Permeation Control Using Enzymatic Reaction of GOD Immobilized onto Oxygen Plasma-pretreated Expanded PTFE Films Grafted with Acrylic Acid

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The film of expanded polytetrafluoroethylene (ePTFE) grafted with acrylic acid (ePTFE-g-PAA) was prepared by the combined use of oxygen plasma treatment and photografting technique changing plasma-treated time, monomer concentration and UV irradiation time. The permeation of insulin through the ePTFE-g-PAA film can be controlled by the pH change in the medium. Since GOD immobilized onto ePTFE-g-PAA film through covalent bonding can enzymatically react with glucose, we have tried to control the permeation of insulin through the GOD immobilized ePTFE-g-PAA film due to pH lowering in medium.

Keywords: oxygen plasma treatment, photo-grafted ePTFE film, acrylic acid, GOD immobilization, permeation control

1. Introduction

We have reported that the PTFE films photo-grafted with electrolytic monomers such as methacrylic acid and 2-(dimethylamino) ethyl methacrylate have sufficient mechanical strength in the swollen state and exhibit the sensitivities in response to the change in temperature and/or pH. [1-5] The introduction of polymer chains onto polymer substrates by means of graft-polymerization has attracted many chemists' attention to an improvement in the surfaces of polymer substrates and preparation of functional polymer membranes.

We have prepared the expanded PTFE films grafted with PAA changing plasma treated time, monomer concentration and UV irradiation time. This study reports the permeation of insulin through ePTFE-g-PAA films using the enzymatic reaction of glucose oxidase (GOD) with glucose, leading to the construction of the drug delivery system useful for diabetic treatment. Furthermore,

immobilization of GOD onto ePTFE-g-PAA film has an advantage of reusing GOD. The high mechanical strength coming from PTFE polymer substrate and pH responsivity as polyelectrolyte on the basis of PAA can be utilized for a functional polymer film.

2. Experimental

2.1. Materials and Reagents

Expanded PTFE films (thickness = 40 μm , average pore size = 0.5 μm , degree of vacancy = 78 %: ADVANTEC Co. Ltd.) as a polymer substrate were washed in methanol and acetone for 10 min in a ultrasonic washing machine, respectively, and then dried under reduced pressure.

Acrylic acid (AA: Wako Pure Chemical Ind., Ltd.) as an electrolytic monomer was purified by distillation under reduced pressure. Benzophenone (BP: Wako Pure Chemical Ind., Ltd.) as a sensitizer, insulin (Wako Pure Chemical Ind., Ltd. for biochemical uses) as a permeant and

potassium dihydrogen-phosphate and disodium hydrogen-phosphate (Wako Pure Chemical Ind., Ltd.) for preparation of buffer solutions were used. GOD (Wako Pure Chemical Ind., Ltd. for biochemical uses) as an enzyme and β -D-glucose as a substrate and 4-aminoantipyrine, phenol and peroxidase (POD) (Wako Pure Chemical Ind., Ltd.) as coloring reagents for the determination of β -D-glucose were used. For GOD immobilization, 1-aminohexyl-3-(2-morpholinoethyl) carbodiimido methoxy-*p*-toluene sulfonate (CMC: Wako Pure Chemical Ind., Ltd.) as a condensation reagent was used. These reagents described above were used without purification, unless otherwise noted.

2.2. Preparation of ePTFE-g-PAA films

Oxygen plasma treatment

The plasma treatment was carried out using a Shimadzu LCDV-20type plasma polymerization apparatus in the manner described in our previous article.[6] The washed ePTFE films were fixed on a sample holder between two plate-electrodes placed 40 mm apart from each other in the glass bell jar with double-sided Scotch tape. Oxygen gas (purity, 99.9 %) was introduced into the glass bell-jar at a flow rate of 20 cm³/min under a vacuum of 6.67 Pa. One side of the ePTFE film was exposed to oxygen plasmas generated at an output of 200 W and a frequency of 15 KHz for 2 - 6 min, while the sample holder was rotated at 60 rpm. After treatment with oxygen plasma, the plasma-treated ePTFE films were stored in an atmosphere of oxygen for 30 min to form oxygen-containing functional groups at the surface of ePTFE film. The oxygen containing groups can act as sites for initiation of graft-polymerization.

Photo-grafting

The plasma-treated ePTFE films were dipped for 1 min in a 0.0275 mol/dm³ acetone solution containing BP. The photografting was carried out using a Riko rotary photochemical reactor RH400-10W (Riko Kagaku Ind.) equipped with a 400W high-pressure mercury lamp. The sensitizer-coated ePTFE films before being dried were immersed in an aqueous monomer solution of AA of 1.5 or 2.0 mol/dm³ in the Pyrex glass tubes. Then, AA was photografted onto the ePTFE surfaces at 60 °C by applying UV rays under a nitrogen atmosphere for 30 - 150 min. The ePTFE-g-PAA films were washed under flowing water and repeatedly immersed in distilled water to remove the unreacted residual monomer and homopolymer, and then

dried under reduced pressure for 24 hours at room temperature. The amount of AA grafted onto ePTFE surfaces was calculated from the weight increase of the samples.

