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Molecular association between flavin derivatives and dendritic polymers at the water|1,2-dichloroethane interface

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ABSTRACT

The ion transfer and adsorption mechanism of flavin derivatives, riboflavin (RF) and flavin mononucleotide (FMN), at the polarized water|1,2-dichloroethane (DCE) interface were studied in the presence of the fourth generation (G4) amino-terminated polyamidoamine (PAMAM) dendrimer or hyperbranched bis-MPA polyester-64-hydroxyl (HBP). The flavin derivatives associated with the positively charged G4 PAMAM dendrimer both in the aqueous solution and at the water|DCE interface. Spectroelectrochemical analysis through potential-modulated fluorescence spectroscopy demonstrated that the dendrimer-bound flavin derivatives were transferred across the water|DCE interface in the positive potential region, while the interfacial adsorption of flavin derivatives in the negative potential region was effectively inhibited by the competitive adsorption of the neutral G4 HBP molecules.

Keywords

Flavin derivatives; Interface between two immiscible electrolyte solutions (ITIES); PAMAM dendrimer; Hyperbranched polymer; Potential-modulated fluorescence (PMF)

1. Introduction

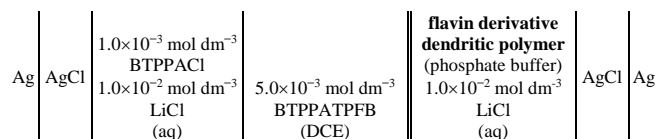
The interface between two immiscible electrolyte solutions (ITIES) is a useful model of biomembrane systems where charge transfer and adsorption processes could be controlled as a function of the Galvani potential difference between two liquid phases [1]. The electrochemical analysis of bioactive substances in biomimetic liquid-liquid systems is significantly important, since it is difficult to elucidate the complicated pharmacokinetics of ionizable species consisting of molecular association, acid-base equilibrium and distribution on or across a biomembrane [2-4]. On the other hand, parallel artificial membrane permeability assay (PAMPA) has been widely used to predict the passive permeation of drug molecules through a biomembrane [5, 6]. The characteristic correlations between the transfer potential of ionizable drugs and the permeability coefficient measured in PAMPA shows a validity of voltammetric data as a drug permeability scale [7]. Although PAMPA is a useful experimental technique providing a high throughput screening ability, the analysis of the transmembrane mechanism of drug molecules remains difficult and, in particular, other surface-sensitive technique is essential to elucidate the interfacial process in detail.

Dendritic polymers such as dendrimer, dendrigraft polymer and hyperbranched polymer have attracted much attention as drug carriers for drug delivery systems (DDS) as well as protein mimetics [8-11]. Among the various dendritic polymers, polyamidoamine (PAMAM) dendrimers with ionic groups and aliphatic polyester hyperbranched polymers based on 2,2-bismethylolpropionic acid (bis-MPA) monomers have structural and physicochemical properties as potential drug carriers, in which small guest molecules are accommodated in the interior cavity with electrostatic and/or nonelectrostatic interactions. Recently, the intermolecular associations of ionic species with the PAMAM dendrimer have been studied at the liquid|liquid interface where

the charge transfer mechanism is effectively influenced by the electrostatic formation of dendrimer-ion associates [12-14]. The molecular association of bioactive substances with the dendritic polymer has, however, rarely been investigated at the liquid|liquid interface [15]. Riboflavin (RF) known as vitamin B2 is composed of isoalloxazine ring and ribitol chain, and flavin mononucleotide (FMN) with a phosphate-ribitol chain is a prosthetic group of oxidoreductases which plays important roles in the electron transfer *in vivo* [16]. Flavoprotein contains those flavin derivatives via either covalent or noncovalent bonds, where the anionic FMN molecule binds to proteins in a different manner than the neutral RF molecule [17-19]. In this study, the interfacial behavior of RF and FMN was spectroelectrochemically studied at the water|1,2-dichloroethane (DCE) interface in the presence of the positively charged PAMAM dendrimer or neutral hyperbranched bis-MPA polyester-64-hydroxyl as a model of DDS and noncovalently protein-bound/lipid-bound flavin derivatives on a biomembrane.

