Artificial Flavolipid. Its Synthesis, Incorporation into Liposomal Membrane, Electron Transport, and Successful Control of Transport Rate

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The artificial flavolipid which has a flavin unit in the vicinity of the phosphatidylcholine moiety has been synthesized and found to be an effective electron transport catalyst across the bilayer membrane; it has also been successfully incorporated into lecithin liposome to give a stable product. The latter was shown to have, from dynamic light scattering and electron microscopic measurements, a single-walled, normal liposome structure of *ca.* 360 Å diameter and was as stable as the parent egg lecithin liposome. Clear biphasic kinetics were observed on reduction of the flavolipid in the bilayer membrane. The faster process was ascribed to reduction of the flavolipid in the outer half layer of the bilayer and the slower to the reduction of the flavolipid in the inner. The electron transport rate across the bilayer membrane from the external $Na_2S_2O_4$ to the internal $K_3Fe(CN)_6$ was much facilitated by the presence of the flavolipid in the membrane: detailed kinetic studies demonstrated that electrons were transported *via* a transient transmembrane channel consisting of two flavolipid molecules. The transport rate across the membrane was controlled by a thermally induced phase transition of the bilayer structure.

Electron transport plays an important role in the respiratory and photosynthetic systems of mitochondria and thylakoid membranes, respectively. Because of its key role in the storage of energy which utilizes O_2 and light as the ultimate sources,[1] such systems have been much investigated.[2,3] One of the difficulties in analysing the natural system arises from the fact that the electron transporting units are tightly bound as a multienzyme complex system to the biological membrane.[4,5] In order to get a clearer understanding based on the molecular mechanism, several model systems have extensively been studied. According to recent and significant progress in this field,[6,7] electron tunnelling from one oxido-reduction centre to the other has been both experimentally verified and discussed interestingly on the basis of the Marcus theory.[8–11] There are two possible mechanisms for electron transfer across the membrane. The first is based on a mobile electron carrier which diffuses across the membrane, and this has been investigated in detail for alkylviologens,[12] benzoquinone, and *inter alia* ferrocene.[13] The second is a membrane penetrating electron channel through which electrons are transported *via* tunnelling. The latter mechanism is interesting in view of its possible significance in biological membranes, where the electron mediator loses its mobility in a highly ordered matrix. This mechanism is of special importance in respect of photochemical charge separation, which is successfully modelled by a carotenoporphyrin-quinone triad.[3] A further interesting approach is provided by poly(ethylenimine)-linked manganese porphyrins[14] which employ porphyrins and their metal complexes as electron transporting centres.

Electron transport across a membrane is sometimes coupled with a proton or other material transport.[15] Biologically important examples of this include ATP synthesis,[16] and *inter alia* visual systems[17] where energy for biological synthesis or information transfer is provided by coupled proton transfer which produces a proton gradient across the membrane. Flavoprotein is an important electron transducing enzyme in the initial stage of many biological metabolic systems, such as amino acid oxidase, NADH dehydrogenase, NADPH cytochrome reductase, and others, where the flavin unit accepts electrons from various reducing substrates and transfers them to acceptors, such as quinones, heme proteins, and iron-sulphur clusters. Here, the flavin unit functions not only as an efficient one- and two-electron transfer catalyst, but sometimes as a hydrogen transfer catalyst, the half and fully reduced flavins having pK_a values close to the physiological pH value.

We have been interested in the construction of an artificial cell for synthesizing ATP[18–20] and to this end we have attached a flavin unit covalently to phosphatidylcholine in the vicinity of the polar head group to give a 'flavolipid'; incorporation of this into a membrane would then allow investigation of transmembrane electron transfer and its control.

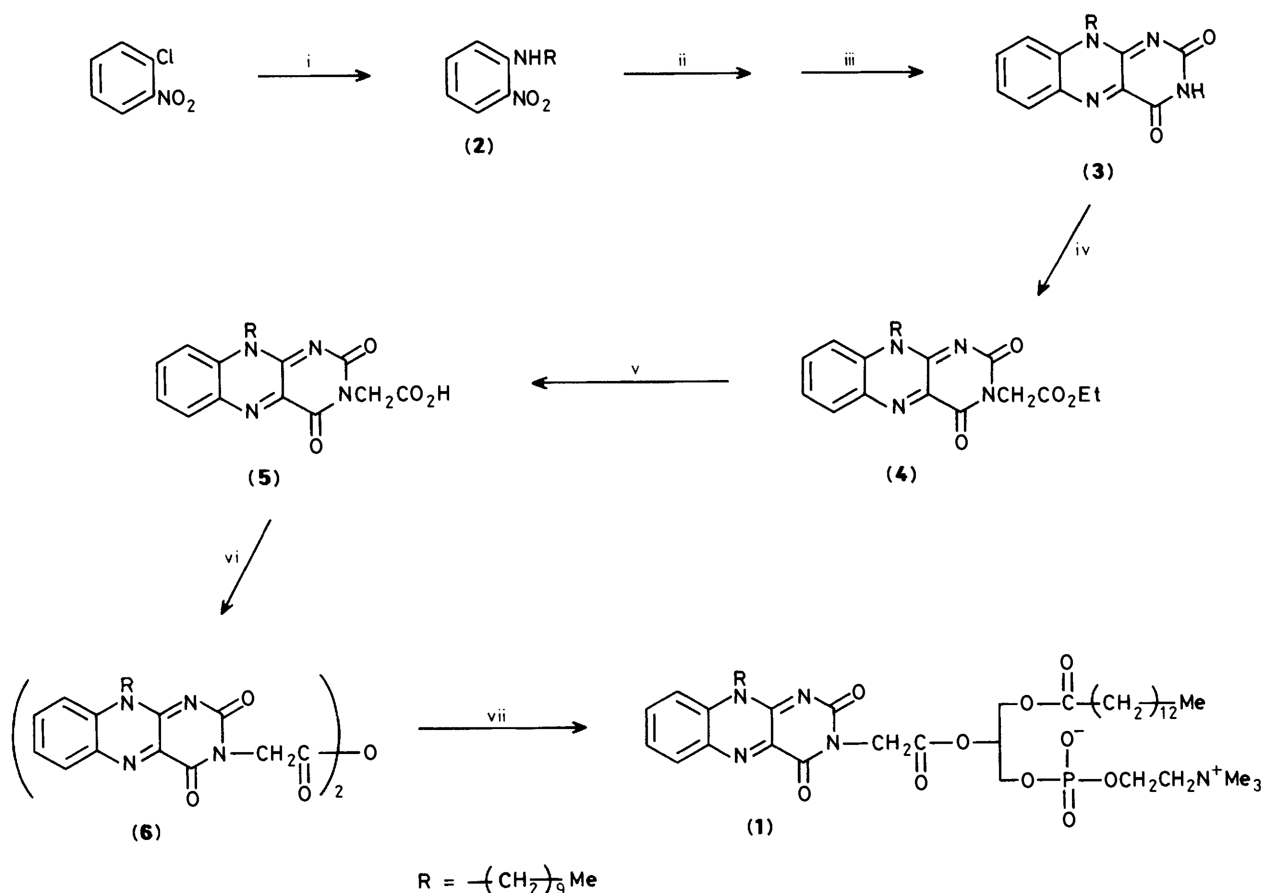
Here, we report both the preparation of the flavolipid and its successful incorporation into egg lecithin liposome. The electron transfer across the bilayer membrane was successfully demonstrated to pass through the oxidation-reduction centre of the flavin unit. This electron transport was shown by kinetic studies to operate through a half-channel mechanism. Furthermore, the idea of on-off control of the electron transport has been introduced.

