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# Enhancement of immobilized lipase activity by design of polymer brushes on a hollow fiber membrane

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**A polymer brush possessing aminoethanol (AE) functional groups for lipase immobilization was grafted onto a hollow fiber membrane by radiation-induced graft polymerization. Almost the AE groups-grafted polymer brushes unfold through positive charge repulsion between the AE groups, enabling multi-layer immobilization of lipase. The hydroxyl groups in AE can also retain water molecules around hydrophilic part of the lipase. In this study, we controlled the length and density of the polymer brushes consisting of the glycidyl methacrylate (GMA) by changing the concentration of GMA monomer during radiation-induced graft polymerization. Immobilized lipase showed the highest activity on the grafted membrane when 5 wt% of glycidyl methacrylate as monomer for the radiation-induced graft polymerization was used. Consequently high efficiency esterification (approximately 1600 mmol/h/g-membrane) was achieved in five-layer lipase on AE polymer brush than that in monolayer lipase on the polymer brush possessing only hydroxyl groups. Moreover, the polymer brush possessing AE functional groups for lipase immobilization maintained high activity on the reuse for several times.**



[**Key words:** Immobilized enzyme; Radiation-induced graft polymerization; Biodiesel fuel; Esterification; *Rhizopus oryzae*; Polymer brush]

Lipase can be categorized as a biopolymer that catalyzes hydrolysis, esterification and esterification reactions (1,2). Enzyme-synthesis of biodiesel fuel has the advantages of mild enzyme reaction conditions and elimination of by-product production (3). Recently, a number of lipase immobilization supports, including microspheres (4), silica gel (5), resin (6) and reversed micelles (7,8), have been reported. Lipase modification systems using surfactants (9–11) and polymer (12–14) have also been described. The utilization of immobilized lipase on supports for industrial applications has some advantages, such as low cost, a relatively high stability and reusability (15). However, these methods suffer from problems such as lipase deactivation and dissolution in organic solution (16,17).

Lipase is activated at the water/oil interface (18,19). Thus, support materials that can provide the water/oil interface are better for efficient utilization of lipase activity.

Radiation-induced graft polymerization (RIGP) is a very useful method for incorporating various molecules and/or functional groups into base materials, and ion-exchange membranes (20,21), chelating resins (22), and catalysis (23) have been developed using this method. A sample solution, driven by a transmembrane pressure difference, can be transported by convection through the pores of a grafted porous hollow fiber membrane. Therefore, the diffusional mass transfer resistance of solutes to the functional groups can be neglected (24,25). Furthermore, a wide variety of functional groups can be introduced at high density on a polymer brush. The low diffusional mass transfer resistance and high functional group density in grafted porous hollow fiber membranes enable high reaction efficiencies and reaction rates.

We surmised that immobilization of lipase on polymer brushes occurred within a hollow fiber membrane by RIGP as shown in Fig. 1. This could be an effective approach to immobilize multi-layers of lipase and to maintain the water/oil interface for lipase activation by subcritical water in an organic solvent. The polymer brush is unfolded by positive charge repulsion between the functional groups (Fig. 2), enabling multi-layer immobilization of lipase (26,27).

We have reported esterification in organic media using lipase immobilized onto a hollow fiber membrane prepared by RIGP (10,26–28). Polymer brushes with positively charged amino groups, such as diethylamine (DEA) and aminoethanol (AE) grafted onto the membrane, can be used for multi-layer immobilization of lipase. For a DEA membrane, the maximum degree of the multi-layer binding of lipase was 20 (26). The lipase immobilized onto DEA membranes showed some multi-layering effect than that on membrane having only OH groups (OH membrane). However, the multi-layering effect was not so high, only 2.2-fold higher at five-layer lipase than the mono-layer OH membrane. According to

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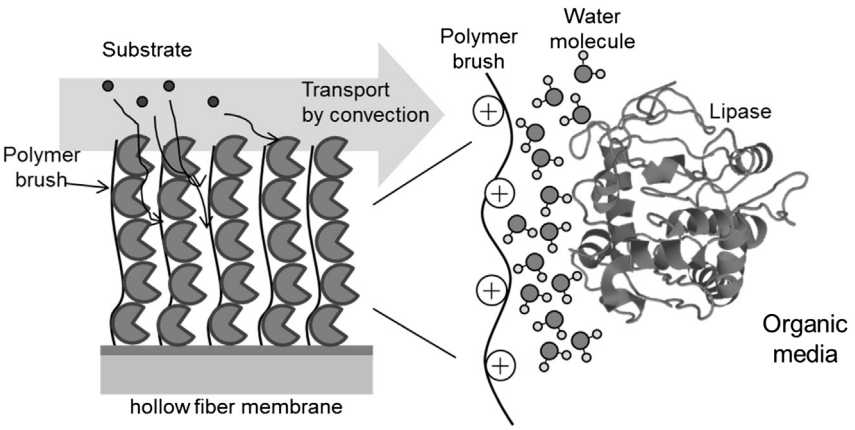


FIG. 1. Strategy for the design of polymers for lipase immobilization. Polymer brushes contain hydroxyl groups and amino groups that bind lipase and water molecules. Substrates in the organic solvent permeate by convective flow.

these results, it was suggested that the lipase structure might be denatured by strong interactions between the DEA and the lipase surface and OH groups play an important role in maintaining the activity of the lipase in organic media (28). Therefore, we employed AE, possessing both OH and positively charged amino groups, as the functional group for immobilization of lipase onto a hollow fiber membrane. In addition the density and length of the AE polymer brush might affect the lipase activity.

The objective of this study was to control and design the length and density of the polymer brush, using AE as a functional group to immobilize lipase, to achieve more efficient utilization of the lipase activity in organic media.