2. Experimental

The cell composition represented in **Cell I** was used for all electrochemical experiments. The flavin derivative and dendritic polymer were dissolved in the aqueous phase, where the pH condition was adjusted at pH 7.1–7.3 by adding $5.0 \times 10^{-3} \text{ mol dm}^{-3}$ $\text{LiH}_2\text{PO}_4/\text{LiOH}$. The fourth generation (G4) dendritic polymers, amino-terminated PAMAM dendrimer with ethylenediamine core and hyperbranched bis-MPA polyester-64-hydroxyl (HBP), are of spherical shape with an



Cell I: Composition of the electrochemical cell.

adequate size to incorporate flavin derivatives [20, 21] and were purchased from Aldrich. RF (Kanto Chemical), FMN (Wako Chemicals) and lumichrome (LC) (TCI) were used as received. All other reagents were of the highest grade available. The supporting electrolytes were LiCl for the aqueous phase and bis(triphenylphosphoranylidene)ammonium tetrakis(pentafluorophenyl)borate (BTPPATPFB) for the organic phase, respectively. The aqueous solutions were prepared with purified water from a Milli-Q system (Millipore Direct-Q3UV). The water|DCE interface with a geometrical area of 0.50 cm² was polarized by a four-electrode potentiostat (Hokuto Denko HA1010mM1A) with platinum wire counter and Ag/AgCl reference electrodes in both aqueous and organic phases. The Galvani potential difference ($\Delta_o^w\phi \equiv \phi^w - \phi^o$) was estimated by taking the formal transfer potential ($\Delta_o^w\phi^{\circ'}$) of tetramethylammonium cation as 0.160 V [22]. In the potential-modulated fluorescence (PMF) measurements, the polarized water|DCE interface was illuminated by a cw laser diode of 50 mW at 404 nm (Coherent CUBE 405-50C) under total internal reflection (TIR). The fluorescence signal from the interfacial region was measured at 522 nm for RF and 523 nm for FMN by a monochromator equipped with a photomultiplier tube (Shimadzu SPG-120S) and analyzed as a function of ac potential modulation by a digital lock-in amplifier (NF LI5640). The ac amplitude was 20 mV at 1 Hz and the linear potential sweep rate was 5 mV s⁻¹. Further details of the PMF setup and analytical procedure are described elsewhere [23, 24]. The ¹H NMR spectra of flavin derivatives in D₂O were recorded on a 400 MHz spectrometer (JEOL JNM-ECS400). All experiments were carried out in a thermostated room at 298±2 K.

3. Results and Discussion

3.1. Molecular association between flavin derivatives and dendritic polymers in aqueous solution

Typical absorption and fluorescence spectra of RF and FMN with the equimolar G4 dendritic polymers in the aqueous solution are shown in **Fig. 1**. In the RF system, the absorption peak at 373 nm was slightly blue-shifted to 370 nm by adding the PAMAM dendrimer, while the

