Activity Evaluation of Antibody Immobilized onto the Self-Assembled Phospholipid Layer Fabricated by Plasma-Assisted Method

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1. Introduction

Considerable interest has focused on the immobilization of functional molecules, such as DNA, enzyme and protein, onto a water-insoluble material, which has been used as a biochip or biosensor.[1-3] Covalent binding method to immobilize proteins onto a material has several advantages and disadvantages.[4-6] It is usually thought to be the stable method to prevent the elution of protein from the material. On the other hand, it is relatively expensive and complicated in procedures involved. Activity yields may be low due to the exposure of the proteins to harsh environments or toxic reagent and the modification of active site. It is also considered that the interaction between material surface and immobilized proteins might make their activities lower. Therefore, the interface to bind proteins and material surface would play an important role to keep their activities.

We have recently reported a novel method to introduce a durable surface wettability and minimize its decay with time on several hydrophobic polymers, such as polyethylene-naphthalate, polyethylene, and nylon-12 (Fig.1).[7-11] This method involves sorption of methylvinylether-maleic anhydride copolymer (VEMA) into the surface layer and immobilization by a plasma-assisted cross-link reaction. Hydrolysis of VEMA follows to generate hydrophilic carboxyl groups on the surface. Durable surface hydrophilicity introduced in this way has been confirmed both by the

![Fig. 1 Schematic illustration of fabrication of durable hydrophilicity on polymer surface](image)
measurement of the water contact angle and by demonstration of the long-term stability of the surface lubricity on the urethane-made catheter.

In the previous paper, we have developed a novel fabrication of self-assembled phospholipid (phosphatidyl choline (PC)) layer on hydrophobic polymer surface by plasma technique.[12] The procedure is as follows: (Fig. 2) Alkyl amines, such as hexamethylene diamine (HMDA), were immobilized on LDPE-VE MAC film by condensation reaction to produce LDPE-HE film. LDPE-HE film was immersed into the suspension of PC to fabricate the self-assembled phospholipid layer. The self-assembled phospholipid layer obtained was thermally stable. We also fabricated the self-assembled phospholipid layer incorporating stearic acid (StA). StA was used as a scaffold to fix proteins. The model protein, albumin, could be immobilized onto this self-assembled layer. It was also shown that the self-assembled phospholipid layer possessed fluidity. The stability of self-assembled phospholipid layer fabricated by this method would depend on the characteristic of grafted alkyl groups acting as anchoring units. It is very important to fabricate the stable self-assembled phospholipid layer to use as interface to immobilize biomolecules. In this paper, we discussed on the effect of the grafted alkyl groups on the thermal stability of self-assembled phospholipid layer based on their grafting ratio and structural characteristics. We also immobilized cytochrome C antibody on the self-assembled phospholipid layer incorporating StA. The activity of cytochrome C antibody immobilized was estimated by sandwich enzyme linked immunosorbent assay (ELISA).

2. Experimental

2.1 Fabrication of self-assembled phospholipid layer

The LDPE-VE MAC film was prepared according to the method reported previously.[12] Several kinds of alkyl groups were introduced into LDPE-VE MAC film by the condensation reaction. The LDPE film grafting alkyl groups was soaked into phosphatidyl choline (PC) suspension (10ml) at 30 °C for 24h. The film was washed with water, and dried in vacuo to obtain LDPE-SA film. The self-assembled phospholipid layer containing stearic acid (StA) was similarly fabricated by using PC and StA suspension to prepare LDPE-StA-PC-SA film.

2.2 Thermal stability of LDPE-PC-SA film

The LDPE-PC-SA film was soaked into 5ml water. The amount of PC eluted from LDPE-PC-SA film was measured by UV spectrometer (210 nm) at various temperatures.

2.3 Immobilization of cytochrome C antibody onto LDPE-StA-PC-SA film

The LDPE-StA-PC-SA film was soaked into 10mM phosphate buffer (pH 4.0, 5 ml) containing 48 mg of EDC at room temperature for 2 h. This film was washed with water. The film was immersed into 0.1 mg/ml cytochrome C antibody-phosphate buffered saline (pH 7.4, 10 ml) at 4 °C for 48h. The film obtained was washed with a phosphate buffered saline (PBS, pH 7.4).

2.4 Activity estimation of cytochrome C antibody immobilized onto LDPE-StA-PC-SA film by sandwich ELISA

0.01 mg/ml Cytochrome C (10 µl) and PBS (190 µl) were added to the LDPE-StA-PC-SA film immobilizing cytochrome C antibody. This solution was kept at room temperature for 2 h and the film was washed with PBS. 0.01 mg/ml Anti-mouse IgG (whole molecule)-peroxidase (10 µl) and PBS (190 µl) were added to this film. This solution was kept at room temperature for 1 h and the film was washed with PBS. 3,3',5,5'-Tetramethylenediamine liquid substrate system for ELISA (200 µl) was added to this film and kept for 30 min. Stop solution (200 µl) was added to this solution. The absorbance at 450 nm was measured.

