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Photo-controlled extraction and active transport of amino acids by functional reversed micelles containing spiropyran derivatives

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Abstract: In a reversed micellar *n*-decane solution, a spiropyran derivative 3′,3′-dimethyl-1′-octadecyl-6-nitrospiro(2H-benzopyran-2,2′-indoline) (PC18) showed normal photochromism, and the reversed micelles provided polar microenvironment increasing stability of a zwitterionic merocyanine (MC) form of PC18. Though the reversed micelles of tetraethyleneglycol dodecylether (TEGDE) alone in *n*-decane had relatively low ability to extract amino acid from aqueous solutions, the PC18-incorporated TEGDE reversed micelles in *n*-decane showed good photo-controlled extraction of zwitterionic amino acid under UV-irradiation and release under VIS-irradiation. Extent of the extracted amino acid was higher for tryptophane (Trp) bearing hydrophobic side chain than alanine (Ala), showing amino acid selectivity. Photo-driven active transport of amino acid across a liquid membrane was attained by the PC18-incorporated TEGDE reversed micellar carrier in a water/*n*-decane/water three-phase system, where one side of an aqueous/organic interface was irradiated by the UV-light and the other side by the VIS-light. When Trp and Ala were present in the aqueous solution, Trp was selectively transported.

Key words: Reversed micelles – spiropyran – photochromic – transport – liquid membrane

Introduction

Though normal and reversed micelles are simple aggregates of amphiphilic molecules in water and apolar media, respectively, they can offer a unique microenvironment that is different from bulk media [1], and their functionality has been the subject of intense studies [2–4]. In particular, use of reversed micelles as a microreactor for enzyme-catalyzed reactions has attracted much interest [5]. However, relatively little of their functionality as a carrier in membrane transport system has been studied. Tondre et al. and other groups reported the transport of alkali metal salts by reversed micelles of AOT (bis(2-ethylhexyl)sulfosuccinate) or tetraethyleneglycol dodecylether (TEGDE) [6–8]. We have reported that transport of fatty acids and steroid hormones by micelles of hexadecyltrimethylammonium bromide (HTAB) showed unique substrate selectivity and bovine serum albumin (BSA)-like transport characteristics [9–11]. Since micelles are molecular aggregates, the functionality of micellar carriers can be easily modified by incorporation of functional molecules without structural modification of the surfactant. Use of functional micellar carrier might open the way for developing a highly functional transport system.

For this purpose, we selected amphiphilic spiropyran derivatives as a photochromic substrate for preparation of photo-responsive reversed micellar carriers. Interconversion between spiropyran (SP) and zwitterionic merocyanine (MC) can be controlled by photoirradiation, syntheses and properties of spiropyran derivatives have been well established [12]. Sunamoto et al.

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reported photo-induced transport of amino acid through a bilayer membrane by association of the MC form of a spiropyran with zwitterionic amino acids [13], and Shimidzu et al. and Winkler et al. showed photo-induced transport of alkali metal salts by a spiropyran derivative [14, 15]. While, use of reversed micelles for extraction and separation of amino acids and proteins has been intensively studied by Luisi et al. [2], in which they pointed out that reversed micelles provided useful hydrophilic microenvironment for solubilization of amino acids and proteins into organic phase. In this report, we will clarify the properties of the spiropyran-incorporated functional reversed micelles and their ability for the photo-controlled extraction of amino acid, and report on the photo-driven active transport of amino acid across a liquid membrane by the photo-responsive reversed micellar carriers.

Experimental

Materials

1′,3′,3′-Trimethyl-6-nitrospiro(2H-benzopyran-2,2′-indoline) (PC1) was obtained from Tokyo Kasei Kogyo Co., and was recrystallized from absolute ethanol. 3′,3′-Dimethyl-1′-octadecyl-6-nitrospiro(2H-benzopyran-2,2′-indoline) (PC18) was synthesized by reference to the synthesis of 1′-benzyl-3′,3′-dimethyl-6-nitrospiro(2H-1-benzopyran-2,2′-indoline) with slight modification [16]. Structure of the product was confirmed by ^{1}H and ^{13}C NMR [14]; yield 12.0%; mp 77–78 °C. Hexadecyltrimethylammonium bromide (HTAB) was obtained from Tokyo Kasei Kogyo Co. and was recrystallized from acetone. TEGDE and AOT were purchased from Wako Pure Chemical Co. and used without further purification. Water was distilled and passed through a Milli-Q system of Millipore Co. All other solvents were UV-grade products from Dojin Chemical Co. Amino acids and other chemicals were obtained commercially, and were purified by conventional methods if necessary.