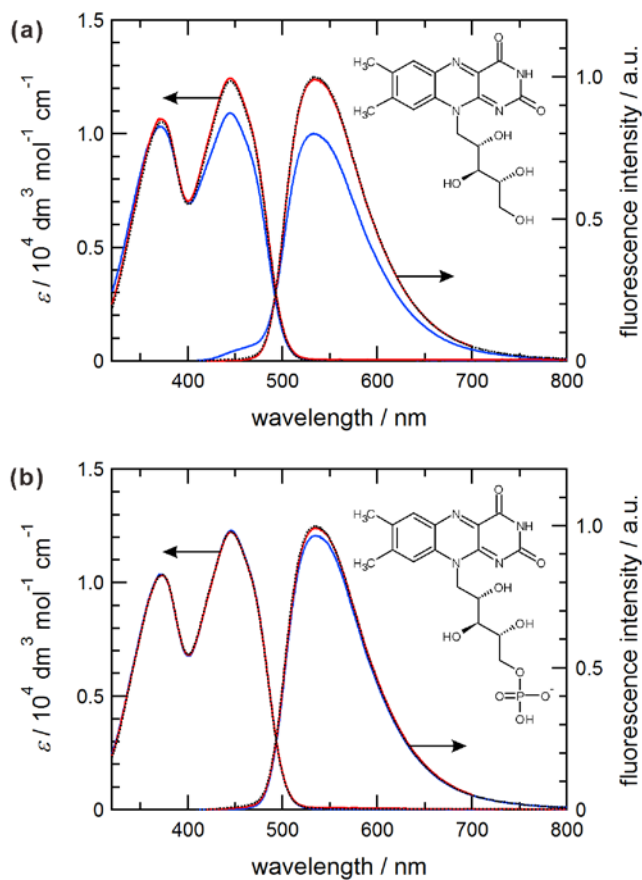


Fig. 1 Absorption and fluorescence spectra of (a) RF and (b) FMN in the aqueous solution at pH 7.1–7.3. The black dotted lines depict the spectra measured in the absence of the dendritic polymers. The blue and red lines refer to the spectra in the presence of the PAMAM dendrimer and HBP, respectively. The concentration of the flavin derivatives and dendritic polymers was $1.0 \times 10^{-5} \text{ mol dm}^{-3}$. The fluorescence spectra were measured at an excitation wavelength of 404 nm.

absorbance at 445 nm and fluorescence intensity at 534 nm were decreased. No obvious effect on the spectra was observed in the presence of HBP. The spectral results indicated a certain interaction between RF and the dendrimer in the aqueous solution. On the other hand, the spectral features of FMN were not significantly changed in the presence of the dendritic polymers. ^1H NMR spectra measured for the PAMAM dendrimer in D_2O demonstrated the specific interaction between the flavin derivatives and the dendrimer, in which four broad peaks attributed to methylene protons in the interior and periphery moieties were shifted upfield by ca. 0.02 ppm and the new peak was observed around 2.72 ppm in the presence of either RF or FMN (**Fig. 2a**) [25, 26]. This upfield shift is not caused by the electrostatic interaction between the flavin derivatives and the dendrimer which induces a decrease of the electron density on methylene protons. The PAMAM dendrimer with the hydrophobic interior has a number of amide and tertiary amine groups as hydrogen

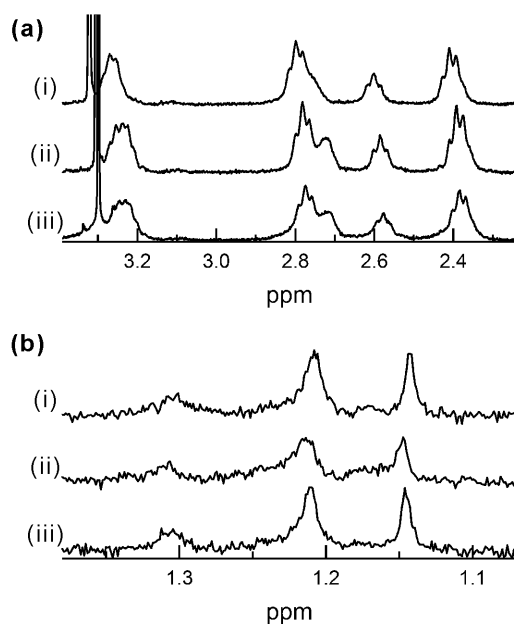


Fig. 2 ^1H NMR spectra of (a) PAMAM dendrimer and (b) HBP in D_2O in (i) the absence and presence of the equimolar (ii) RF and (iii) FMN. The concentrations of (a) PAMAM dendrimer and (b) HBP were $5.0 \times 10^{-5} \text{ mol dm}^{-3}$ and $1.0 \times 10^{-5} \text{ mol dm}^{-3}$, respectively.

bonding sites. The NMR spectra, thus, suggest that uncharged moieties of flavin derivatives associate with the nonpolar interior or periphery of the dendrimer through the hydrophobic interaction and/or hydrogen bonding as reported in the DNA and cyclodextrin systems [27-29]. Although the electrostatic interaction with the positively charged dendrimer should also be considered for the FMN anions in the aqueous solution, the downfield shift attributed to the electrostatic interaction was not clearly observed under the present experimental condition. As shown in **Fig. 2b**, the ^1H NMR spectra of HBP in D_2O were only slightly shifted by adding RF or FMN, indicating that HBP hardly interacts with the flavin derivatives in aqueous solution.

