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Detection of highly oriented aggregation of L-glutamic acid-derived lipids in dilute organic solution†

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Aggregation structures in organic gels and xerogels formed from L-glutamic acid-derived lipids were investigated by scanning and transmission electron microscopies, X-ray analyses, and ¹H NMR and IR spectroscopic methods. These analyses showed that the gels were produced through the formation of highly oriented aggregates based on a single layer and a remarkable development of their fibrous morphology. We also describe how the critical aggregation concentration can be observed at a concentration below the critical gel concentration by using a dye-complexation method with a cyanine dye, NK-77.

1. Introduction

Hydrogels have been investigated for biomedical uses and related applications such as drug delivery, controlled-release and enzyme immobilization. If the gel system were developed to organic media, their possible applications would be further expanded. Recently, it has been reported that some special lipids form organic gels in organic solvents. These findings are very interesting because of the fact that lipids produce highly oriented aggregates like aqueous lipid membranes. The driving forces for the aggregation are much different, dependent upon their chemical structures. For example, we have shown that L-glutamic acid-derived lipids **1a** and **2** produce organic gels through formation of lipid aggregation by hydrogen bonding interaction among the amide moieties [1–5]. Similar observations were made by Yamada *et al.* [6] and Yasuda *et al.* [7]. On the other hand, Lin *et al.* [8] and Murata *et al.* [9, 10] have used π - π^* interaction as a driving force for the aggregation and Ishikawa *et al.* [11, 12] have focused on the strong solvophobic property of perfluoroalkyl groups. The most attractive property of these organic gels is related to their micro-environment. In the case of L-glutamic acid-

derived lipids, the chirality was remarkably enhanced through the molecular orienting effect, and the enhanced chirality induced distinct enantioselectivity for the elution of amino acids through them [2, 4]. In addition, spiropyran- or azobenzene-containing lipids are attractive as light-responsive organic gel-forming materials [5]. Shinkai *et al.* have also synthesized organic gel-forming lipids with head groups as functional organic media [13, 14].

Formation of organic gels can be visually observed. For example, lipid **1a** shows a sol-gel transition at concentrations between 0.1 and 1mM in benzene solution. However, we estimate that the lipid forms aggregates even in the sol-forming concentration range and so to evaluate the exact critical aggregation concentration is very important because the suprafuctions are probably brought about through molecular aggregation. In this paper, we study the aggregation structures of lipid **1a** and **2** in organic solvents using scanning and transmission electron microscopies, X-ray analyses, ¹H NMR and IR spectroscopic methods and a dye-complexation method.

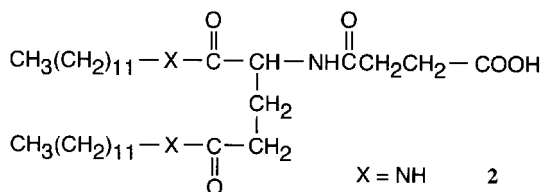
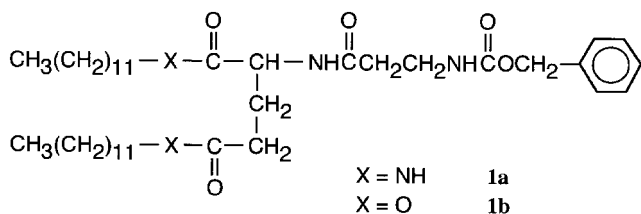
2. Experimental

L-Glutamic acid-derived lipids **1a**, **1b** and **2** were synthesized according to the procedure reported previously

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† Functional organic gels.

[3]. Cyanine dye NK-77 (Nippon Kanko Shikiso Laboratory) was used without further purification.



Organic gels were prepared by dissolving lipids in hot organic solvents and allowing the solution to stand at room temperature.

Differential scanning calorimetry (DSC), UV-vis spectrophotometry and circular dichroism spectrophotometry were carried out with a Seiko I & E DSC-10/SSC-580, a JASCO UVIDEC-660 and a JASCO J-500C, respectively. Electron micrographs were obtained using a JEOL JSM-5310LV or JEOL JSM-1200EX, and X-ray diffraction patterns using a Rigaku Denki RAD-1.