Determination of amino acid concentration

L-Tryptophan (Trp), L-phenylalanine (Phe), and L-tyrosine (Tyr) in aqueous solutions were analyzed directly by a Hitachi HPLC system (L-6000 and UV-4200) with a Hitachi #3161 column ($\phi 4 \times 150$ mm/aqueous sodium chloride solution (0.1–0.01 M) (1 M = 1 mol dm^{-3})) for Trp and Phe, and with a Tosoh TSK-gel ODS-80 column ($\phi 4.6 \times 150$ mm/ethanol (12v/v%)-water) for Tyr. Flow-rate was 1 ml min^{-1} and peaks were monitored at 287 nm for Trp, 257 nm for Phe, and 275 nm for Tyr. L-Alanine (Ala) was analyzed with a Jasco LC-800 HPLC system after derivatization with dabsyl chloride according to the standard procedure [17] (Hitachi #3161/acetonitrile-25 mM ammonium acetate buffer (pH 6.5) containing 4% dimethyl formamide; 15/85 (v/v) to 35/65). Flow-rate was 1 ml min^{-1} and peaks were monitored at 436 nm.

Kinetic measurements and determination of water content

A sample solution in a quartz-made cell (path length 10 mm) was irradiated by a high-pressure mercury lamp (Ushio Sen HL-100, 100W) through a glass filter (Toshiba UV-D35, 300 nm $< \lambda <$ 400 nm, for UV-light irradiation; and Y-46, 440 nm $< \lambda$, for VIS-light irradiation) and photo-induced coloration and thermal decoloration was spectrophotometrically recorded with a Photal MCPD-1000 system at 25 °C. Half-life times ($t_{1/2}$) of the MC to PC form for PC1 and PC18 were determined from the decay curves of the visible MC absorption in the dark. No effort was made to exclude oxygen.

For denaturation study of merocyanine, a sample solution in the cell thermostated at 25 °C was irradiated with a high-pressure mercury lamp (Riko UVL-400, 400 W) through a glass filter (Toshiba UV-D35), and electronic spectra were monitored simultaneously from the perpendicular direction during UV-light irradiation. The denaturation half-life time ($t_{d\,1/2}$) was evaluated from the visible absorption decay.

Water contents in the reversed micellar solutions were measured with a Hiranuma automatic aqua counter model AQ-6 based on the Karl Fischer method [18].

Extraction of amino acids in two-phase system

Transfer of amino acids across an aqueous/organic interface was examined in a cylindrical glass

vessel (ϕ13 mm). The organic phase was 2.0 ml of a *n*-decane solution containing 100 mM AOT, a *n*-decane solution containing 100 mM TEGDE and *n*-hexanol (1:1 w/w), or a *n*-decane and *n*-hexanol (1:1 v/v) solution containing 100 mM HTAB. Equal volume of an aqueous buffer containing 0.10 mM amino acid and 250 mM potassium chloride was placed under the organic phase. The aqueous phase was gently stirred (100 rpm) by magnetic stirrer for 18 h at 20 °C, and amounts of amino acid transferred to the organic phase were determined by measuring the amino acid concentration in the aqueous phase by HPLC. Buffer solutions used were 0.2 M KCl-HCl (pH 2.0), 0.2 M KH_2PO_3-NaOH (pH 6.0), 0.2 M H_3BO_3-KCl-NaOH (pH 9.0), and 0.2 M KCl-NaOH (pH 13.0).

When the spiropyran derivative (1.0 mM) was present in the organic phase, the sample solutions were subjected to UV irradiation for 20 min, and subsequently to VIS irradiation for 10 min. The aqueous phase was stirred at 800 rpm and 400 rpm for a system using PC18 alone and a PC18-incorporated reversed micellar system, respectively. Amino acid concentration in the aqueous phase was determined immediately after UV or VIS irradiation.

Photo-driven active transport in three-phase system

All measurements were carried out at 20 °C with a Pyrex-made H-shaped tube (ϕ10 mm) where two aqueous phases were separated by an organic phase. The two aqueous phases (3.0 ml) were the identical buffered solution (pH 6.0) containing 2.0 mM of amino acid and 250 mM potassium chloride, and the organic phase (6.0 ml) was the same solution used for the extraction experiment. One side of the aqueous/organic interface was irradiated by the UV-light and the other side by the VIS-light. Aliquots of the two aqueous phases were sampled at appropriate intervals and the amino acid concentrations were determined with the HPLC system.