3.2. Interfacial mechanism of flavin derivatives affected by dendritic polymers

The fundamental behavior of the flavin derivatives at the water|DCE interface was investigated under neutral pH conditions through the electrochemical measurements. At pH 7.1, the noncharged RF ($\text{p}K_{\text{a,RF}} = 9.88$) is the dominant species, whereas FMN ($\text{p}K_{\text{a1,FMN}} = 6.18$, $\text{p}K_{\text{a2,FMN}} = 10.08$) exists mainly as a monoanionic form (FMN^-) [30]. In the RF system, the cyclic voltammograms (CVs) and capacitance curves were almost identical to the base electrolyte system (**Figs. 3a** and **3c**), because of no charge transfer and adsorption at the interface. The capacitance in the FMN system was slightly increased at $-0.22 \text{ V} < \Delta\phi^{\text{w}}$, while the ion transfer response was not clearly observed within the potential window (**Figs. 3b** and **3d**). The electrochemical data indicated that FMN^- is not transferred across the interface under neutral pH conditions but its weak interfacial adsorption takes place over the potential window. In the presence of the dendrimer, electrochemical responses of the flavin derivatives were buried in the ion transfer and adsorption responses of positively charged dendrimers accompanied by the facilitated transfer of the organic electrolyte anion, tetrakis(pentafluorophenyl)borate (TPFB^-) at the positive edge of the potential window [31, 32]. The capacitance measured in the presence of HBP was effectively decreased at