Molecular structures were modelled by Sony-Tektronix CACHE-mechanics. The calculations were carried out until the energy changes were below $0.001 \text{ kcal mol}^{-1}$.

3. Results and discussion

3.1. Microscopic observation of aggregate morphology

When **1a** was dissolved in hot benzene at concentrations of 1mM and 10mM and the solutions were then allowed to stand at room temperature, the solutions produced clear gels, figure 1(a). Freeze-drying of this benzene gel provided a xerogel with almost no change in the volume, figure 1(b). Scanning electron microscopy (SEM) showed that the xerogel was constructed from a network of microfibrillar aggregates, figures 2(a) and 2(b). No similar gel formation was observed at concentrations below 0.1mM. This indicates that the critical gelation concentration (c_{gc}) lies between 1 and 0.1mM in benzene at room temperature. However, SEM observations showed that the white powders obtained by freeze-drying the 0.01 mM solution included microfibrillar aggregates, figure 2(c). These results indicate that lipid **1a** can form aggregates in benzene solution even in the sol state and thus that the c_{gc} does not agree with the critical aggregation concentration (c_{ac}).

In addition, figure 2(d) shows the SEM image of the powders obtained by recrystallization from a methanol

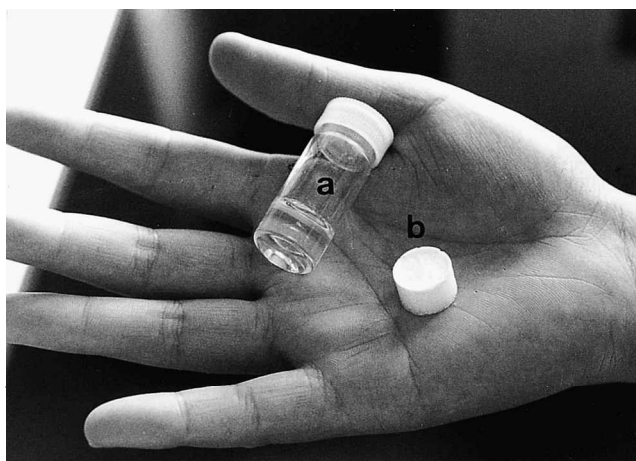


Figure 1. Photograph of a benzene gel (a) and a xerogel (b) containing 10mM of **1a**.

solution of **1a**. Only needle-like aggregates were observed. Therefore, the gel formation is attributable to the development of fibrous aggregates and subsequent formation of an aggregate network.

3.2. Aggregation structure in the xerogel

The xerogel from the **1a**–benzene gel (10mM) gave a sharp endothermic peak with a peak-top temperature (T_c) of 172.3°C by DSC. A very similar DSC thermogram was obtained from recrystallized **1a** ($T_c = 173.0^\circ\text{C}$). However, X-ray diffraction provided slightly different patterns: the xerogel provided a pattern with $2\theta = 2.86^\circ$ (30.7 \AA) and 21.64° (4.10 \AA), while the corresponding diffractions were observed at $2\theta = 3.40^\circ$ (26.7 \AA) and 21.46° (4.24 \AA) in the case of recrystallized **1a**. A difference was also detected by IR spectroscopy. The N–H stretching ascribed to amide bonding was observed at 3288 cm^{-1} in the xerogel and at 3298 cm^{-1} in recrystallized **1a**. In general, hydrogen bonding interactions among amide groups cause a shift to lower wave numbers [15]. On the basis of these observations, we believe that the fibrous aggregates of **1a** in the xerogel are based on a single-layer structure and that the stacking among the lipids is rather tighter compared with that for the recrystallized **1a** aggregates (figure 3).

