CHEMISTRY LETTERS, pp. 2227-2230, 1991.

Immobilization of Myoglobin in a Multilayer Film of Two-Dimensional Polymer Network[1)]

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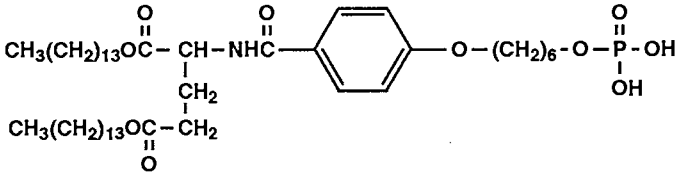
Myoglobin, dioxygen storage protein, was effectively entrapped without denaturation in a multilayered, two-dimensional polymer network that was prepared by using a cast film of a phosphate bilayer membrane as molecular template. The spatial orientation of Mb observed in the presence of the bilayer matrix was lost by its solvent extraction.

We recently achieved layered arrangement of anisotropically oriented myoglobin molecules in hydrophilic interbilayer regions of cast films of synthetic bilayer membranes.[2,3)] The membrane/protein film should be attractive from the practical standpoint. This film, however, is readily dissolved in aqueous and organic solvents. True immobilization of protein molecules in two-dimensional molecular systems would be important fundamentally as well as practically. We decided for this purpose to make use of a two-dimensional polymer network that could be synthesized in a cast film of a synthetic bilayer membrane as a reaction template.[4,5)] Protein molecules should become entrapped effectively in the 2D-polymer network, by simultaneous incorporation of protein and monomer/polymer in the interbilayer region of a regular, multilayer template.

A water-soluble hemoprotein, metmyoglobin (met-Mb), was used again in this study as a representative protein for immobilization. The heme site of Mb acts as a convenient monitor for the state of Mb by UV-visible and electron spin resonance (ESR) spectroscopies.[6)] Amphiphile **1** was dispersed homogeneously in 10 mM Tris-HCl buffer by sonication (pH=7.5, [**1**]=33.3



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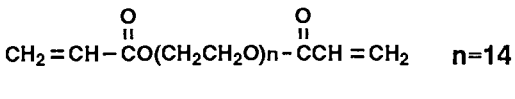

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mM).[7] An equimolar amount of bis-acrylate monomer **2** (Shin-Nakamura Chemicals) and varying amounts of met-Mb (from horse heart, Sigma, **1**:Mb (mol/mol) = 1:0.025-0.0016) were added to the dispersion. The clear reddish dispersions were cast on solid supports (Fluoropore membrane filter from Sumitomo Electric, Type FP-010, Pore size 0.10 μm, or glass plate) and dried for a few days at 25 °C. The resultant cast film was subjected to polymerization by γ-ray irradiation (^{60}Co, 1 Mrad at 0 °C under Ar atmosphere). A flexible film was formed as shown in Fig. 1.

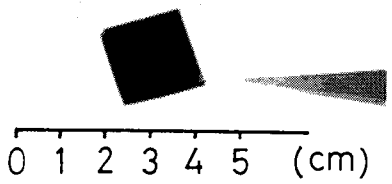


Fig. 1. Photograph of a polymerized composite film.

An absorption spectrum of the composite film was identical with that of native met-Mb in Tris-HCl buffer (Soret band = 409 nm, Q band = 500, 540, 630 nm).[8] The g values of an ESR spectrum of the film were also essentially the same as those of a powder sample of native met-Mb ($g_{\parallel}$ = 2.0, $g_{\perp}$ = 5.9).[9] It is clear that the monomer incorporation and its γ-ray polymerization did not induce denaturation. ESR spectra of the composite film were dependent on angle ϕ between the applied magnetic field and the normal of the film plane, as shown in Fig. 2a. The $g_{\parallel}$ component (g = 2.0) is weak but the $g_{\perp}$ (g = 5.9) peak is intense, when the film plane is parallel to the magnetic field (ϕ = 90°). As the film plane is rotated, $g_{\parallel}$ is intensified to a maximum at ϕ = 30° , and $g_{\perp}$ decreases gradually from ϕ = 90° to 0°. This macroscopic ESR anisotropy clearly indicates that the protein molecule is oriented against the film plane in high regularity. The tilt angle of the heme plane of met-Mb against the film plane can quantitatively be estimated to be 30° (standard deviation, 25°) by computer simulation (Fig. 2b).[10-12] This orientation is identical to that in the film before polymerization.[13] Thus, polymerization proceeded without disturbing the Mb orientation.