Results and Discussion

Preparation and Structure of Flavolipid.—As a reversible redox active site, we chose a flavin[21] unit which was incorporated into the bilayer membrane and facilitated electron transfer across the membrane. Synthesis of the flavolipid (**1**) is outlined in Scheme 1. 10-Decylisoalloxazin-3-ylacetic acid was converted into its anhydride using ethyl chloroformate followed by esterification with myristoyl-L-α-lysophosphatidylcholine in the presence of 4-(*N*,*N*-dimethylamino)pyridine to produce (**1**).[22] The flavolipid (**1**) thus obtained has the following characteristics. (i) A phosphatidylcholine entity as a polar head group which, since it is identical with that of egg lecithin, should fit to the membrane surface of the latter with minimal perturbation of the surface properties of the bilayer membrane. (ii) A flavin chromophore bound covalently in the vicinity of the polar head group as a redox centre in the membrane. The depth of the flavin from the interphase was adjusted to 7 ± 1 Å‡ as estimated from a CPK model of the extended form in the

† Deceased March 22, 1987.

‡ The distance was measured from the centre of P atom of the phosphate group.



Scheme 1. *Reagents:* i, Decylamine; ii, H_2/Pt; iii, Alloxan; iv, $BrCH_2CO_2Et$; v, conc. HCl; vi, $ClCO_2Et$; vii, Myristoyl lyso-Pc

membrane phase. This distance, close to the interphase, is expected to function as an efficient redox centre to accept and release electrons from reductants and to oxidants, respectively, both present in the aqueous phase. (iii) The flavin entity is attached additionally by a long alkyl chain and the second acyl part of the lipid consists of a hydrophobic myristoyl residue. These two hydrophobic chains may serve to provide greater affinity to the phospholipid bilayer and to stabilize the bilayer structure of the liposome.

Structure and Stability of Artificial Liposomes Functionalized with Flavolipid.—After purification by centrifugation followed by gel filtration, artificial liposomes modified with flavolipid [$^{(o)}$|Fl-Lip|$^{(i)}$] showed absorption maxima at 336 and 444 nm with a shoulder at 472 nm. All of these absorptions are characteristic of the flavin chromophore. Artificial liposomes modified with the flavolipid incorporating ferricyanide in its internal aqueous phase showed absorptions at 304 and 425 nm, characteristic of ferricyanide, 336 and 472sh nm, characteristic of the flavin chromophore, and 215 nm, characteristic of the liposome. The absorption at 425 nm of ferricyanide was somewhat overshadowed by a tail of the absorption of flavolipid at 444 nm. A quantitative determination of the concentration of both species was obtained from the absorption at 425 nm aided by the absorption at 472 nm which arises only from the flavolipid.

As shown in Table 1, the electronic spectra of the flavin chromophore are sensitive to the solvent used. Comparison with the electronic spectra of 10-pentylisoalloxazine in various solvents (water, 0.25M Tris HCl, methanol, and chloroform) indicates that the absorption maxima of the flavolipid incorporated into the egg lecithin liposome appear at positions almost identical with those in chloroform. In contrast, the absorptions in the membrane differ significantly from those in pure water or Tris buffer. This evidence suggests that the flavin entity is located neither in the bulk aqueous phase nor at the bilayer interphase, but is incorporated into the membrane phase. In the fluorescence spectra, the solvent effect for the flavin chromophore in various solvents shows a trend similar to that of the electronic spectra.

A dynamic light scattering study of the purified liposomes

Table 1. Solvent dependence of electronic and fluorescence spectra of the flavin chromophore[a]

Flavin	Medium	Electronic spectrum (nm)		Fluorescence spectrum[b] (nm)
10-Pentylisoalloxazine	H_2O	347	433	512
	0.25M Tris HCl[c]	347	433	512
	MeOH	333	433	507
	$CHCl_3$	336	442, 415 (sh), 470 (sh)	498
(1)	Liposome[c]	336	444, 418 (sh), 472 (sh)	507

[a] At 25 °C. [b] Excitation at 340 nm. [c] pH = 7.0. [d] sh Means a shoulder.

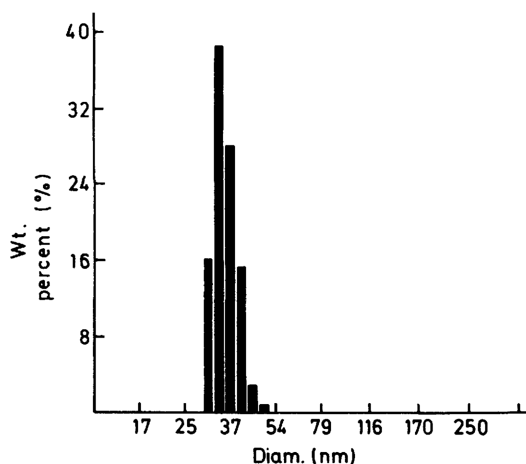


Figure 1. Dynamic light scattering of the artificial liposome modified with flavolipid (3 mol% flavolipid, at pH 7.0, 25 °C)