3.3. Driving force for molecular aggregation

The benzene gel of **1a** also provided an endothermic peak ($T_c = 68^\circ\text{C}$) in the DSC thermogram. This remarkable decrease of T_c (from 172.3 to 68°C) can be explained by proposing that the **1a** aggregates are solvated with benzene, but are still in a highly-oriented state like a crystalline state. In support of this, we have reported that the **1a**–benzene gel showed a remarkable enhancement of optical activity [1, 2]. It is known that the formation of highly oriented structures is accompanied

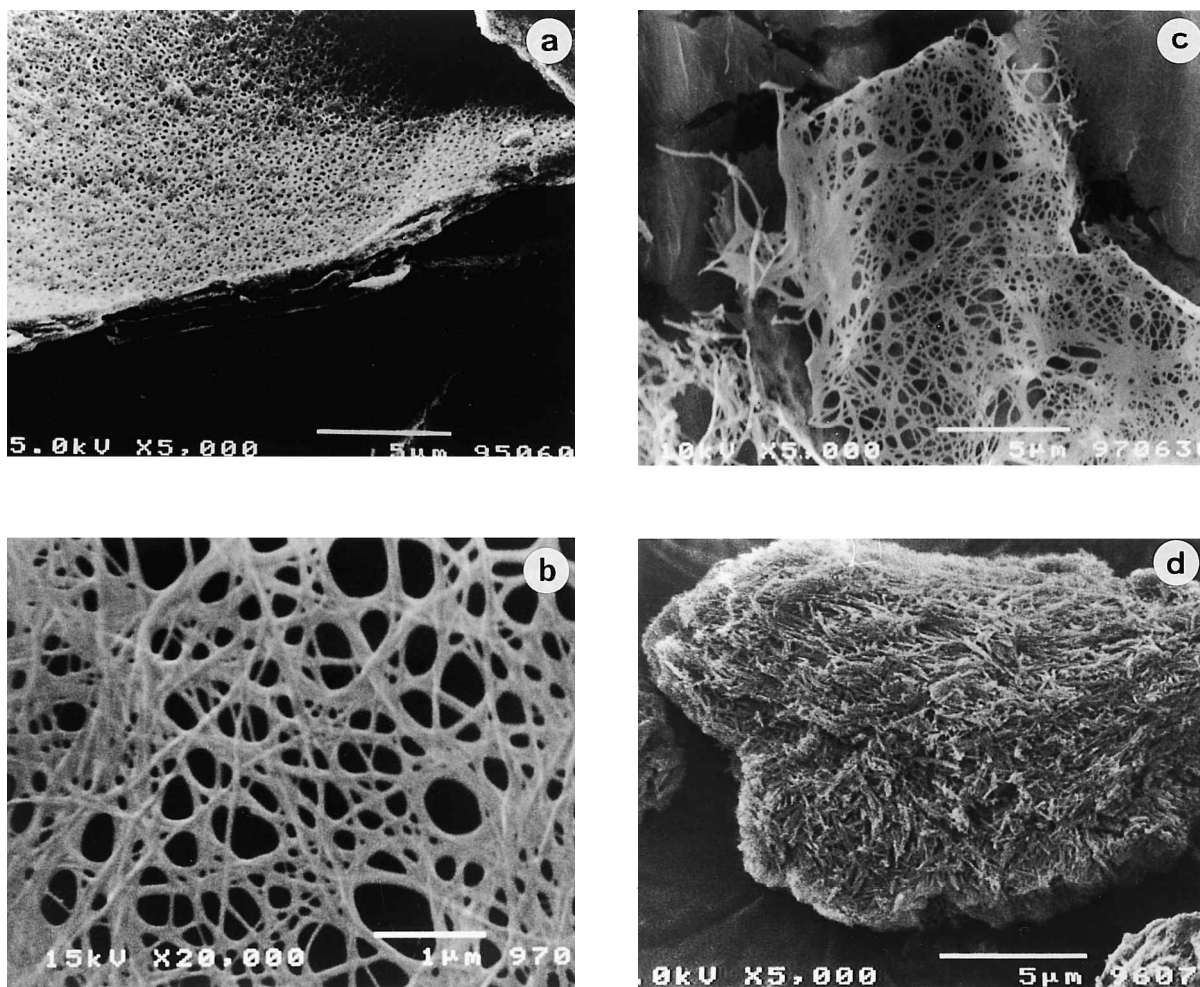
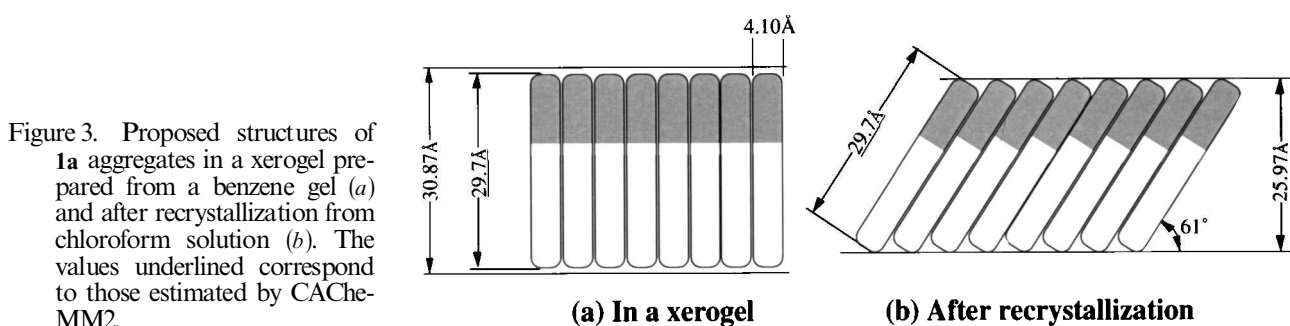