The polymerized composite film was then soaked in water-saturated $CHCl_3$ for 15 min at room temperature. The film weight decreased from 10.2 mg to 5.3 mg during the extraction process. This weight loss corresponds approximately to the sum of weights of the amphiphile (4.4 mg) and the Tris buffer (0.6 mg). In differential scanning calorimetry (Seiko Instruments SSC 5200, scan speed 1 °C/min), the composite film gave endothermic peaks due to the gel-to-liquid crystal phase transition at 52 °C (ΔH = 4.8 kJ/mol) and 66 °C (ΔH = 18.3 kJ/mol). These peaks disappeared almost completely after the $CHCl_3$ extraction. The difference in peak intensities before and after soaking indicates that about 95% of the amphiphile was removed. This is consistent with a decrease in the phosphorus content of

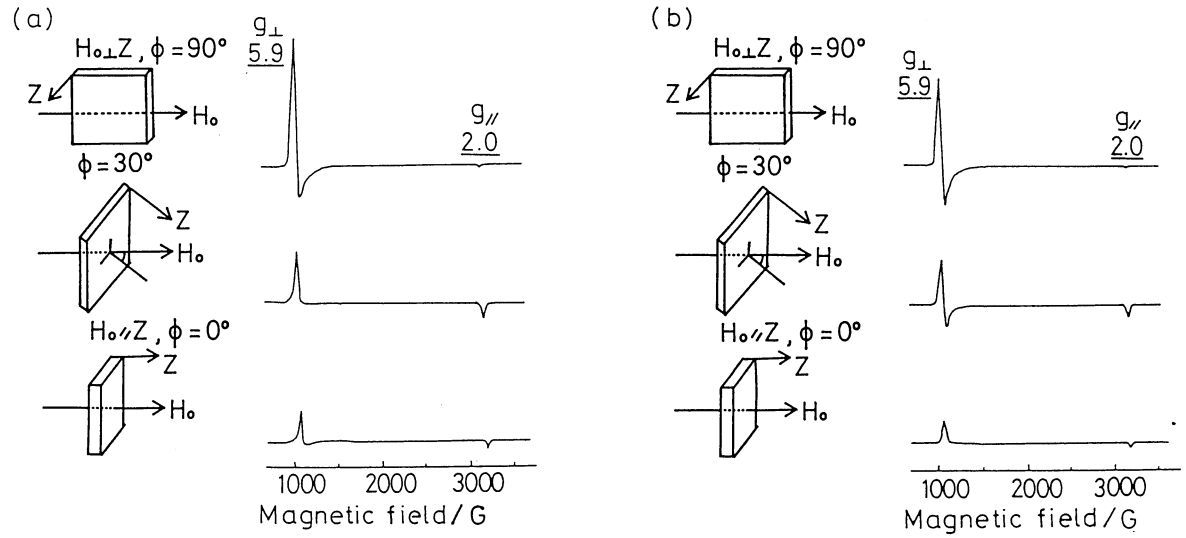


Fig. 2. Observed (a) and simulated (b) ESR spectra of the polymerized composite film (JEOL JES 2X X-band spectrometer, at 4 K, microwave power 5 mW, microwave frequency 9.044 GHz, modulation amp. 7.9 G).

the composite film that comes only from the amphiphile component from 1.39% (calcd content, 1.58) to 0.0-0.2% by $CHCl_3$ extraction.[14]

In contrast, Mb molecules were effectively immobilized in the film. The UV-visible absorption intensity at 409 nm (Soret band of met-Mb) showed that about 10% of Mb leaked within 5 min of soaking, but further leakage was not observed. Most Mb molecules (80-90%) were maintained in the film even after 30 min immersion of the film. As expected, the composite cast film before polymerization was completely dissolved in $CHCl_3$ within 2-3 min. It is clear that the 2D-polymer network that is produced in the interbilayer region of the template can effectively entrap Mb molecules after removal of the template membrane.