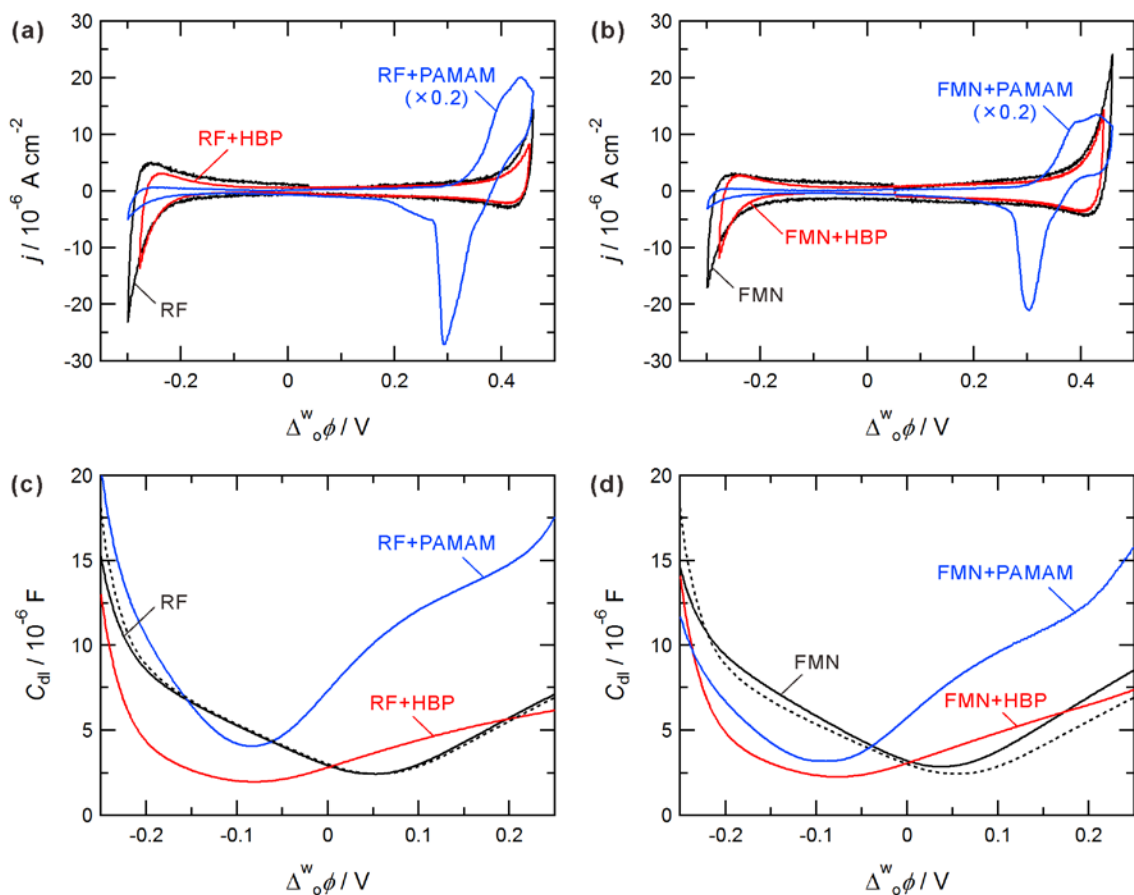


Fig. 3 CVs and capacitance curves of (a, c) RF and (b, d) FMN at pH 7.1–7.3. The potential sweep rates were (a, b) 50 mV s^{-1} and (c, d) 5 mV s^{-1} , respectively. (c, d) The dotted lines refer to the base electrolyte system at pH 7.1. The amplitude of ac potential modulation was 10 mV at 7 Hz . The concentration of the flavin derivatives and dendritic polymers was $1.0 \times 10^{-5} \text{ mol dm}^{-3}$.

$\Delta_o^w \phi < 0 \text{ V}$ due to the preferential adsorption of neutral HBP molecules (Figs. 3c and 3d). The intrinsic electrochemical responses of the dendrimer and HBP at the water|DCE interface are summarized in Supplementary material: S1.

The PMF analysis was carried out to elucidate the interfacial mechanism of the fluorescent flavin derivatives in the presence of the dendritic polymers. The high selectivity of PMF

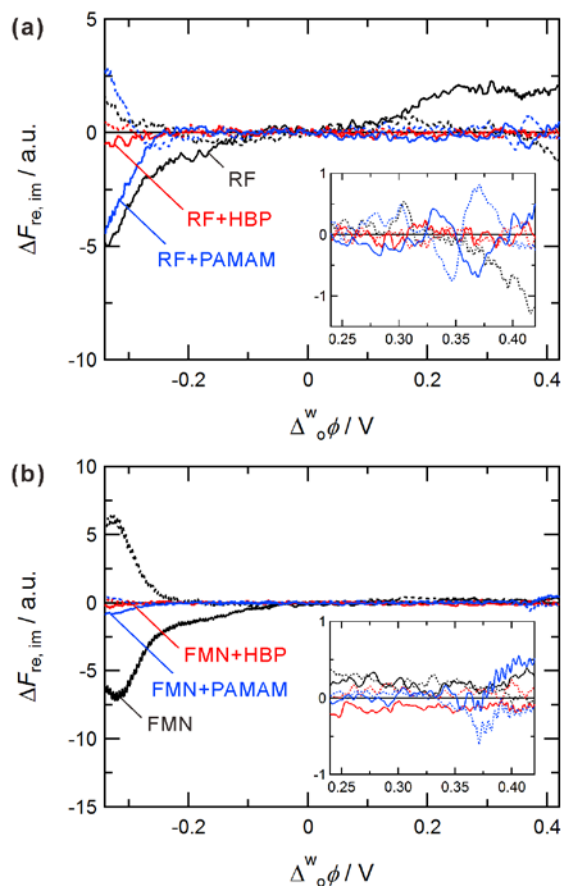


Fig. 4 Potential dependences of the PMF responses for **(a)** RF and **(b)** FMN at pH 7.1–7.3. The solid and dotted lines depict the real and imaginary components, respectively. The concentration of flavin derivatives and dendritic polymers was $1.0 \times 10^{-5} \text{ mol dm}^{-3}$.