Figure 2. SEM images of **1a** aggregates in xerogels, ((a) and (b) 10mM, and (c) 0.01mM); and after recrystallization from chloroform solution, ((d) 10mM).



by chirality enhancement in the case of chiral lipids in aqueous systems [16–19]. Therefore, we consider that the **1a**–benzene gel is constructed from developed fibrous aggregates with highly oriented structures similar to that observed in the xerogel.

We have also discussed the driving force for aggregation in benzene. Results of calculations have indicated that the three amide moieties around the *L*-glutamic acid

moiety can act as hydrogen bonding interaction sources for formation of the aggregate network (figure 4). In support of this, when an amount of trifluoroacetic acid, equimolar to **1a**, was added to the benzene solution as an inhibitor of hydrogen bonding interactions, gel formation was not observed and there was a remarkable decrease of optical activity [1, 3]. In the present study, ^1H NMR spectroscopy was applied to detect hydrogen bonding

interactions. Since the protons of benzene have similar chemical shifts to those of the amide bonds, a chloroform–tetrachloromethane mixture was used as solvent. As a result, the peaks at 5.90 and 6.86 ppm observed with a chloroform solution shifted to 5.95 and 7.03 ppm with addition of 50 vol % of tetrachloromethane. These protons are ascribed to the amide protons of the dodecyl-amide moieties. In general, hydrogen bonding interaction among amide groups induces down field shifts of the amide protons [20]. On the other hand, the addition of tetrachloromethane induced a sol-to-gel transition. In addition, the lipid **1b** corresponding to an ester type lipid did not form a gel in benzene and tetrachloromethane. These results strongly support the view that the two amide groups of the didodecyl amide moieties act as hydrogen bonding interaction sources for lipid aggregation.

3.4. Evaluation of critical aggregation concentration

As described in §3.1, the **1a**–benzene sol (prepared at a concentration below 0.1mM) provided a SEM image including fibrous aggregates. This shows that the critical gelation concentration ($0.1\text{mM} < c_{gc} < 1\text{mM}$) does not agree with the critical aggregation concentration (c_{ac}). The disagreement was observed with several organic

