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p-Phenylenediamine Epoxy Resin Film for Redox Enzyme Detection

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Summary: Phenylenediamine derivatives (PDs) are environmentally hazardous, though very useful for chemical analysis. To minimize release into the environment, a PD-containing epoxy resin film was developed that retained redox enzyme activity. *p*-Phenylenediamine and 2,2-bis(4-glycidyloxyphenyl)-propane were cured to produce a violet film. This film was reactive against peroxidase, a redox enzyme that oxidizes phenylenediamine to afford an imine. Enzymatic oxidation caused the film to change color from violet to deep green. The film exhibited fluorescence at 394 nm under excitation at 350 nm, and the fluorescent intensity decreased with greater oxidation. Thus, the film could be used to detect redox enzyme activity.

Keywords: biomaterial; film; fungi; phenylenediamine; redox enzyme

Introduction

Phenylenediamine derivatives (PDs) are strong electron donors and are therefore used as dyes in biochemical and environmental analyses.[1] For instance, the oxidation of *N,N,N′,N′*-tetramethylphenylenediamine (TMPD) by peroxidase (POD), a redox enzyme that oxidizes various organic compounds in living cells, yields a TMPD radical cation that imparts a blue color (absorption at 564 and 606.5 nm in water).[2] In addition, diaphorase (Dp), a redox enzyme that reduces quinone to produce hydroquinone in living cells, reduces the radical cation to yield colorless TMPD. Most PDs are considered environmentally hazardous, but are nonetheless useful for many chemical analyses. Thus, if PDs could be processed into a film, they could be recycled easily, thus minimizing pollution.

In this study, we attempted to prepare a film that contained PDs and maintained reactivity against redox enzymes such that changes in film color or fluorescence intensity could be exploited in a variety of analyses. Such PD-containing films may be useful not only for determining the redox enzyme activity but also for detecting certain types of bacteria on various film-coated surfaces.

Epoxy resins and epoxylated monomers such as epoxyacrylate are useful for coating surfaces such as floors, walls, and glasses.[3] In addition, amine-epoxide thermosets have been extensively studied and have been used to manufacture many functional materials.[4] Thus, we prepared an epoxy resin film with *p*-phenylenediamine as one monomer. *p*-Phenylenediamine and 2,2-bis(4-glycidyloxyphenyl)-propane were cured on laboratory dishes to obtain a violet-colored thin film (Scheme 1). Here, we examined the enzymatic activity of the violet film and studied the structural changes in the film on the basis of its IR spectra. The oxidation of PDs in the film by POD produced a deep-green color. The

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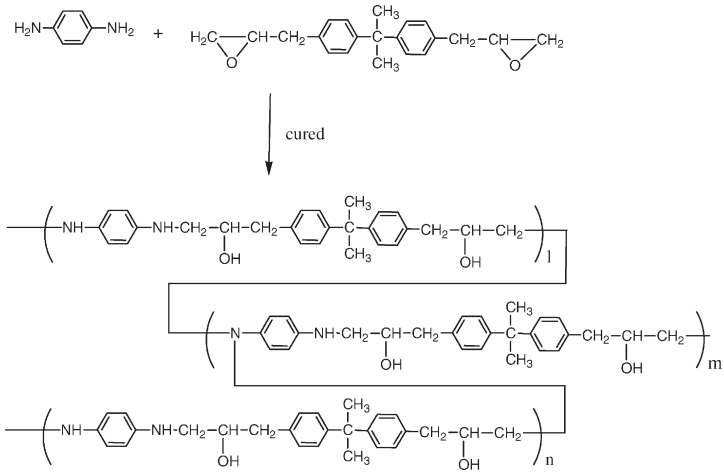


Scheme 1.
Epoxy resin.

color depth depended on the concentration of the enzyme donor, i.e., NADH coenzyme for Dp and H_2O_2 for POD. In this study, we attempted to show that the epoxy resin film containing phenylenediamine would retain reactivity against redox enzymes and that this film could be used for biochemical analyses.