[$^{(o)}$|Fl-Lip|$^{(i)}$,$^{(o)}$|Fl-Lip|$^{(i)}$$K_3Fe(CN)_6$] indicated that the diameter of liposome particles was distributed in the range 300—500 Å with a mean value at *ca.* 360 Å (Figure 1). The electron micrograph of the flavoliposome [$^{(o)}$|Fl-Lip|$^{(i)}$] demonstrated a closed, single compartment bimolecular liposomal structure with a diameter of *ca.* 400 Å and a membrane thickness of *ca.* 40—50 Å. The artificial liposome modified with flavolipid [$^{(o)}$|Fl-Lip|$^{(i)}$,$^{(o)}$|Fl-Lip|$^{(i)}$$K_3Fe(CN)_6$] showed gel permeation behaviour similar to that of the unmodified artificial liposomes [$^{(o)}$|Lip|$^{(i)}$$K_3Fe(CN)_6$], and in so doing demonstrated that the flavolipid did not perturb significantly the structure and the size of liposome. The artificial liposomes modified with the flavolipid in less than 5 mol% produced no precipitate when set aside for 7 days after preparation. Furthermore, a dynamic light scattering study of the particle size distribution of the liposome modified with the flavolipid showed no change over 7 days. As much as 50 mol% of the flavolipid can be incorporated into the bilayer membrane with retention of stability for at least 4 days. These results demonstrate the high affinity of the flavolipid for the phospholipid.

Reduction of Flavolipid Incorporated into the Bilayer Membrane by Dithionite in the External Aqueous Phase.—A solution of the liposome modified with the flavolipid [$^{(o)}$|Fl-Lip|$^{(i)}$] was reduced with aqueous $Na_2S_2O_4$, the decrease of the oxidized flavolipid (fl^{ox}) in the bilayer membrane being monitored by the absorbance at 444 nm. As shown in Figure 2, the change of fl^{ox} concentration was observed to be clearly biphasic, where *ca.* 50—60% of the total change of the fl^{ox} absorbance decreased faster than the remaining 50—40%. The present preparative method for artificial liposomes modified with flavolipid is expected to distribute the flavolipid both in the outer and in the inner layer of the egg lecithin membrane. Therefore, from the particle size (360 Å) and the thickness (40—50 Å), the flavolipid is estimated to be distributed in the outer and the inner layer in the ratio 6:4. The flavin moiety is located at a fixed distance from the surface ($\simeq$7 Å inside the membrane from the P atom of the phosphatidyl unit).* Therefore the redox centres should be located at the inner membrane, one near to the exterior surface and the other near to the interior. In such a situation, topological differences in the flavin moiety in the membrane may be a determining factor in the reduction rate of the flavolipid by $Na_2S_2O_4$, although the effect of

* See footnote ‡ on p. 383.

Table 2. Biphasic reduction rate constants of flavolipid incorporated into the bilayer membrane

Temp. (°C)	fl^{ox} (mM)	$Na_2S_2O_4$ (mM)	$k_{o\to m}$ (s^{-1})	k_m (s^{-1})
25	0.037	1.0	6.2	0.63
5	0.037	1.0	2.9	0.56
5	0.019	0.052	0.34	*a*
5	0.019	0.26	0.95	*a*
5	0.019	0.52	1.48	0.13
5	0.019	1.0	1.85	0.14

[a] Monophasic reduction.

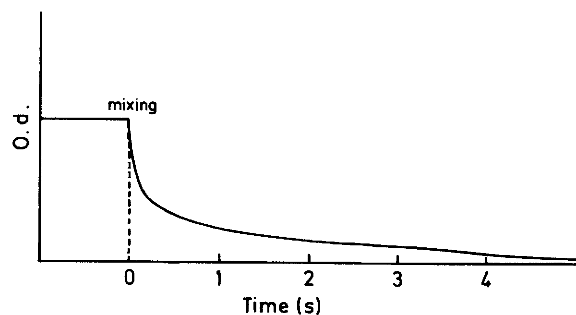


Figure 2. Reduction of flavolipid in the liposomal membrane. Change of absorption at 444 nm, characteristic of fl^{ox}

microenvironments and/or different binding sites in the liposomal membrane have been suggested in the interphasic reactions of several substrates in liposome systems.[23] The above experiment shows that flavolipids located both in the outer and in the inner layers are completely reduced by $Na_2S_2O_4$. The flavolipid located at the outer layer of the membrane, fl^{ox}(out), seems to be easily reduced. Therefore the faster process could be ascribed to the interphasic electron flux, $J_{out\to m}$, from the external aqueous phase into the membrane phase (Scheme 2).

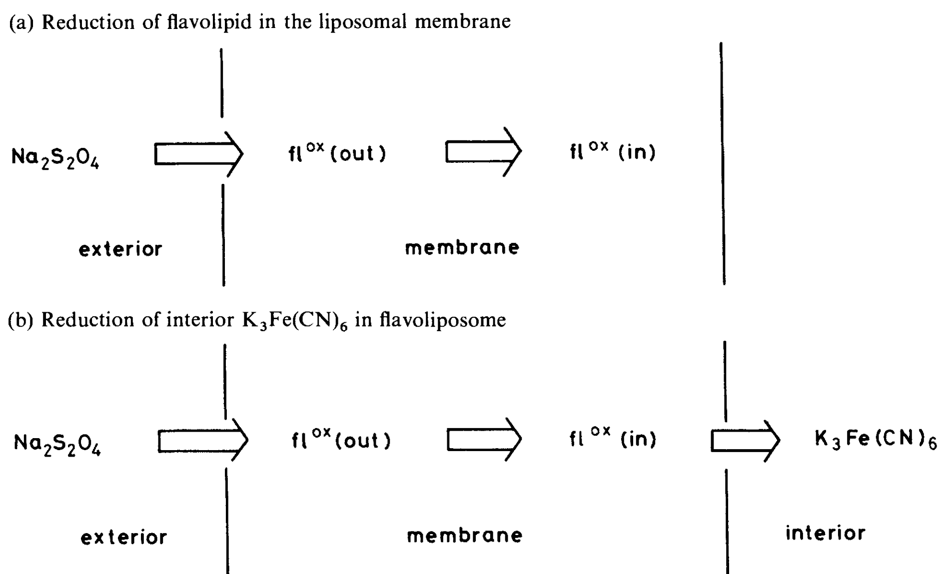
On the other hand, the reduction of the flavolipid located at the inner half layer of the membrane, fl^{ox}(in), follows a slower pseudo first-order process. The rate constants here obtained are given in Table 2. Careful investigation of the rate at different initial concentrations of $Na_2S_2O_4$ indicates that the rate of the fast process is 1/2 order with respect to the concentration of $Na_2S_2O_4$. With a decrease in the initial concentration of $Na_2S_2O_4$, the reduction of fl^{ox} was observed to be almost monophasic and first order with respect to the concentration of fl^{ox}. This may be explained by the idea that the rate-determining step is shifted from the flavin-flavin electron transfer step to the interphasic electron influx step from the external $Na_2S_2O_4$ to the fl^{ox}(out) in the membrane. In the clear biphasic region, the rate constants of the slower process were independent of the initial concentration of $Na_2S_2O_4$.