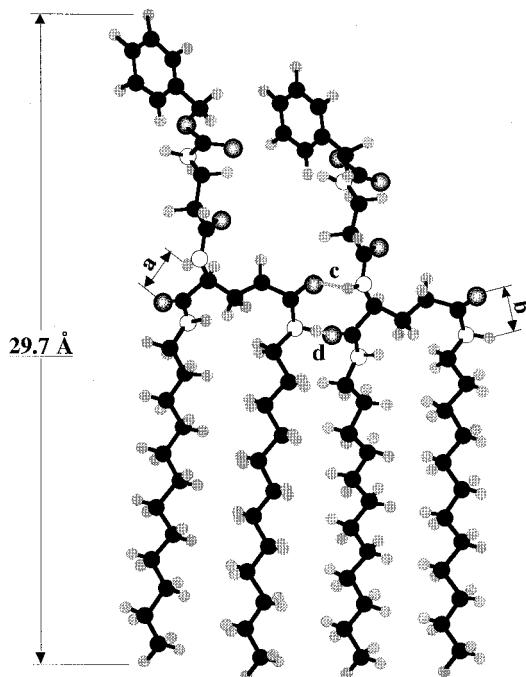


Figure 4. Proposed structures showing the hydrogen bonding interaction around three amide groups of lipid **1a**. The values (centre-to-centre distances between the atoms) were estimated with CACHE-MM2 [4]: the bonds *c* and *d* represent hydrogen bonds. The distances between the two atoms at *a* and *b* were determined to be 2.36 and 2.22 Å, respectively.

solvent systems. Toluene, *p*-xylene, cyclohexane and tetrachloromethane, as well as benzene gave the formation of clear gels at 1mM, but no similar gel formation was

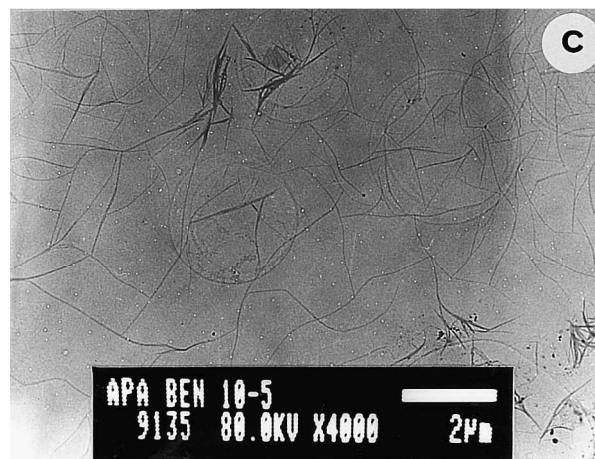
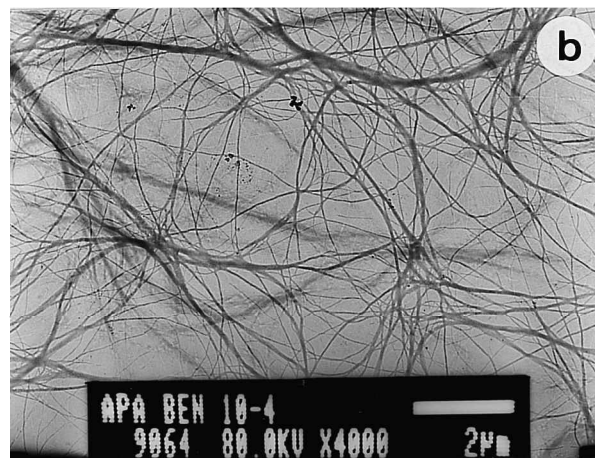
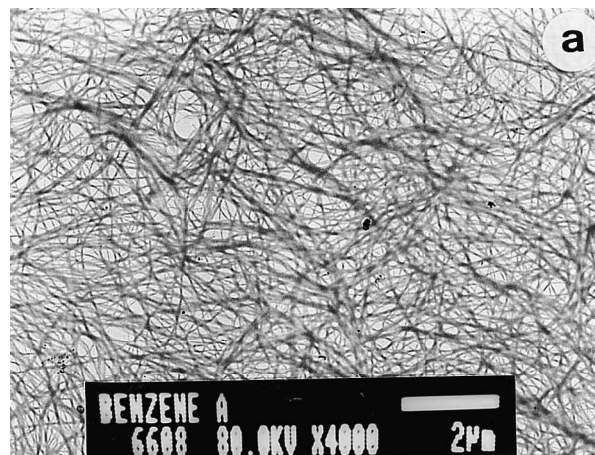


Figure 5. TEM images of **1a** aggregates in the cast films from benzene: (a) 1mM, (b) 0.1mM, and (c) 0.01mM.

observed with tetrahydrofuran, chloroform and ethanol at this concentration. However, as shown in the transmission electron micrographs (TEM) of figures 5 and 6,

these organic solvents did provide microfibrillar aggregates of **1a**, although their detailed morphologies were different in their lengths and widths.