Figure 3 shows ESR spectra of the polymer film after the $CHCl_3$ immersion. The g values of met-Mb in this film are identical with those of native met-Mb. Denaturation apparently did not occur by the $CHCl_3$ treatment. However, macroscopic anisotropy that was observed in the original composite film (Fig. 2a), was completely lost, since the ESR spectral pattern does not display any change associated with the orientation of the film in the magnetic field. We have shown for Mb-bilayer composite films (without polymer network) that the spatial orientation of Mb is determined by optimized electrostatic

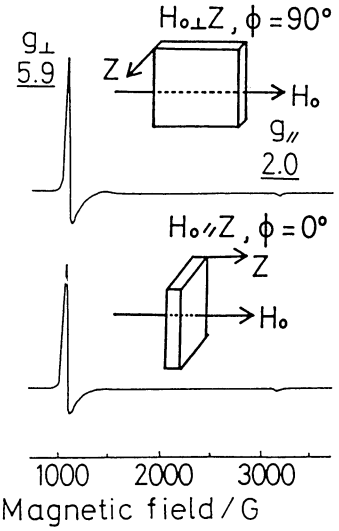
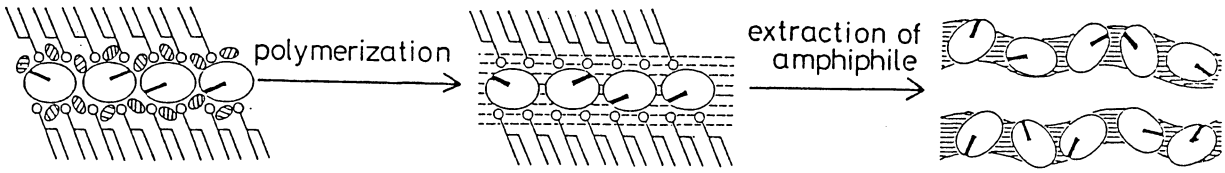


Fig. 3. ESR spectra of the film after $CHCl_3$ immersion.



Scheme 1.

attraction of the positive charges of the protein surface and the negative phosphate unit of the bilayer surface.[2,13] This is the case in the present system. The spatial containment of Mb molecules in the 2D-polymer network is not sufficient to maintain their anisotropy.

In conclusion, a novel multilayer film comprising Mb molecules within a 2D-polymer network was prepared by use of a multibilayer film as a reaction template (Scheme 1). This film is unique in that the individual layer is exposed because of its molecular level thickness. The unique functions associated with this film are under investigation in these laboratories

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7) Amphiphile **1** was synthesized according to the procedure described in ref. 12. Colorless fine crystals, mp 61-63 °C, Anal Found: C, 65.80; H, 9.82; N, 1.61%. Calcd for $C_{48}H_{82}O_{12}NP$: C, 65.76; H, 9.84; N, 1.61%.
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9) It is known that the heme unit of native met-Mb is in the ferric high spin state. T. Yonetani and H. Schleyer, J. Biol. Chem., **242**, 3926 (1967).
10) ESR simulation was conducted by using a program developed by Y. Ishikawa for determination of the tilt angle and its standard deviation.[11,12]
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12) I. Hamachi, S. Noda, and T. Kunitake, J. Am. Chem. Soc., in press.
13) This orientation is slightly different from that in the Mb/bilayer composite film without monomer (tilt angle, 15°, standard deviation, 25°).
14) The apparent chemical formula of the polymerized film was calculated as a sum of the amphiphile (1 equiv.), monomer (1 equiv.), Mb (0.006 equiv.), buffer (0.2 equiv.) and water (1.53 equiv.). Elemental analysis of phosphorus was performed at the Center of Elemental Analysis, Kyoto University. We are grateful to Prof. H. Ogoshi for this analysis.

(Received September 27, 1991)