$$\frac{-d[fl^{ox}]}{dt} = k_{obs}[fl^{ox}]$$
$$k_{obs} = k_{o\to m} + k_m$$
$$k_{o\to m} = k[Na_2S_2O_4]$$
$$k_m = k_2[fl^{red}(out)] \quad (1)$$

For the following reasons, this slow reduction is reasonably ascribed to a long distance electron-transfer process from fl^{red}(out) (the reduced form of the flavolipid at the outer half

(a) Reduction of flavolipid in the liposomal membrane

(b) Reduction of interior $K_3Fe(CN)_6$ in flavoliposome



Scheme 2.

layer of the membrane)* to the fl^{ox}(in). (i) The flavolipid having a polar head group is a constituent of the bilayer membrane, and its estimated vertical diffusion rate, *i.e.* the flip-flop rate constant, $k = 10^{-5}\ s^{-1}$ for the normal phospholipid, is too slow to be accounted for the observed process.[24] (ii) The direct reduction of fl^{ox}(in) by the $Na_2S_2O_4$ seems to be improbable because the distance between the exterior interphase and the nearest edge of fl^{ox}(in) is *ca.* 30 Å as judged from a CPK model.† (iii) The lateral diffusion of the flavolipid is expected to be relatively free (10^{-8}—10^{-9} $cm^2\ s^{-1}$) in the membrane and certainly accommodates the idea of fl^{red}(out)→fl^{ox}(in) electron transfer on their mutual approach. (iv) The nearest edge-edge distance between the fl^{red}(out) and the fl^{ox}(in) is estimated to be *ca.* 16 ± 1 Å from a CPK model. The observed rate constants are compatible with those for electron transfer across a similar distance in porphyrin complexes.[6,7,25]

Electron Transport across the Bilayer Membrane Modified with Flavolipid.—The artificial liposome modified with the ferricyanide-containing flavolipid in its internal aqueous phase [$^{(o)}$|Fl-Lip|$^{(i)}K_3Fe(CN)_6$] was treated with $Na_2S_2O_4$ added to the external aqueous phase. As described previously,[26] a rapid reduction of fl^{ox} to fl^{red} on mixing with the aqueous $Na_2S_2O_4$ was observed and this was followed by a slower pseudo first-order decrease of ferricyanide. Under pseudo first-order

$$\frac{-d[Fe(CN)_6^{3-}]}{dt} = k[K_3Fe(CN)_6] \qquad (2)$$

conditions, the rate dependence on the concentration of $Na_2S_2O_4$ was investigated. The pseudo first-order rate constant was found to be 1/2 order with respect to the concentration of $Na_2S_2O_4$ in the range of 1.0—4.0mM. Therefore, the total rate expression is given by Equation (2′):

$$\frac{-d[Fe(CN)_6^{3-}]}{dt} = k_2[Na_2S_2O_4]^{\frac{1}{2}}[K_3Fe(CN)_6] \qquad (2')$$

* In these experiments, a semiquinone form of the flavolipid was not observed spectroscopically.

† The distance was estimated from an all-*trans* extended form of the present flavolipid.

Table 3. Reduction rate constants of $K_3Fe(CN)_6$ in the flavoliposome[a,b]

Concentration			
Flavolipid (mM)	$Na_2S_2O_4$ (mM)	$K_3Fe(CN)_6$ (mM)	k (s^{-1})
0	4.3	0.40	0.001
0.0015	4.3	0.40	0.0025
0.0068	4.3	0.40	0.025
0.011	1.1	0.40	0.031
0.011	2.1	0.40	0.043
0.011	4.3	0.40	0.053
0.011	8.4	0.40	0.056
0.014	4.3	0.40	0.083
0.070	3.0	0.41	0.44

[a] All reactions were performed at 25 °C, pH 7.0. [b] Analytical local concentration of internal $K_3Fe(CN)_6$ = 0.75M.

The half-order dependence on the $Na_2S_2O_4$ concentration is normally observed in both its homogeneous and heterogeneous reductions (micelle or liposome) for a variety of substrates.[27] This is interpreted in terms of a rapid homolytic cleavage of $S_2O_4^{2-}$ to $2{\cdot}SO_2^-$ followed by the electron-transfer process. In the concentration range for $Na_2S_2O_4$ >4.0mM, saturation kinetics with respect to $Na_2S_2O_4$ were observed. The results are summarized in Table 3.

The rapid reduction of fl^{ox} to fl^{red} was clearly observable only when the concentration of $Na_2S_2O_4$ was >4mM. The amount of fl^{red} corresponded to *ca.* 50—60 mol% of the total fl^{ox} initially employed. This value corresponds roughly to the flavolipid in the outer half layer of the membrane. At a lower concentration of $Na_2S_2O_4$ (<4mM), the initial rapid reduction of fl^{ox} to fl^{red} was not clearly discernible, the reduction of ferricyanide proceeding without the apparent fast reduction of fl^{ox}. Therefore, in the lower concentration range, the rate-determining step of the internal ferricyanide reduction must be the interphasic influx ($J_{out \to m}$). At a higher concentration of $Na_2S_2O_4$, the interphasic reduction becomes faster compared with fl^{red}(out)→fl^{ox}(in) electron transfer, *i.e.* now the rate-determining step. When the concentrations of $Na_2S_2O_4$ and flavolipid were kept high, 4mM and 70 μM, respectively, rapid

Table 4. Electron transport rate across the liposomal membrane modified with various electron mediators