Experimental Part

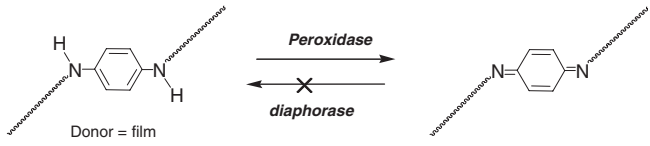
Polymerization

In a glove box, a mixture of *p*-phenylenediamine (260 mg, 2.41 mmol) and 2,2-bis(4-glycidyloxyphenyl)-propane (820 mg, 2.41 mmol) in methanol (50 ml) was poured into a 3.5cmΦ laboratory dish under nitrogen atmosphere. The mixture was cured under nitrogen atmosphere at 26 °C for one week in a glove box (Scheme 2). The obtained film was washed with water, methanol, and chloroform. The polymerization reaction afforded a violet film.

Enzymatic Reaction

POD from *horseradish* (EC.1.11.1.7) was purchased from WAKO (450 unit/mg of activity). POD solution (2 ml) in phosphate buffered saline (pH 6.4, 1 mg/ml) was poured onto the film-coated laboratory dish. The, 0.1 ml of 0.3% H_2O_2 was added to the solution. After incubation at 30 °C for 5 h, the film was successively rinsed with water, methanol, and chloroform.

Dp from *Clostridium kluyveri* (EC.1.6.99.X) was purchased from WAKO (139 unit/mg of activity). 5 ml of 89 mM Nicotine amide adenosine dinucleotide reduced form (NADH) in phosphate buffered saline (pH 7.4) was poured on to the film after oxidation with POD on a laboratory dish. To the solution was added 0.1 ml of Dp solution in phosphate buffered



Scheme 2.
Enzymatic oxidation of the film.

saline (pH 7.4, 65 mg/ml). After incubation at 30 °C for 5 h, the film was successively rinsed with water, methanol, and chloroform.

Trichoderma Viride

Trichoderma viride was purchased from Riken Bioresource Center. The film was coated with an approximately 1-mm-thick layer of potato dextrose agar (PDA) culture medium on a laboratory dish. *T. viride* was cultured in the medium and incubated for 3 days at 28 °C. Then, the culture medium was removed and the film was successively rinsed with water, methanol, and chloroform.

Results and Discussion

Polymerization

A 1:1 molar mixture of *p*-phenylenediamine and 2,2-bis(4-glycidyloxyphenyl)-propane was dissolved in methanol. The polymerization reaction afforded a violet film which was insoluble in any solvent we examined; DMF, DMSO, Chloroform, THF, diethylether, methanol, water, and so on. The polymer may have a network structure such as an epoxy glue. The structure of the film was studied on the basis of its IR spectra (Figure 1A). The IR spectrum indicated the following peaks: 3400–3200 cm^{-1}, ν(NH,OH); 1600 cm^{-1}, ν(CC); 1470 cm^{-1}, ν(CC in benzene); and 1280 cm^{-1}, ν(CN in amines). These peaks suggest an epoxy resin including a phenylenediamine unit as shown in Scheme 1.

Enzymatic Reaction

A film cured in methanol solution was used for the enzymatic reactions. A violet-colored thin film was oxidized with POD from horseradish to afford a deep-green film. The absorption spectra exhibited specific double absorption peaks of the oxidized form of the PD at 590 nm and 639 nm (Figure 2). The IR spectra indicated absorption peaks corresponding to C=N at 1650 cm–^{1} and –HC=CH– out-of-plane at 750 cm–1 after the enzymatic reaction (Figure 1B). These results suggested that the amine repeating unit of the epoxy resin film formed an imine due to the oxidation reaction. Moreover, the epoxy resin film exhibited fluorescence at 394 nm under excitation at 350 nm (Figure 3). The fluorescence intensity decreased after the oxidation reaction. Thus, the film could be useful for detecting oxidants such as redox enzymes and bacteria.

We attempted reduction of a film oxidized with POD in order to see if it could be reused. The reduction was carried out with Dp because it reduces quinodiimine derivatives such as the oxidized form of *N,N,N′,N′*-tetramethyl-phenylenediamine to give PDs. However, the oxidized

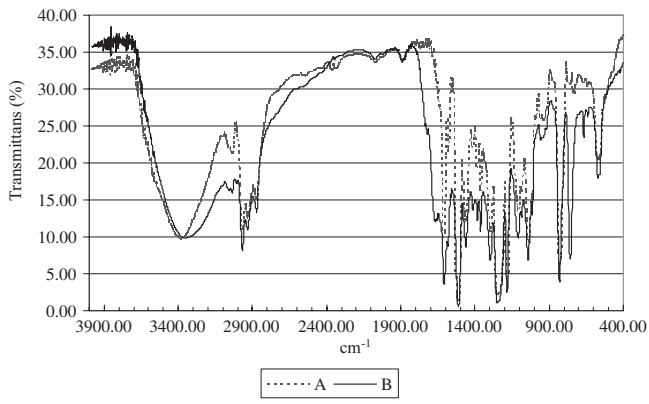


Figure 1.
IR spectra of film. A: before oxidation with POD (dotted line), B: after oxidation with POD (solid line).