spectroscopy to potential-dependent interfacial phenomena enables us to analyze the transfer and adsorption mechanism of charged fluorescent species. The PMF responses (ΔF) are expressed as functions of the faradaic ac current for an ion transfer and the ac potential-modulated surface coverage for an adsorption, respectively (Supplementary material: **S2**) [23, 24]. **Fig. 4** shows the potential dependences of the PMF responses measured for RF and FMN in the presence and absence of the equimolar dendritic polymer. Although, in principle, the PMF signal should not be observed for the neutral RF which is not transferred and adsorbed depending on $\Delta_0^w \phi$, the broad

PMF response was obtained even in the absence of the dendritic polymer. It is known that the ionizable photoproducts such as lumichrome (LC) are generated from RF under UV-vis irradiations [33-35]. LC coexists with a small abundance of its monoanionic form at neutral pH [36-38]. In fact, LC displayed the similar PMF responses to the RF system (Supplementary material: **S3** and **S4**). Therefore, the PMF responses observed in the RF system are considered those for the LC species which is generated under the TIR excitation and adsorbed at the interface. The PMF responses were totally vanished by adding the surface-active HBP, indicating that the adsorption of LC^- could be inhibited by the competitive adsorption of HBP. In the presence of the positively charged PAMAM dendrimer, the weak PMF signals were measured as positive real ($\Delta F_{re} > 0$) and negative imaginary components ($\Delta F_{im} < 0$) at 0.33 V and $\Delta F_{re} < 0$, $\Delta F_{im} > 0$ at 0.36 V, respectively (**Fig. 4a**, inset). These PMF responses were attributed to the ion transfer of the dendrimer-bound fluorescent RF (or LC) and its adsorption at the organic side of the interface [32]. In the FMN system (**Fig. 4b**), the bell-shaped PMF responses ($\Delta F_{re} < 0$, $0 < \Delta F_{im}$) obtained around -0.32 V demonstrated that the adsorption process of the anionic FMN^- occurs at the aqueous side of the interface [24]. The PMF intensity was drastically decreased in the presence of HBP similarly to the RF system, where the preferential adsorption of HBP depressed the adsorption of FMN^- . The addition of the PAMAM dendrimer also weakened the PMF responses around -0.32 V, where the positively charged dendrimer shows no affinity for the interface [14]. It has been reported that the transfer potential of anionic species is negatively shifted as a function of the stability of the PAMAM dendrimer-anion associate in the aqueous phase [12, 13]. A negligible potential shift of PMF response implies a rather weak association in the present system. The weakened PMF response, therefore, reflects a decrease in the effective diffusion coefficient of free FMN^- in the interfacial region due to the electrostatic interaction with the positively

charged dendrimer. In addition, the complicated PMF signals at $0.35 \text{ V} < \Delta_o^w \phi$ (**Fig. 4b**, inset) could be attributed to the partial dissociation of FMN^- from the dendrimer-FMN associates during its transfer process across the interface. These PMF results exhibit the specific molecular association between the dendritic polymers and FMN in the interfacial region.

4. Conclusions

The transfer and adsorption of the flavin derivatives in the presence and absence of the dendritic polymers were studied at the polarized water|DCE interface. The PMF analysis uncovered the specific interactions between the flavin derivatives and the dendritic polymers in the interfacial region, where the adsorption processes of the flavin derivatives were effectively inhibited by the competitive adsorption of surface-active HBP molecules. On the other hand, the flavin derivatives formed the ion-associates with the positively charged PAMAM dendrimer, and the dendrimer-bound flavin derivatives could be transferred across the interface at the positive edge of the potential window. The present results demonstrated that the dendritic polymers specifically interact with both ionized and neutral flavin derivatives and affect the charge transfer mechanism at the polarized liquid|liquid interface. The interfacial molecular association involving dendritic polymers observed in this study allows us to estimate the fundamental transmembrane features of dendritic polymer-based drug carriers for DDS as well as noncovalently biomacromolecule-bound substances, i.e., flavoproteins. Furthermore, the examined dendritic polymers will be potential modifiers of the pharmacokinetics of both charged and noncharged bioactive substances.

Acknowledgments

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