Electron donor	Electron acceptor	Mediator[a]	(M)	k (s^{-1})	k/[mediator] (s^{-1} M^{-1})	Ref.
Ascorbate	$FeCl_3$	Chloroplast-pigment	*b*	0.007		*c*
$Na_2S_2O_4$	$K_3Fe(CN)_6$	UQ_{10}	19.5	1.277	650 × 10^2	28
		PQ	19.5	0.293	150 × 10^2	28
		UQ_1	25	0.028	11 × 10^2	28
Ascorbate	$K_3Fe(CN)_6$	Ferrocene/FCCP	20	0.013	6.5 × 10^2	13
		Benzoquinone	20	0.013	6.5 × 10^2	13
$Na_2S_2O_4$	$K_3Fe(CN)_6$	C_4V^{2+}	4	0.0023	5.8 × 10^2	19
		cyt-c	8.7	0.0075	8.6 × 10^2	19
		cyt-c_3	10.8	0.230	210 × 10^2	19
$Na_2S_2O_4$	$K_3Fe(CN)_6$	Flavolipid	70	0.45	64 × 10^2	*d*

[a] Abbreviations: UQ_{10}, ubiquinone with ten isoprene units; UQ_1, ubiquinone with one isoprene unit; PQ, platoquinone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; C_4V^{2+}, *N,N'*-dibutylviologen. [b] There is no description of the mediator concentration. [c] M. Mangel, *Biochim. Biophys. Acta*, 1976, **430**, 459. [d] This work.

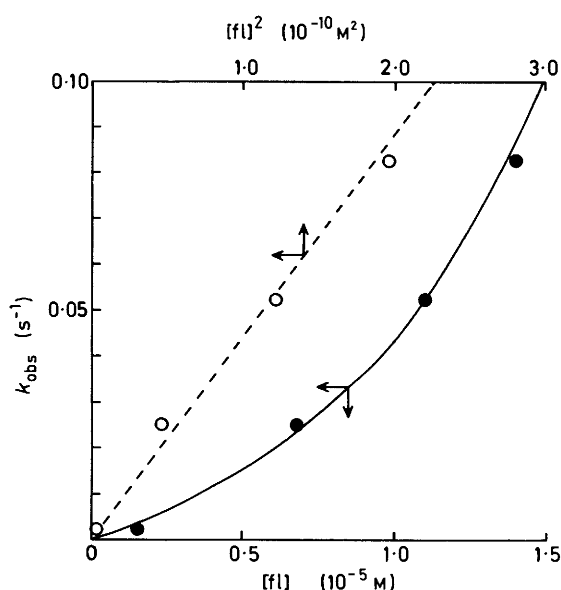
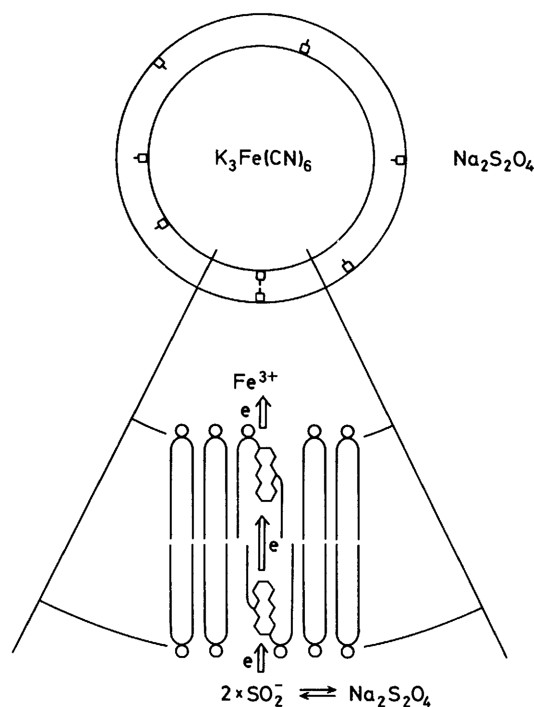


Figure 3. Dependence of k_{obs} on the concentration of flavolipid



Scheme 3.

reduction of fl^{ox} to fl^{red} proceeded further to give an accumulation of up to 80 mol% of fl^{red}. This suggests that the rate of the fl^{red}(out)→fl^{ox}(in) electron-transfer step becomes faster than the interphasic electron efflux into the internal aqueous phase ($J_{m\rightarrow in}$). In order to focus on the fl^{red}(out)→fl^{ox}(in) electron-transfer step, the reaction conditions in the following were chosen so that the concentration of $Na_2S_2O_4$ was 4.2mM (the saturated range) and the concentration of the flavolipid was changed from 1.5 to 14 μM (*i.e.* less than 70 μM). The observed rates were found to depend on the 2nd power of the flavolipid concentration (see Figure 3).

The second-order dependence with respect to the flavolipid concentration strongly supports the view that the electron transfer from fl^{red}(out) to fl^{ox}(in) in the bilayer membrane plays a key role in transmembrane electron transfer. Here two molecules of flavolipids are thought to associate for at least a short period to form an electron channel, through which electrons are transported. The carrier mechanism by a flip-flop of flavolipids can account for neither the observed molecularity nor the transport rate. The flip-flop rate is too slow to account for the electron transport process of 10^{-1} s^{-1} here observed (Scheme 3). The present electron transport rate constants are compared with those in the literature (Table 4), in which transmembrane electron transport is observed.

The pseudo first-order rates are reported at different mediator concentrations, so making direct comparisons difficult. Since exact concentration dependences were not obtained in all cases, k_{obs}/[mediator] values were compared in order to get a rough comparison. As shown in Table 4, the apparent electron transport rate for the flavolipid is much faster than those observed in any liposomes modified with ferrocene, benzoquinone,[13] *N,N'*-dibutylviologen (the most efficient electron carrier of all the alkyl viologens reported[12]), or cytochrome-c which plays a key role in the natural electron-transfer systems. The liposomes modified with ubiquinone-10, plastoquinone,[28] and cytochrome-c_3 [19] exhibited the fastest transmembrane electron transfer. All of the last listed class of mediators are important oxido-reduction centres in the respiratory chain of the mitochondria or the photosynthetic electron transfer of chloroplast. The present artificial flavolipid

Table 5. Reduction rate constants of $K_3Fe(CN)_6$ in DPPC–flavoliposome at various temperatures[a]

Concentration of flavolipid (mM)	Temp. (°C)	k (s^{-1})
0	10	0.001
0	25	0.001
0	40	0.002
0.02	10	0.007
0.02	20	0.018
0.02	25	0.065
0.02	30	0.19
0.02	35	0.55
0.02	40	1.57
0.02	50	1.62

[a] pH 7.0, concentration of $K_3Fe(CN)_6$ = 0.25mM, concentration of $Na_2S_2O_4$ = 4.2mM.

competes with those natural systems as the fastest member of the electron-transporting catalyst across the bilayer membrane.