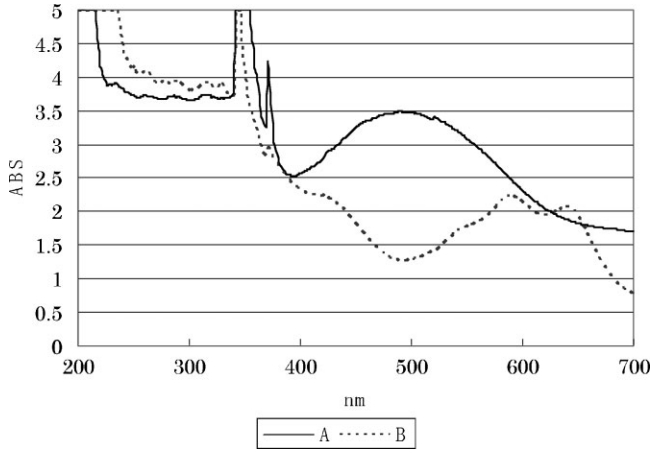


Figure 2.
UV-Vis spectra of film. A: before oxidation with POD (solid line), B: after oxidation with POD (dotted line).

film was not reduced under our reaction conditions (Scheme 2). Two significant reasons exist for why the reaction did not proceed: First, steric hindrance may reduce accessibility for the enzyme. POD is a heme-enzyme, whereas Dp is a flavin mononucleotide (FMN)-enzyme. The accessibility may be different between POD and Dp. A second reason is the fact that the phenylenediamine repeating unit in the film may have higher redox potential than NADH. The electrochemical measurements supported the later reason. As shown in Figure 4, cyclic voltammetry revealed that both reduction peaks and oxidation peaks of the film shifted to more negative potential than the peaks of NADH, which suggested that the phenylenediamine repeating unit in the film has lower redox potential than NADH. The results suggested that stronger reducing agent was required for reducing the oxidized film.

Biological Response with *Trichoderma Viride*

To examine the oxidation of the film by microbes, we tested the incubation of *T. viride* on the film. *T. viride* is a type of mold that contains a redox enzyme. In the absorption spectra, no absorption was observed at 590 nm and 639 nm. Moreover, in the IR spectra shown in Figure 5, no

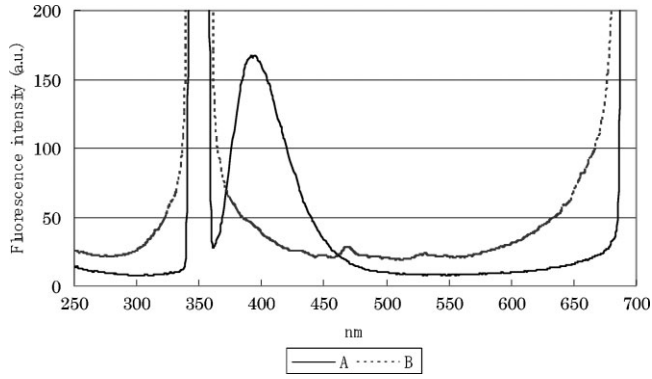


Figure 3.
Fluorescence spectra of film. A: before oxidation with POD (solid line), B: after oxidation with POD (dotted line).

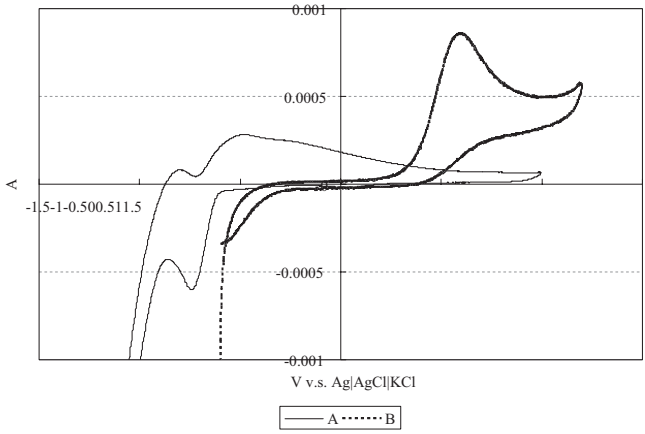


Figure 4.
Cyclic voltanmetry of film and NADH. A: film (solid line), B: NADH (dotted line).

absorption was observed at 1650 cm–1 ν(C=N). These results suggest that the oxidation of the film by *T. viride* did not proceed or that the degree of oxidation was quite small.