2.3. Thickness of the ePTFE-g-PAA films

The ePTFE-g-PAA films were immersed in phosphate buffer solutions of pH 7.35 and 8.00 for 24 hours, and then the grafted films were taken out from the solutions and blotted with filter paper to take away the water attached on their surfaces. The thickness of each film with a minimum of 5 values at any points was measured using a micrometer (Mitsutoyo Co. Ltd.) and the averaged value was calculated.

2.4. Permeability of insulin

Through ePTFE-g-PAA films

The ePTFE-g-PAA films of AA grafted amounts of 2.35 - 6.61 mmol/g swollen in a buffer solution of pH 7.35 or 8.00 were clamped between the two halves of the cell (100cm³). A phosphate buffer solution (ionic strength = 0.01 mol/dm³) of pH 7.35 or 8.00 was put in one side of the cell and insulin containing buffer solution of the same pH in the other side of the cell, and then both solutions were stirred. The amount of insulin permeated through the ePTFE-g-PAA films was determined by measuring the absorbance at a wavelength of 276 nm, the wavelength of maximum absorption of insulin, and the sample solutions measured were recovered immediately to the cell.

GOD immobilization onto ePTFE-g-PAA film

The modification by condensation reaction between the amino groups of enzyme and the carboxyl groups of the grafted PAA was carried out for GOD immobilization onto the ePTFE-g-PAA film. GOD 25mg and CMC 117mg were put into 50cm³ of the phosphate buffer solution of pH 7.00, and made to react at 4 °C for 18 hours.[7,8]

Determination of GOD enzymatic activity

Into 200cm³ of a pH 7.00 phosphate buffer solution, 4-aminoantipyrine 32mg, phenol 4.2mg, and peroxidase (POD) 2.5mg were added (Solution 1). The pH 7.00 phosphate buffer solution with glucose of 10 mmol/dm³ was prepared (Solution 2).

The GOD immobilized ePTFE-g-PAA film was immersed in the phosphate buffer solution (pH7.00) for 24 hours and then made to react with glucose in Solution 2 at 37 °C, which was added to 2 cm³ of Solution 1 after a predetermined time. After 5 min the absorbance in 505nm was measured.

Permeation control of insulin using enzymatic reaction

Glucose was added into the cell of the feed side at 150 min after the permeation experiments started.

3. Results and Discussion

3.1. Preparation of ePTFE-g-PAA films

Oxygen plasma treatment and photo-grafting

The graftings of AA onto ePTFE films plasma-treated for 2 min were carried out changing the UV irradiation time at the monomer concentration 1.5 or 2.0 mol/dm³ (Figure 1). The amount of grafted AA markedly increased in the monomer concentration of 2.0 mol/dm³. Therefore, to follow the relation of the plasma-treated time to the grafted amount, the change in the grafted amount of AA onto ePTFE exposed to oxygen plasma for different times are shown in Figure 2, keeping UV irradiation time (150 min) and monomer concentration (2.0 mol/dm³) constant.

The ePTFE-g-PAA films of higher amounts of grafted AA were obtained in the range of the lengthened plasma-treated time investigated here without discoloration.

Figure 1 Changes in the amount of AA grafted onto ePTFE films with UV irradiation time
Plasma-treated time: 2 min
Monomer concentration: 1.5 (○) and 2.0 (●) mol/dm³

3.2. Thickness of the ePTFE-g-PAA films

Figure 3 shows that the thickness of the ePTFE-g-PAA films equilibrated with the buffer solutions of pH 7.35 and 8.00 increased with an increase in the grafted amount as expected.

It is considered that a slight difference of thickness between pH 8.00 and 7.35 solutions comes from the expansion of grafted AA polymer

Figure 2 Changes in the amount of AA grafted onto ePTFE films with plasma-treated time
UV irradiation time: 150 min
Monomer concentration: 2.0 mol/dm³

chains due to the dissociation of carboxyl groups attached to AA monomers.

Figure 3 Changes in the thickness of ePTFE-g-PAA films in the phosphate buffer solutions of pH 7.35 (○) and 8.00 (●) with AA grafted amounts

3.3. Permeability of insulin

Through ePTFE-g-PAA films

The dependence of the permeation of insulin through ePTFE-g-PAA films on the amount of grafted PAA and pH of the buffer solutions was investigated. This is a prerequisite experiment to be made before the permeation experiment of insulin through the films in response to pH changes of aqueous solutions caused by the enzymatic reaction of GOD with glucose.

Figure 4 shows the changes in the amount of permeated insulin through the ePTFE-g-PAA films with grafted amount of 2.35, 4.61, 5.96 and 6.61 mmol/g-dry film with time at pH 7.35 and 8.00.