Results and discussion

Photochromic properties of spiropyrans in reversed micellar systems

In all solvents and reversed micellar solutions examined, PC1 and PC18 showed normal photochromism (Scheme 1), i.e., SP to MC by UV-light irradiation (100-W high-pressure mercury lamp, 300 nm $< \lambda <$ 400 nm) and MC to SP by thermal process or VIS-light irradiation (420 nm $< \lambda$) (Fig. 1). Though PC1 and PC18 in polar media in the dark showed faint color, indicating the presence of the MC form, proportion of the MC form was negligibly small. Table 1 shows the absorption maxima (λ_{max}) in the VIS region of the photo-induced MC form of PC1 and PC18 (MC1 and

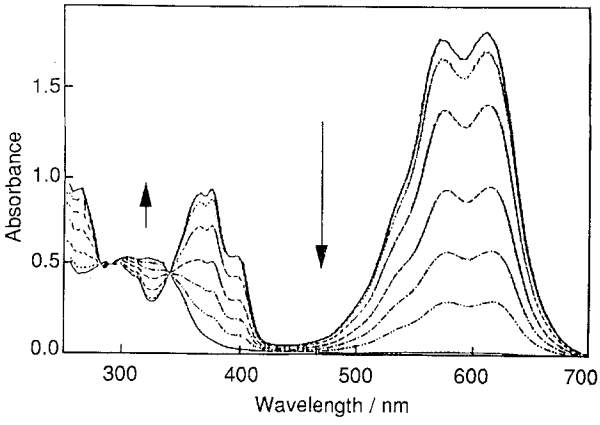
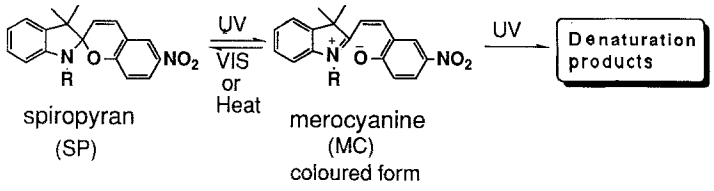


Fig. 1. Spectral change of the photo-induced MC18 in cyclohexane (3×10^{-5} M) by the thermal reverse reaction in the dark at 25 °C; time intervals are 0, 4, 17, 42, 72, 120 and 270 s after UV-irradiation



Scheme 1.

Table 1. Absorption maxima (λ_{max}) and half-life times ($t_{1/2}$) of the MC1 and MC18 (3×10^{-5} M) in various solvents at 25 °C

	MC1		MC18	
Solvent	λ_{max}/nm	$t_{1/2}$/s	λ_{max}/nm	$t_{1/2}$/s
Methanol	525	3000	533	
Ethanol	538		548	1100
Acetone	568		569	870
Chloroform	579	130	580	620
Benzene	602		604	
Cyclohexane	577, 615	30	578, 615	44
n-Hexane	578, 615		578, 615	
n-Decane	580, 616		580, 615	16

MC18, respectively), together with their half-life times ($t_{1/2}$) for the thermal reverse reaction to the SP form in various organic solvents.

Increase of solvent polarity caused a blue shift of the absorption of the MC1 and MC18 and concomitant increase of their half-life times [19, 20]. On the other hand, the SP form of PC1 and PC18 showed a small red shift as the polarity was increased from cyclohexane to methanol (λ_{max} 318 to 330 nm and 320 to 341 nm for PC1 and PC18, respectively).

As the λ_{max} and the $t_{1/2}$ of the MC forms are sensitive to the nature of the solvents, they can be a good measure of the microenvironment around the MC unit in reversed micellar solutions [21]. Table 2 shows the λ_{max} of the characteristic MC absorption in the AOT/cyclohexane reversed micellar solutions ([PC]/[AOT] molar ratio = 3×10^{-3}). Large blue shift was observed for the MC1 as the water content was increased, suggesting that methyl-substituted PC1 in the MC form was solubilized around the hydrophilic water core of the reversed micelles. The MC18 having long alkyl chain also showed a similar blue shift in the AOT reversed micellar systems, indicating the incorporation of MC18 in the reversed micelles, but the extent of the shift was much smaller. Therefore, the zwitterionic MC unit of PC18 anchored in the reversed micelles might not be located in the water core, but may be slightly apart from the core.

The $t_{1/2}$ slightly increased as the water content was increased. As polar microenvironment stabilizes the MC form, the results qualitatively coincide with those indicated from the λ_{max}.