After incubation with and without *T. viride*, the film color became deeper, suggesting that some structural change had occurred. In fact, the absorption in the visible region decreased without the appearance of double peaks at 590 nm and 639 nm (Figure 6). The incubation was carried out in a nitrogen atmosphere under sunlight, which accelerated the oxidation of phenylenediamine. The chemical oxidation of phenylenediamine has been extensively studied because it is used in hair coloring and for color photography.[5] Although the nature of transformation that occurred on treatment with *T. viride* is not clear, the substitution reaction of the amine by the oxygen of the water molecule or the oligomerization of the phenylenediamine moiety may have proceeded. These reactions should yield a different structure because of the POD oxidation reaction.

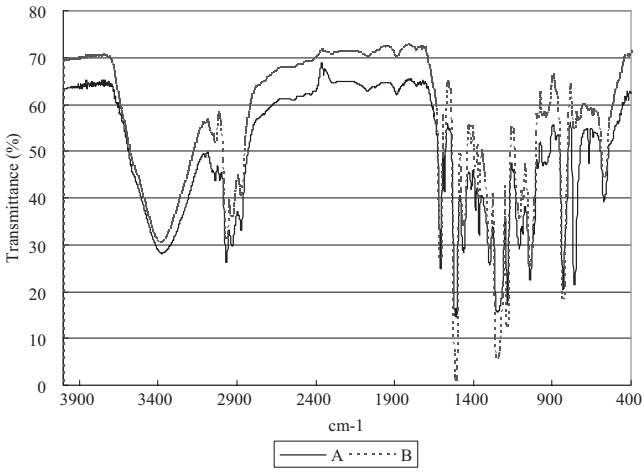


Figure 5.
IR spectra of film. A: before incubation of *Trichoderma viride* (solid line), B: after incubation of *Trichoderma viride* (dotted line).

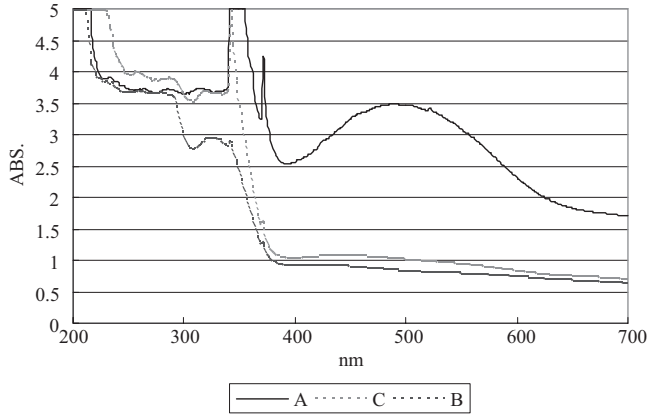


Figure 6.
UV-Vis spectra of film. A: before incubation of *Trichoderma viride* (solid line), B: after incubation of *Trichoderma viride* (dotted line), C: control: incubation with PDA medium.

Conclusion

In this study, we have shown the biological oxidation of an epoxy resin film consisting of phenylenediamine and 2,2-bis(4-glycidyloxyphenyl)-propane. PDs are oxidized by POD to produce a colored compound, and thus, can be used in many analytical reactions. We found that a phenylenediamine film reacted with POD and became deep green in color. Moreover, the fluorescence of the film (e.g., excitation at 350 nm, emission at 394 nm) decreased with increasing oxidation.

In the future, we will attempt to achieve reversible colorization of the film, given that PDs are environmentally hazardous. Therefore, we will focus on the reduction of the oxidized film and thus make the film reusable. Toward the goal of reusability, we are studying various *N*-substituted PDs. Many PDs are unstable under air and light. The epoxy resin film containing phenylenediamine was also unstable under air and light, although the film was stable under nitrogen atmosphere and dim light.

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