3.2 Effect of zirconium layer existence

To estimate the role of the Zr layer in recognizing the phosphate group MLCT films, with or without Zr as the final layer, were constructed on the same glass surface. Figure 4 shows the microarray images and plot of fluorescence intensity according to the probe concentration on the MLCT film with or without Zr. In the case with Zr condition, the fluorescence intensity started to increase remarkably from a probe concentration of 20 uM, due to the anchoring of the oligonucleotide probe. However, in the case without Zr condition, fluorescence intensity keep the baseline level until the probe concentration of 20 uM, and total increase of fluorescence intensity at the maximun probe concentration was very small. Figure 4 shows that the fluorescence intensity at the same probe concentration was higher in the substrate surface with Zr, compared to that without, as a recognition layer for the phosphate group. The S/N ratio at the highest probe concentration was 5.052 and 2.041 in the conditions with and without the Zr layer, respectively. As the Zr layer is terminated in oxide and hydroxide ions in the aqueous condition, we understand that the Zr surface can interact to the terminal phosphate group with strong binding and to the backbone phosphate group with weak interaction.

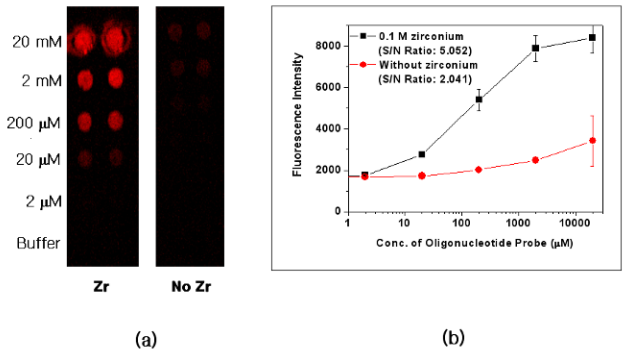

Fig. 4. Scan images and plot of fluorescence intensity corresponding to the recognition of the phosphate group on the multilayer charged thin film with and without zirconium layer. (a) Scan images of microarray. (b) Plot of fluorescence intensity.

3.3 Effect of positively charged polymer contents

To optimize the condition of the positively charged polymer layer, Zr substrates with two different PDDA concentrations (1 and 10 wt,%) were fabricated and tested. The 10 wt% PDDA substrates showed a remarkable increase in fluorescence intensity due to the recognition of oligonucleotide probes compared to the 1 wt% PDDA substrate (figure 5). From the numerical values of the fluorescence scanning images, the S/N ratio at the highest probe concentration was 11.98 and 2.21 for 10 and 1 wt % PDDA, respectively. This remarkable difference confirmed that the PDDA content can affect the construction of the next layer in MLCT films. These results presented in figure 5 indicated that the low PDDA content affected and disturbed the fabrication of the stable, phytic acid/Zr layer.

Multilayer charged thin film was constructed in the condition of 0.1 M phytic acid, and 0.05 M zirconium with 20 min dipping time. The prepared MLCT films have charged polymer contents with 1 wt% and 10 wt% PDDA, respectively. The concentration of oligonucleotide probe ranged from 50 mM to 5 M.

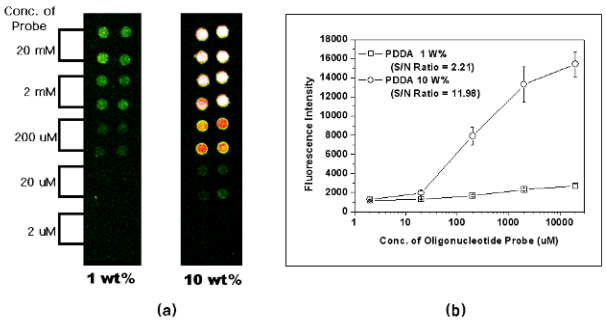

Fig. 5. Microarray images and plot of fluorescence intensity according to the positively charged polymer (PDDA) contents. (a) Microarray images scanned by fluorescence chip scanner. (b) Plot of fluorescence intensity corresponding to various concentrations of Cy5-labeled oligonucleotide probe onto the multilayer charged thin film with two different contents of positively charged polymer.

3.4 Variation in fluorescence intensity as a function of dipping time

Dipping time is very important in the fabrication of a charged thin film layer. Too short dipping time results in an unstable or insufficient layer, while too long results in the elution of the already constructed, charged thin film. In this work, three different dipping times were applied for the fabrication of Zr substrates. As shown in figure 6, the fluorescence intensity, which reflects the recognition ability of the Zr layer for the oligonucleotide probe, was very similar at 20- and 60-min dipping times, although slightly decreased for the latter, but was markedly reduced for the 90-min dipping time. These differences in fluorescence intensity appeared clearly from 20 μ M concentration. The averaged numerical value of fluorescence intensity from each spot was processed and is shown in figure 6b as a plot-chart. The optimum condition of dipping time for the fabrication of a stable charged thin film layer was determined to range from 20 to 60 mins. The S/N ratio at the maximum probe concentration was 11.98 and 11.91 at 20- and 60-min dipping time, respectively.

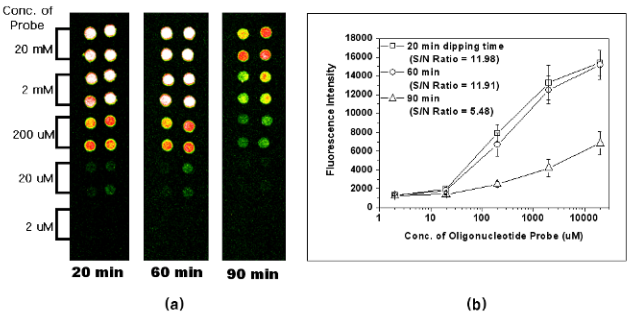

Fig. 6. Microarray images and a plot of fluorescence intensity corresponding to various dipping times. (a) Microarray images scanned by fluorescence chip scanner. The time under each image indicates the dipping time (unit: min) for the construction of each charged thin film layer. (b) Plot of fluorescence intensity corresponding to various concentrations of Cy5-labeled oligonucleotide probe onto the multilayer charged thin film prepared at three different dipping times (20, 60, and 90 min). Multilayer charged thin film was constructed in the condition of 10 wt% PDDA, 0.1 M phytic acid, and with (or without) 0.05 M zirconium. Rectangles, circles, and triangles indicate the 20, 60 and 90 min dipping times, respectively. The concentration of oligonucleotide probe ranged from 50 mM to 5 M.

4 CONCLUSIONS

In this study, we fabricated a MLCT film and evaluated its recognition ability for the phosphate group. In the results, the presence of a Zr layer on the substrate surface induced a MLCT film which could recognize the phosphate groups. In addition, the fabrication process was optimized with respect to the quantity of charged polymer, dipping time, and the number of alternated, charged layer (phytic acid/Zr) pairs. This approach is the first step for phosphate recognition using a MLCT film for biochip application. Further studies are presently in progress. The phosphate recognition system described in this paper using MLCT film has the advantages of being an easy, cost-effective and simple process which does not require any equipment. Therefore, we believe that these experimental results support the potential application of multilayer charged thin films in various biochip and biosensor fields.

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