Table 2. Absorption maxima (λ_{max}) and half-life times ($t_{1/2}$) of the MC1 and MC18 (3×10^{-5} M) in the AOT reversed micellar system in cyclohexane at 25 °C

		MC1		MC18	
AOT/mM	Water/mM	λ_{max}/nm	$t_{1/2}$/s	λ_{max}/nm	$t_{1/2}$/s
0	0	577	30	578	44
10	0	556	65	576	50
10	3	556	65	575	50
10	10	538	108	571	56
10	20	532	126	567	77

Table 3. Absorption maxima (λ_{max}) and half-life times ($t_{1/2}$) of MC form of PC18 in different reversed micellar systems at 25 °C

	λ_{max}/nm	$t_{1/2}$/s
Ethanol	548	1100
Acetone	569	870
Cyclohexane	578	44
n-Decane	580	16
AOT	564	60
AOT[a])	540	180
TEGDE	570	27
TEGDE[a])	563	30

[PC18] = 3×10^{-5} M, [surfactant] = 100 mM
[a]) Reversed micellar solutions are saturated with pH 6.0 buffer solutions

Table 3 shows the λ_{max} and the $t_{1/2}$ of MC18 in the AOT and TEGDE reversed micelles in *n*-decane. In the case of TEGDE, *n*-hexanol was added as a co-surfactant in order to stabilize the reversed micellar system [22]. When the reversed micelles were saturated with water (pH 6.0 buffer solution), the λ_{max} of the MC18 showed a similar blue shift and the $t_{1/2}$ increased. The water contents in the reversed micellar phase were found to be 2.5% and 0.25% in the AOT and TEGDE systems, respectively. Therefore, the water-rich AOT reversed micelles might provide more polar microenvironment to the MC unit compared with the TEGDE system. These results confirmed that the MC form of PC18 in the AOT and TEGDE reversed micelles was incorporated in the reversed micelles.

In the cases of SP1 and SP18, however, clear shifts of the absorption maxima were not observed in the reversed micellar solutions compared with those in *n*-decane solutions. At this

point, we could not determine whether the SP was incorporated in the reversed micelles or not.

Denaturation of MC18 by UV-light irradiation

Though spiropyran undergoes reversible structural transformation upon photoirradiation, longer UV-irradiation is known to induce irreversible denaturation of the MC into a nonphotochromic substrate. Especially in apolar solvents or in basic media, the MC form is prone to undergo denaturation [19]. When PC18 was irradiated by the UV-light in *n*-decane, the characteristic visible absorption rapidly increased at an early stage due to the SP-to-MC transformation. However, a relatively slow decrease of the absorption was observed by further UV-irradiation, and the solution became colorless after sufficient time of irradiation. The resultant colorless solution did not show photochromic properties, thus confirming the irreversible denaturation of PC18. Denaturation half-life times ($t_{d\,1/2}$) of MC18 in different systems are summarized in Table 4. For this experiment, a 400-W high-pressure mercury lamp was used instead of the 100-W lamp. The $t_{d\,1/2}$ values in the reversed micellar systems were much larger than that in *n*-decane, which is attributed to the higher polarity of the microenvironment around MC18 in the reversed micelles (see Table 3). Thus, it was shown that the reversed micelles provided polar microenvironment in *n*-decane, thus increasing the stability of the MC form of PC18.

Table 4. Denaturation half-life times ($t_{d\,1/2}$) of the MC form of PC18 (3×10^{-5} M) under UV-irradiation at 25 °C[a])

	$t_{d\,1/2}$/min	(relative value)
Ethanol	90	22.5
Acetone	10	2.5
Chloroform	0.3	0.075
n-Decane	4.0	1.0
0.1 M AOT reversed micellar system[b])	55	13.0
0.1 M TEGDE reversed micellar system[b,c])	35	8.75

[a]) conditions: see Experimental section
[b]) saturated with a buffer solution (pH 6.0) containing 250 mM KCl
[c]) TEGDE/*n*-hexanol = 1/1 (w/w)

Table 5. Extraction of Trp in aqueous buffer solutions (0.1 mM) to different reversed micellar *n*-decane phases or MC18/*n*-decane phase at 20°C[a])

	Yield of transfer (%)[b])			
pH	AOT	HTAB	TEGDE	MC18[c])
2.0	95	9.0	8.8	1.0
6.0	19	10	6.5	7.0
9.0	13	25	10	3.2
13.0	0.6	63	7.6	0.6