Control of the Rate of Transmembrane Electron Transport by Thermally Induced Phase Transition.—Dipalmitoylphosphatidylcholine (DPPC) in place of the egg lecithin was used to prepare artificial liposomes showing a phase transition at a definite temperature (T_c). This liposome was successfully modified with the present flavolipid. The phase transition temperature of the flavoliposome [$^{(o)}$|Fl-Lip(DPCC)|$^{(i)}$] containing the flavolipid in 3 mol% quantity was determined as 38—39 °C by differential scanning calorimetry (d.s.c.) measurement and a pre-transition peak was also observed at 35 °C. The d.s.c. measurement was obtained after centrifugation without a gel filtration to remove the multilayer liposomes, in which, however, less than 5% of the total lipid molecule was contained. The data are in the range of T_c values (35—40 °C) reported for the parent DPPC liposome.[29] No other peak corresponding to the phase transition of flavolipid domain was detected in the temperature range of 8—65 °C.

The DPPC-liposome modified with the flavolipid containing ferricyanide in its internal aqueous phase [$^{(o)}$|Fl-Lip-(DPPC)|$^{(i)}K_3Fe(CN)_6$] was treated with a reducing agent by the addition of aqueous $Na_2S_2O_4$ to its external aqueous phase. The decrease of the internal ferricyanide concentration was traced by monitoring the absorbance at 425 nm. A rapid reduction of fl^{ox} to fl^{red} was observed and this very rapid conversion was followed by a slower pseudo first-order decrease of ferricyanide. This kinetic behaviour was similar to that observed for the liposome derived from the egg lecithin. The pseudo first-order rate constants thus obtained in the range 10—50 °C are summarized in Table 5. As shown in Table 5, the electron transport rate was very slow below the T_c and increased dramatically at the temperature slightly lower than the T_c and reached a limiting rate which corresponded to that observed for the egg lecithin liposome functionalized with the flavolipid. Thus the rate enhancement below and above the T_c amounted to *ca.* 10^2. The electron transport rate across the DPPC membrane without modification by the flavolipid was observed to be as negligibly small as 10^{-3} s^{-1} in the temperature range examined. In this case, no abrupt change of the transport rate was observed near the T_c. Therefore, the dramatic enhancement of the electron transport rate here observed can reasonably be related to an increase of the lateral diffusion rate of the flavolipid in the phospholipid bilayer. The lateral diffusion rate of phospholipids in the bilayer membrane has been reported to increase by a factor of (1.4—5.5) × 10^2 above the T_c than below the T_c by using a technique of a fluorescence recovery after photobleaching.[30] The increased rate of the lateral diffusion of lipid molecules may lead to an increase of collision frequency for the two flavolipid molecules, one at the inner half layer and the other at the outer half layer of the membrane. In other words, the flavolipids provide a 'transient channel' (Scheme 3), through which electrons are transferred across the membrane. It has been reported previously that the permeability of inorganic as well as organic materials through the bilayer membrane is controlled by the temperature.[31] However, all of these permeation controls were interpreted by a 'leakage' through a disordered region of the bilayer membrane. In contrast to such a mechanism, the present system provides the first demonstration that the electron channel controls the rate of electron transport across the membrane by a gel-liquid crystal-phase transition.

Experimental

Instruments.—^{1}H N.m.r. spectra were obtained with a JEOL JNM PMX 60SI NMR spectrometer, a JEOL JNM FX90Q FT NMR spectrometer or a JEOL JNM GX400 spectrometer. Mass spectra were obtained with a JEOL JMS-DX 300 mass spectrometer. I.r. spectra were recorded on a Hitachi model 260-50 spectrophotometer. Microanalyses were performed at the Microanalytical Centre of Kyoto University. Electronic absorption spectra were measured with either a Union SM-401 high sensitivity spectrometer or a Hitachi U-3400 spectrophotometer. Sonication was performed with an ultrasonic disruptor, Model UR-200P (Tomy Seiko). Centrifugation was carried out with a refrigerated centrifuge, Tomy Seiko Model RS-20III. pH Measurement was performed on a Toa pH meter, Model HM 5-ES instrument.

Materials.—Unless stated otherwise, all reagents and chemicals were obtained commercially and used without further purification. The egg yolk lecithin was carefully purified according to the literature method[32] and stored at −75 °C under argon in the dark. Dry dimethylformamide was obtained by stirring over BaO at room temperature overnight, followed by distillation under reduced pressure under argon. Tetrahydrofuran was purified by refluxing first over sodium for 12 h then with lithium aluminium hydride for 1 h, followed by distillation under nitrogen. Dry triethylamine was obtained by stirring over potassium hydroxide overnight, refluxing with calcium hydride for 5 h, followed by distillation under nitrogen.

N-*Decyl-2-nitroaniline* (**2**).—A mixture of 1-chloro-2-nitrobenzene (27.5 g, 0.18 mol), decylamine (25 ml, 0.13 mol), and anhydrous sodium acetate (15.2 g, 0.19 mol) was stirred at 110 °C for 24 h. After cooling to room temperature, the orange coloured mixture was diluted with water (40 ml) and diethyl ether (60 ml) and the ether layer separated. The aqueous layer was extracted with further ether (2 × 60 ml) and the combined ether extracts were dried ($MgSO_4$) and evaporated; excess of 1-chloro-2-nitrobenzene was then removed by distillation under reduced pressure (68—72 °C/1 mmHg). Further distillation yielded the title compound (25.5 g, 73%), b.p. 145—150 °C (1 mmHg); δ_H(60 MHz; $CDCl_3$; standard Me_4Si), 0.73—1.05 (3 H, t-like, CH_3), 1.05—1.90 [16 H, m, $(CH_2)_8$], 3.27 (2 H, q-like, CH_2NH), 6.82 (1 H, dd, J_{65} 8 Hz, J_{64} 2 Hz, 6-H), 6.58 (1 H, ddd, $J_{43} = J_{45}$ = 8 Hz, J_{46} 2 Hz, 4-H), 7.41 (1 H, ddd, $J_{54} = J_{56}$ = 8 Hz, J_{53} 2 Hz, 5-H), 7.86—8.23 (1 H, br s, NH), 8.13 (1 H, ddd, J_{34} 8 Hz, J_{35} 2 Hz, 3-H).