Fig. 2 Schematic illustration for the fabrication of self-assembled phospholipid layer onto a hydrophobic material.
3. Results and Discussion

3.1 Effect of the density of introduced alkyl groups on the thermal stability of LDPE-PC-SA film

We prepared LDPE-HE films possessing various densities of alkyl groups. Figure 3 shows the elution ratio of PC from the self-assembled phospholipid layer (LDPE-PC-SA) fabricated with LDPE-HE introducing various densities of alkyl groups against temperature. In our experimental condition, it was difficult to obtain LDPE-HE film possessing more than 0.33 nmol/cm² of alkyl groups. The elution ratio was suppressed with increasing the density of alkyl groups. The LDPE-PC-SA possessing 0.33 nmol/cm² of alkyl groups did not release PC up to 80 °C within a detectable extent.

3.2 Effect of the structure of alkyl groups on the thermal stability of LDPE-PC-SA film

The structure of alkyl groups might also affect on the thermal stability of the self-assembled phospholipid layer. We introduced trimethylene diamine (TMDA), HMDA or dodecamethylene diamine (DMDA) as alkyl groups into LDPE-VEMAC film. The densities of alkyl groups were 0.33 nmol/cm² in each case. Figure 4 shows the effect of the chain length on the thermal stability. LDPE-PC-SA using TMDA as alkyl groups released PC at 40 °C. However, LDPE-PC-SA using HMDA and DMDA did not release PC up to 80 °C. It was suggested that more than C6 chain length was effective to obtain thermal stable LDPE-PC-SA.

We have used the alkyl diamine as grafted alkyl groups. It is considered that the terminal group, amino group, may effect on the thermal stability of LDPE-PC-SA. Three kinds of amines, HMDA, n-hexylamine (HMMA) and 5-amino-1-pentanol (5AP), were used as alkyl groups to investigate the effect of terminal group on the thermal stability.

We prepared the LDPE-VEMAC introducing these alkyl groups with the almost same density (0.3±0.03 nmol/cm²). Figure 5 shows the effect of the terminal group on the thermal stability of LDPE-PC-SA. LDPE-PC-SA with HMMA easily released PC with increasing temperature. Although PC was slightly released at 40 °C from the LDPE-PC-SA with 5AP, the eluted ratio of PC tended to gradually level off to ca. 5%. As described above, LDPE-PC-SA with HMDA did not release PC within a detectable extent. These results suggest that hydrophilic group as a terminal is effective for the fabrication of thermally stable LDPE-PC-SA.
3.3 Activity estimation of cytochrome C antibody immobilized onto self-assembled phospholipid layer by sandwich ELISA

It is difficult to bind biomolecules to PC layer directly, because PC has no efficient functional group to react with other compounds. In the previous paper, we tried to incorporate stearic acid (StA) as a scaffold to immobilize biomolecules into the self-assembled PC layer.[12] StA could easily be incorporated into PC layer by the immersion of LDPE-HE film into the suspension of PC and StA to obtain LDPE-StA-PC-SA film.

We studied the activity of antibody immobilized onto LDPE-StA-PC-SA film by sandwich ELISA. Sandwich ELISA is one of the effective detection methods of target antigen. Figure 6 shows the representative illustration of sandwich ELISA. Cytochrome C antibody was immobilized onto LDPE-VEMAC and LDPE-StA-PC-SA film with EDC as a model antibody for sandwich ELISA. Table 1 shows the density of antibody and the absorbance of colored product produced by sandwich ELISA. Although the density of immobilized antibody was almost same in two samples, the absorbance in LDPE-StA-PC-SA was larger than that in LDPE-VEMAC. This result suggests that the antibody immobilized onto LDPE-StA-PC-SA film possessed higher activity than that onto LDPE-VEMAC.

![Image](https://example.com/image.png)

**Fig. 6** Representative illustration of sandwich ELISA.

<table>
<thead>
<tr>
<th>Density of antibody (pmol/cm²)</th>
<th>Absorbance (450nm)</th>
</tr>
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<tbody>
<tr>
<td>LDPE-VEMAC</td>
<td>6.0</td>
</tr>
<tr>
<td>LDPE-StA-PC-SA</td>
<td>7.0</td>
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4. Conclusion

The conclusions drawn from the present study can be summarized as follows.

The thermal stability of the self-assembled phospholipid layer depended on the density and structure of grafted alkyl groups. It is considered that HMDA or 5AP might be useful for the preparation of thermally stable LDPE-PC-SA film due to the effective anchoring units and the suitable solubility in water. Cytochrome C antibody was immobilized onto LDPE-StA-PC-SA film in which stearic acid was incorporated as a scaffold to immobilize antibody. It was suggested that the immobilization of antibody onto LDPE-StA-PC-SA film would be more useful than the direct immobilization onto LDPE-VEMAC film.

We are now actively elaborating the activity estimation of antibodies and enzymes immobilized onto LDPE-StA-PC-SA film.

**References**