Figure 4 Changes in the insulin permeabilities through ePTFE-g-PAA films of grafted amounts of 2.35 (○), 4.61 (●), 5.96 (□) and 6.61 (■) mmol/g with time in the phosphate buffer solutions of pH 7.35 and 8.00

The amounts of permeated insulin through ePTFE-g-PAA films in the buffer solution of pH 8.00 were depressed except for the film with the grafted amount of 2.35 mmol/g, while those at pH 7.35 increased clearly with time. Grafted PAA chains at pH 8.00 can be extended due to repulsion among ionized carboxyl groups of PAA chains and so insulin be prevented from passing through channels present in ePTFE films. The grafted amount of 2.35 mmol/g-dry film is so low that

insulin can pass through the channels even if the medium at pH 8.00 promotes more dissociation of AA monomers and so expansion of grafted chains. However, grafted PAA chains at pH 7.35 can be forced to contract due to the decreased ionization of carboxyl groups of PAA chains, and so insulin can pass through the channels in the films.

The largest difference in the permeated amount of insulin with time between pH 7.35 and 8.00 solutions was observed in the grafted amount of 5.96 mmol/g-dry film. Therefore, the ePTFE-g-PAA film of grafted amount of 5.96 mmol/g-dry film can be the most advantageous to control the permeation of insulin in response to the pH change in the medium in this system.

Since the grafted chains on ePTFE-g-PAA films in a pH 8.00 buffer solution can develop by electrostatic repulsion of -COO^- anions, and so make the channels in the film narrow, the permeation of insulin is considered to be depressed. And those in a pH 7.35 buffer solution will contract in comparison to pH 8.00 due to decreased dissociation. Therefore, the contracted grafted chains favor making channels in the film open and the permeation of insulin is promoted.

Determination of GOD enzymatic activity

From the above results, the ePTFE-g-PAA film with the grafted amounts of 5.96 mmol/g as a film for the insulin permeation control by the enzymatic reaction was selected, and then GOD was immobilized onto the film.

To estimate the enzymatic activities of immobilized GOD with the allowed time the absorbance measurement in 505 nm was made using 4-aminoantipyrine as a coloring agent. The results of absorbance measurements made immediately and at 7 weeks showed no difference (Figure 5). Therefore, the enzymatic activities of GOD immobilized ePTFE-g-PAA films can be reserved if stored under mild conditions not to cause deterioration or denaturation of immobilized GOD.

Through GOD immobilized ePTFE-g-PAA film

The amount of permeated insulin through the film with time is shown in Figure 6. The permeabilities of insulin in the buffer solutions of pH 7.35 and 8.00 through the film were similar to the behavior observed in Figure 4. Therefore, this film can be applied to the experiment on the insulin permeation in response to pH changes brought about by the enzymatic reaction of GOD with glucose.

Figure 5 Enzymatic activities of GOD immobilized onto ePTFE-g-PAA film measured immediately (○) and 7 weeks later (●)
Grafted amount of AA = 5.96 mmol/g
Original pore size = 0.5 μ m

Figure 6 Changes in the insulin permeabilities through GOD immobilized onto ePTFE-g-PAA films of grafted amounts of 5.96 mmol/g with time in the phosphate buffer solutions of pH 7.35 (○), and 8.00 (●)

Permeation control of insulin using pH change by enzymatic reaction

Figure 7 shows the changes in insulin permeabilities through the films by addition of glucose with time to the aqueous solution of pH 8.00. The insulin permeation through the film in the absence of glucose was little observed within 150 minutes, and so the solutions in both cells were kept at pH 8.00.

It is important to this experiment for GOD immobilized surface of the film to be set to face

toward the permeation side. Immediately after addition of glucose into permeation side cell at 150 minutes, the amount of permeated insulin increased rapidly. It is considered that the pH fall of the film circumstance occurred quickly by the enzymatic reaction between the glucose added and the GOD immobilized onto the film.

Figure 7 Changes in the insulin permeabilities through GOD immobilized onto ePTFE-g-PAA films of grafted amounts of 5.96 mmol/g by addition of glucose with time to the aqueous solution of pH 8.00
Grafted chains toward permeation side

Figure 8 is different from Figure 7 in making GOD immobilized surface of the film set to face toward the insulin feed side.

Figure 8 Changes in the insulin permeabilities through GOD immobilized onto ePTFE-g-PAA films of grafted amounts of 5.96 mmol/g by addition of glucose with time to the aqueous solution of pH 8.00
Grafted chains toward feed side

The amount of permeated insulin increased slowly during 120 minutes after addition of glucose into the feed side cell at 150 minutes.

This shows that it takes a considerable time for glucose to permeate through the film and react with the immobilized GOD in the insulin feed side compared with the experiment shown in Figure 7.

We have made it clear that the permeation of insulin using GOD immobilized onto ePTFE-g-PAA films can be controlled in response to pH changes caused through the enzymatic reaction with glucose.

4. Conclusions

The control permeation of insulin was examined through the GOD immobilized onto ePTFE-g-PAA films. From the results, we can draw following conclusions.

- 1) The amount of grafted AA of ePTFE films can be controlled with the plasma-treated time, monomer concentration and UV irradiation time,
- 2) The permeability of insulin through the GOD immobilized onto the ePTFE-g-PAA film increases in the pH 7.35 and decreases in pH 8.00,
- 3) This system will be able to contribute to the control release of insulin in response to the enzymatic reaction of GOD with glucose.

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