10-*Decylisoalloxazine* (**3**).[33]—*N*-Decyl-2-nitroaniline (13.9 g, 49.9 mmol) was dissolved in absolute ethanol (80 ml) and to this

solution was added platinum oxide (0.15 g). The catalytic hydrogenation was conducted with vigorous stirring at ambient temperature and pressure in the dark for 10 h. When the calculated amount of hydrogen gas (3.6 l, 0.15 mol) had been absorbed, 1M HCl (40 ml) was added to the reaction mixture. Platinum powder was filtered off and to the filtrate was added alloxan monohydrate (9.2 g, 57.5 mmol); the mixture was then refluxed in the dark for 15 min. After the mixture had cooled to 0 °C, the resulting brown precipitate was filtered off and washed with 5 ml of cold ethanol to give a yellow-green solid (5.7 g). The filtrate, on concentration (to 30 ml), gave a second crop of product (2.3 g); total yield of crude 10-decylisoalloxazine amounted to 49%. An analytical sample was recrystallized from ethanol, m.p. 245—247 °C; δ_H(90 MHz; solvent [$^2H_6$]DMSO; standard Me_4Si) 0.73—1.03 (3 H, t-like, CH_3), 1.33—1.96 [16 H, m, $(CH_2)_8$], 4.40—4.76 (2 H, t-like, CH_2N), and 7.46—8.26 (4 H, m, isoalloxazine ring); $\nu_{max.}$ 3 580 and 3 540 (NH) and 1 720 and 1 670 cm^{-1} (CO) (Found: C, 67.47; H, 7.21; N, 15.65%; M^+, 354. $C_{20}H_{26}N_4O_2$ requires C, 67.77; H, 7.39; N, 15.81%; M, 354).

10-Decyl-3-ethoxycarbonylmethylisoalloxazine (**4**).[34]—To a mixture of crude 10-decylisoalloxazine (5.0 g, 14.1 mmol), powdered anhydrous potassium carbonate (5.9 g, 42.7 mmol), and dry dimethylformamide (60 ml) was added dropwise, ethyl bromoacetate (5.2 ml, 46.9 mmol); the mixture was then stirred at 40 °C for 6 h under argon in the dark. Dimethylformamide and the excess of ethyl bromoacetate were distilled off under reduced pressure and to the residual brown solid were added dichloromethane (120 ml) and 1M HCl (100 ml). The organic layer was separated, washed with water (2 × 20 ml), dried ($MgSO_4$), and evaporated to give a crude brown product (6.7 g). Recrystallization of this twice from ethanol yielded the pure yellow product (3.8 g, 61%), m.p. 160—163 °C; $\delta_H(CDCl_3)$ 0.73—1.03 (3 H, t-like, CH_3), 1.27 (3 H, t, J 7 Hz, OCH_2CH_3), 1.03—2.08 [16 H, m, $(CH_2)_8$], 4.24 (2 H, q, J 7 Hz, OCH_2CH_3), 4.50—4.90 (2 H, t-like, CH_2N), 4.83 (2 H, s, NCH_2CO_2), and 7.43—8.42 (4 H, m, isoalloxazine ring); $\nu_{max.}$ 1 735, 1 715, and 1 660 cm^{-1} (CO) (Found: C, 65.6; H, 7.3; N, 12.5%; M^+, 440. $C_{24}H_{32}N_4O_4$ requires C, 65.43; H, 7.32; N, 12.72%; M, 440).

3-Carboxymethyl-10-decylisoalloxazine (**5**).[34]—10-Decyl-3-ethoxycarbonylmethylisoalloxazine (3.15 g, 7.14 mmol) was dissolved in concentrated HCl (12 ml) and the solution was stirred at room temperature for 36 h in the dark. It was then poured into cold water (400 ml) and the yellow precipitate so formed was filtered off, washed with water (3 × 50 ml) and dried *in vacuo* [at room temperature, for 24 h (0.1 mmHg) over P_2O_5] to yield the yellow carboxylic acid (2.68 g, 91%). The analytical sample was recrystallized from ethanol, m.p. 88—90 °C; δ_H([$^2H_6$]DMSO) 0.71—1.01 (3 H, t-like, CH_3), 1.03—2.00 [16 H, m, $(CH_2)_8$], 4.38—4.83 (2 H, t-like, CH_2N), 4.58 (2 H, s, NCH_2CO_2), and 7.58—8.33 (4 H, m, isoalloxazine ring); $\nu_{max.}$ 3 450 (OH) and 1 750 and 1 655 cm^{-1} (CO) (Found: C, 64.1; H, 6.9; N, 13.5%; M^+, 412. $C_{22}H_{28}N_4O_4$ requires C, 64.06; H, 6.84; N, 13.58%; M, 412).

3-(10-Decylisoalloxazyl)acetic Anhydride (**6**).[22]—3-Carboxymethyl-10-decylisoalloxazine (0.54 g, 1.3 mmol) thus obtained was dissolved in dry THF (8 ml) and to the solution was added dry triethylamine (0.25 ml, 1.5 mmol). The resulting solution was cooled to −20 °C and a solution of freshly distilled ethyl chloroformate (0.15 ml, 1.6 mmol) dissolved in dry THF (5 ml) was added dropwise with external cooling during 15 min. The mixture was stirred further for 2 h and then warmed to room temperature and stirred for an additional 1 h. The mixture was again cooled to −20 °C and 3-carboxymethyl-10-decylisoalloxazine (0.54 g, 1.3 mmol) in dry THF (8 ml) and dry triethylamine (0.25 ml, 1.5 mmol) in dry THF (3 ml) were added dropwise simultaneously. The reaction mixture was stirred at room temperature overnight and then evaporated under reduced pressure. The crude product was dissolved in chloroform (150 ml) and the solution was washed with water (50 ml), dried ($MgSO_4$), evaporated, and dried [room temperature for 24 h (0.1 mmHg)] to afford the yellow anhydride (0.96 g, 92%). The product was used for the next reaction without further purification; $\nu_{max.}$ 1 830, 1 760, 1 710, and 1 660 cm^{-1} (CO).