[a]) conditions: see Experimental section
[b]) 100% transfer corresponds to 0.1 mM
[c]) [MC18] = 1.0 mM, UV irradiation for 20 min, solutions are vigorously stirred (800 rpm)

Extraction of amino acid by the PC18-incorporated reversed micelles

Before the study of the photo-controlled extraction by the PC18-incorporated reversed micelles, we tested to what extent amino acid was extracted by reversed micelles [2] or by MC18 alone (Table 5). As different buffer solutions were used to cover the pH range from 2 to 13, 250 mM of potassium chloride was added to ensure that intrinsic ionic strength of each buffer solution was not significantly different. We selected *n*-decane/*n*-hexanol = 1/1 (v/v) as a solvent of HTAB in order to stabilize the reversed micellar phase [2]. It is obvious that positively charged L-tryptophane (Trp) in low pH was effectively extracted into the anionic AOT reversed micelles, while the cationic HTAB reversed micelles were effective for extraction of negatively charged Trp in high pH (Table 4). But, extraction by the nonionic TEGDE reversed micelles was less than 10% and only slightly affected by pH. These results indicated that an electrostatic force worked effectively for extraction of amino acid into the reversed micelles [2].

In the presence of PC18 alone in *n*-decane after sufficient UV-irradiation, Trp was extracted at pH 6.0 where both MC18 and Trp were zwitterionic, but practically no extraction was observed at pH 2 or 13. Therefore, association of the zwitterionic MC18 with Trp was important for transfer of Trp into *n*-decane phase. No transfer of Trp was observed without UV-irradiation, confirming that the SP form of PC18 was ineffective. As the MC form of PC1 was partly distributed in

Table 6. Proportion of amino acids in the PC18-incorporated reversed micellar phase after equilibrium extraction from an aqueous buffer solution (pH 6.0) at 20 °C[a])

Surfactant	Amino acid	Yield of Transfer (%) UV[b])	VIS[b])	Without PC18[c])
TEGDE	Trp	16.0	8.0	6.5
TEGDE	Phe	14.3	7.6	5.9
TEGDE	Tyr	7.5	3.8	2.0
TEGDE	Ala	2.3	1.4	0.5
AOT	Trp	27.0	22.5	19.3
AOT	Phe	24.5	20.1	16.0
none[d])	Trp	7.0	0.0	
none[d])	Phe	6.0	0.0	

[a]) Conditions: see experimental section
[b]) UV-light irradiation for 20 min, and subsequent VIS-light irradiation for 10 min
[c]) Reversed micellar systems without PC18
[d]) PC18 alone

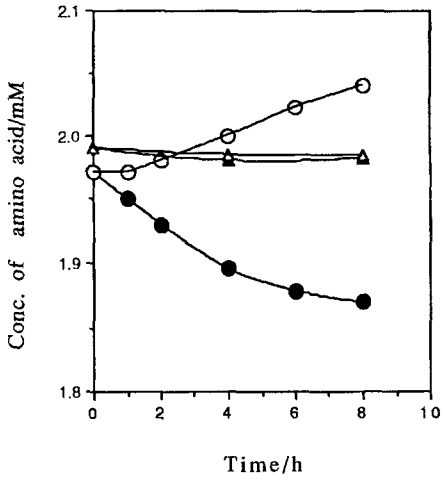


Fig. 2. Time-course of the Trp (●) and Ala (▲) concentrations in the UV-irradiated and the Trp (○) and Ala (△) concentrations in the VIS-irradiated aqueous phases during the photo-driven transport by the PC18-incorporated TEGDE reversed micellar carrier at 20 °C. Initial amino acid concentrations in both aqueous solutions were 2 mM. Detailed experimental conditions, see Experimental section

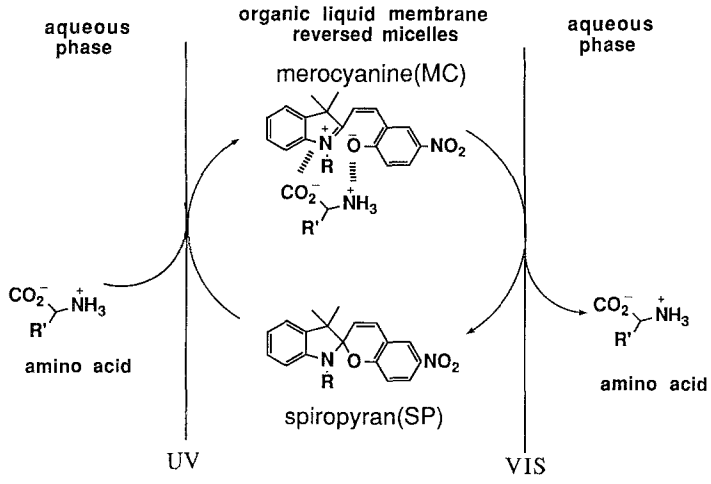
aqueous phase, PC1 was not suitable for extraction experiments.