## MATERIALS AND METHODS

**Chemicals** A porous hollow fiber membrane made of polyethylene with inner and outer diameters of 2.0 and 3.1 mm, respectively, a pore size of 500 nm, and a porosity of 70% was used as a trunk polymer for grafting of the polymer brush. This membrane was supplied by Asahi Kasei Corporation, Tokyo, Japan. Glycidyl methacrylate (GMA) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and used without further purification. Other reagents were of analytical or higher grade. Lipase derived from *Rhizopus oryzae* (F-AP15) was gifted from Amano Enzyme Inc. (Aichi, Japan). In all experiments, the purified lipase by centrifugation were used and their concentrations were determined by a Lipase Kit S (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan).

**Preparation of the polymer brush on the hollow fiber membrane** A scheme for the preparation of the polymer brush on the hollow fiber membrane is shown in Fig. 3. The membrane was irradiated at 200 kGy to form radicals by an electron beam. The irradiated membrane was then immersed in 10% (v/v) GMA/ethanol solution at 313 K for 5, 10 and 15 min, respectively. The resulting GMA-introduced porous hollow fiber membrane was referred to as GMA membrane. The degree of GMA grafting (DG) was defined as:

$$\mathrm{DG}\ (\%) = 100\ (W_1 - W_0)/W_0 \tag{1}$$

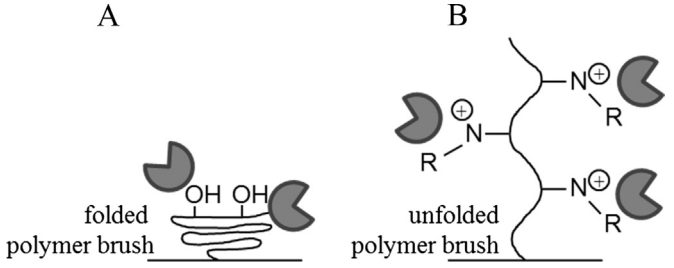


FIG. 2. Conceptual images for the folding and unfolding of the polymer brushes: (A) folded polymer brush possessing hydroxyl groups without charge repulsion; (B) unfolded polymer brush with amino groups possessing positive charges.

where $W_0$ and $W_1$ are the masses of the trunk and GMA membranes, respectively. The resulting membrane was referred to as a GMA membrane. The DG was set in the range of 30–111%.

The GMA membrane was reacted with 0.5 M AE solution at 40 °C for 12 h to introduce AE as a functional group onto the polymer brush. Molar conversion of the epoxy group in the polymer brush to the functional group was defined as:

$$\text{Molar conversion}\ (\%) = \frac{(\text{moles of functional group after functionalization})}{(\text{moles of epoxy group before functionalozation})} \tag{2}$$

where the moles were calculated from the membrane mass change after grafting. The resulting AE-introduced porous hollow fiber membrane was referred to as AE membrane.

**Immobilization of lipase onto the hollow fiber membrane** Lipase from *R. oryzae* was immobilized onto the hollow fiber membrane and the lipase activity determined by esterification of oleic acid to the oleic acid methyl ester in organic media. The prepared lipase solution (0.2 g/L) which using the purified lipase (>150,000 U/g, pH 7.0, 40 °C) by centrifugation in 10 mM phosphate buffer (pH 5.5) was forced to permeate from the inside to the outside of the membrane at a pressure of 0.04 MPa at 303 K. The concentration of lipase in the effluent flowing to the outside surface of the membrane was determined by UV absorption at 280 nm and a Lipase Kit S (Dainippon Pharmaceutical Co. Ltd.).

The amount of lipase adsorbed onto the membrane was calculated as follows:

$$\text{Amount of lipase adsorbed}\ (\mathrm{mg/g}) = \int_0^{V_e} (C_0 - C)dV/W \tag{3}$$

where $C_0$ and $C$ are the concentrations of the feed and effluent, respectively, and $V$, $V_e$, and $W$ are the effluent volume, the effluent volume when $C$ reached $C_0$, and the mass of the membrane, respectively.

The lipase-adsorbed membrane was immersed in 0.01% (v/v) glutaraldehyde solution dissolved in 10 mM phosphate buffer (pH 5.5) at 303 K for 24 h to covalently cross-link the lipase molecules. Subsequently, uncross-linked lipase was eluted by permeating with 0.5 M NaCl solution and small amount of 50% (v/v) ethanol solution. The amount of lipase immobilized onto the membrane and the cross-linking percentage were defined as follows:

$$\text{Amount of lipase immobilized}\ (\mathrm{mg/g}) = Aa - Ae \tag{4}$$

$$\text{Cross}-\text{linking percentage}\ (\%) = \frac{100\ (\text{amount of lipase immobilized})}{(\text{amount of lipase adsorbed})} \tag{5}$$

where $Aa$ and $Ae$ are the amount of lipase adsorbed onto the membrane and the amount of lipase eluted from the membrane, respectively. The structural properties of the lipase from *R. oryzae* were determined by X-ray and other analysis methods (PDB ID: 1TIC) (29). According to this information, the size and molecular weight of the lipase are about 5 nm and 40 kDa, respectively. We confirmed the molecular weight of the lipase by analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), obtaining good agreement (data not shown).

**Esterification reaction by immobilized lipase** The substrate solution between oleic acid (10 mM) and methanol (10 mM) was permeated from the inside of the membrane to the outside at 313 K to determine the lipase activity. The concentration of the oleic acid methyl ester product in the effluent was determined by gas chromatography (HP-5890, Hewlett Packard, Palo, Alto, CA, USA) equipped with a flame ionization detector (FID) and a 15 m capillary column DB-1 (J&W Scientific Columns, Agilent Technologies, Palo Alto, CA, USA). The flow rates of helium carrier