1-Myristoyl-2-(10-decylisoalloxazin-3-yl)acetyl-L-α-phosphatidylcholine (**1**).[22]—Myristoyl-L-α-lysophosphatidylcholine (88 mg, 0.19 mmol) and the anhydride (**5**) (308 mg, 0.38 mmol) were dissolved in dry chloroform (10 ml). To the mixture was added 4-(dimethylamino)pyridine (50 mg, 0.41 mmol) and the mixture was carefully degassed with argon and then stirred at 45 °C in the dark for 36 h. Evaporation of solvent under reduced pressure gave a residual yellow solid which dissolved in $CHCl_3$–CH_3OH–H_2O (4:5:1, v/v; 5 ml) and the solution was passed through an AG-501-X8(D) column (1 cm diam. × 20 cm). The lipid remaining on the adsorbent was removed with eluant (100 ml) of the above composition. The fractions containing the product were then combined and the solvent was removed under reduced pressure. The crude lipid obtained as a yellow solid was further chromatographed on a silica gel column (2.5 cm diam. × 25 cm) with $CHCl_3$–CH_3OH as eluant (gradient from 10:0 to 5:5). Fractions containing the main product [R_F 0.30, $CHCl_3$–CH_3OH–H_2O (56:25:4, v/v)] were collected. Removal of the solvent under reduced pressure followed by drying *in vacuo* (0.1 mmHg) for 24 h at room temperature afforded the yellow flavolipid (108 mg, 68%). This was further purified by reprecipitation from $CHCl_3$–hexane; $\delta_H(CDCl_3)$ 0.87—0.89 (6 H, t-like, CH_3 × 2), 1.23—1.86 [38 H, m, (CH_2)], 2.30 (2 H, t, J 8 Hz, CH_2CO_2), 3.39 [9 H, br s, $-N^+(CH_3)_3$], 3.84—5.28 (13 H, m, CH_2O-3, HCO, CH_2N, and NCH_2CO_2), and 7.64—8.32 (4 H, m, isoalloxazine ring); $\nu_{max.}$ 1 730, 1 705, and 1 660 cm^{-1} (CO) (Found: C, 57.0; H, 8.5; N, 7.35; P, 3.25. $C_{44}H_{72}N_5O_{10}P{\cdot}4H_2O$ requires C, 56.58; H, 8.63; N, 7.50; P, 3.32%); m/z (f.a.b.) 862 [$(M + H)^+$]; $\lambda_{max.}(CHCl_3)$ 290 (42 000), 336 (9 500), 444 (9 900), and 472sh nm (6 700).

Preparation of Artificial Single-wall Bilayer Liposome.—Artificial egg lecithin liposomes functionalized with the flavolipid were prepared according to a slightly modified procedure of ours.[19] A solution of 75—80 mg of the carefully purified egg yolk lecithin and an appropriate amount of the flavolipid in $CHCl_3$ (15 ml) was gently evaporated under reduced pressure under argon. The resulting thin film was dried *in vacuo* for 8 h in the dark at room temperature. Into the dried lipid film was added 5mM aqueous Tris HCl (pH 7.0; 5 ml) and the lipid film was suspended in the solution. The suspended mixture chilled in an ice-bath was sonicated in a box filled with argon for 5 min. The sonication was repeated three times at an interval of 10 min. The resulting mixture was centrifuged (1.9 × 10^3 r.p.m.) for 15 min at 4 °C and the yellow supernatant was applied to a Sepharose 4B column (1.0 cm diam. × 40 cm), which was eluted with 5mM aqueous Tris HCl buffer (pH 7.0) at 4 °C. Single-wall liposomes were obtained from the eluates between 34 to 45 ml (±2 ml). Artificial liposomes functionalized with the flavolipid in its membrane phase and containing $K_3Fe(CN)_6$ in its internal aqueous phase were prepared starting from the Tris HCl solution containing 0.75M of $K_3Fe(CN)_6$.

Artificial dipalmitoylphosphatidylcholine, DPPC (Sigma) liposomes functionalized with the flavolipid and containing $K_3Fe(CN)_6$ in its internal phase were prepared by the same method as described above except that the suspended solution of the lipid film was sonicated five times with external cooling in an argon box.

Reduction of Flavolipid Incorporated into the Bilayer Membrane of the Functionalized Liposome by $Na_2S_2O_4$.—A freshly prepared liposome solution (2.0 ml) [$^{(o)}$|Fl-Lip|$^{(i)}$], kept at pH 7.0 by the use of a 5mM Tris•HCl buffer, was placed in a 10-mm quartz cell equipped with a three-way stopcock. The solution was deaerated through careful substitution of air by argon *via* evacuation and the introduction of argon at room temperature. The procedure was repeated 20 times for each sample. Into the deaerated liposome solution at 25 °C, 0.2 ml of freshly prepared $Na_2S_2O_4$ (0.052—1.04mM) [by titration with $K_3Fe(CN)_6$(aq)] was added using a specially designed syringe.[19] The reduction of flavolipid present in the bilayer membrane phase was traced by monitoring the change of the absorbance at 444 nm.

Reduction of $K_3Fe(CN)_6$ *in the Internal Aqueous Phase of the Functionalized Liposome by* $Na_2S_2O_4$.—Reduction of internal $K_3Fe(CN)_6$ by $Na_2S_2O_4$ was carried out by using the same method as described above. The reduction of internal $K_3Fe(CN)_6$ was traced by the intensity change of the visible adsorption at 425 nm. That the adsorption of the flavolipid at 425 nm did not seriously interfere with the above measurements, was supported by the following evidence. (i) The reduction rate of flavolipid was independently estimated by monitoring the intensity change of the characteristic flavin chromophore adsorption at 472 nm. Thus the net decrease of $K_3Fe(CN)_6$ was easily subtracted from the overall change. (ii) The change in the $K_3Fe(CN)_6$ concentration was measured in the region where the concentrations of the oxidized and the reduced form of flavolipid were practically constant in the steady state.

Differential Scanning Calorimetry.—The thermal analysis of the DPPC liposome functionalized with the flavolipid was performed with a Seiko DSC calorimeter (I & E Mode DSC-10 calorimeter combined with a Seiko SSC/580 thermal controller). The flavoliposome employed was obtained through centrifugation after sonication without gel filtration. The sample (50 µl, the total lipid concentration was *ca.* 10 mg/ml) was sealed in an aluminium pan with a sealer. The heating rate was 1 °C/min. The scanning temperature range was 8—65 °C.

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