The photo-controlled extraction and release of amino acid by the PC18-incorporated reversed micelles were studied at pH 6.0 where amino acid was zwitterionic. Table 6 shows proportions of amino acids in the organic phase after UV-irradiation for 20 min and subsequent VIS-irradiation for 10 min. Under the experimental conditions we employed, no coloration of the organic phase was observed before UV-irradiation, and UV-irradiation for 20 min or VIS-irradiation for 10 min was sufficient for equilibrium extraction or release, respectively, of amino acid. The equilibrium proportions of amino acids transferred to the PC18-incorporated reversed micellar phase under UV-irradiation were only slightly larger than the total of those by reversed micelles and MC18 alone, showing only a small synergistic effect. Upon VIS-irradiation, the organic reversed micellar solution became colorless, indicating the MC-to-SP transformation of PC18, and a portion of the amino acids, presumably extracted by the MC, was efficiently released back to the aqueous phase. Though contribution of the photochromic PC18 for the extraction of amino acid in the PC18-incorporated AOT system was small because of the high extraction ability of the AOT reversed micelles alone, it was much higher in the TEGDE reversed micellar systems and approximately twice the amounts of amino acid was transferred into the organic phase under UV-irradiation compared with those under VIS-irradiation. Therefore, the PC18-incorporated reversed micelles can serve as a good photo-responsive extraction system for amino acid.

Extraction by the reversed micellar system showed amino acid selectivity. The selectivity is roughly coincided with the hydrophobicity of the side chain of amino acid [23], indicating the importance of the side chain structure. It is interesting that the selectivity of the PC18-incorporated reversed micelles was essentially the same as that of the reversed micelles alone.

Photo-driven active transport across a liquid membrane

Photo-driven active transport of amino acid by the PC18-incorporated reversed micelles through a *n*-decane liquid membrane was carried out in an H-shaped tube. The two identical aqueous solutions containing 2.0 mM of Trp and 2.0 mM of Ala were separated by the PC18-incorporated TEGDE reversed micellar solution. Photoirradiation was performed after the two aqueous phases and the reversed micellar liquid membrane phase



Scheme 2. Schematic representation of amino acid transport through a reversed micellar liquid membrane

reached equilibrium in the dark. During this initial period in the dark, a small decrease of the amino acid concentration was observed in both aqueous phases, which was presumably due to the intrinsic extraction by the TEGDE reversed micelles. Figure 2 shows the Trp and Ala concentration profiles of the UV and VIS-irradiated aqueous phases. Upon photoirradiation, the Trp concentration of the UV-irradiated side started decreasing. While that of the VIS-irradiated side started increasing after certain time of induction period and became higher than the initial concentration by 4 h of irradiation, demonstrating the photodriven active transport of Trp by the functional reversed micelles. Mechanism of the transport is shown in Scheme 2. On the other hand, the Ala concentration of the both sides remained unchanged during photoirradiation, indicating that the functional TEGDE reversed micellar carrier has high amino acid selectivity. This selectivity in the transport system was essentially the same as that observed for the amino acid extraction (Table 6).

The active transport of Trp by the AOT reversed micellar system was unsuccessful. As the AOT reversed micelles alone has high ability to extract Trp into the organic phase, contribution of the PC18 might be too small to induce active transport. Besides, the transport by PC18 alone without surfactants was failed because PC18 was denatured completely within 3 h of irradiation under the experimental conditions.

Thus, active and selective transport of Trp by the PC18-incorporated reversed micellar carrier was achieved. Photo-induced concentration gradients of the SP and MC forms of PC18 must be serving as the driving force of the active transport. While, the TEGDE reversed micelles provided relatively polar environment in the *n*-decane phase, which stabilized the MC form of PC18 suppressing denaturation and helped the selective solubilization of the amino acids. Functional reversed micellar carrier can easily be prepared by incorporating functional molecules into micellar aggregates. Therefore, further elaboration of the reversed micelles as well as functional molecules may lead to the development of a highly functional transport system.

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