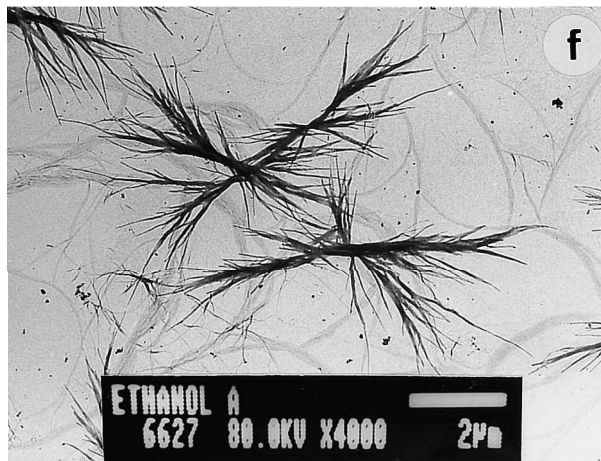
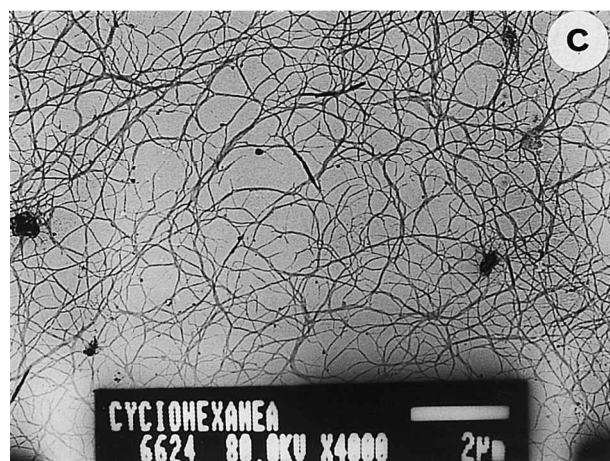
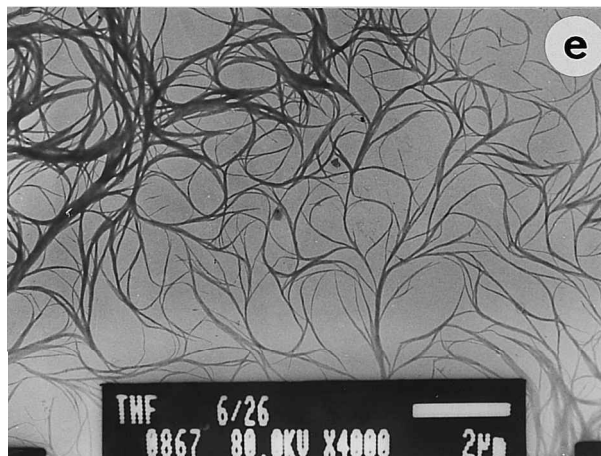
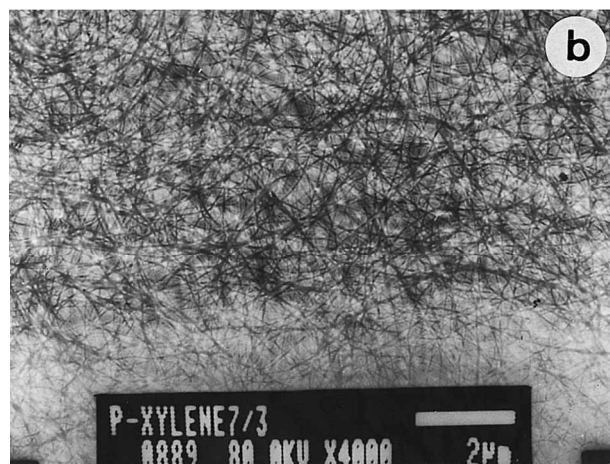
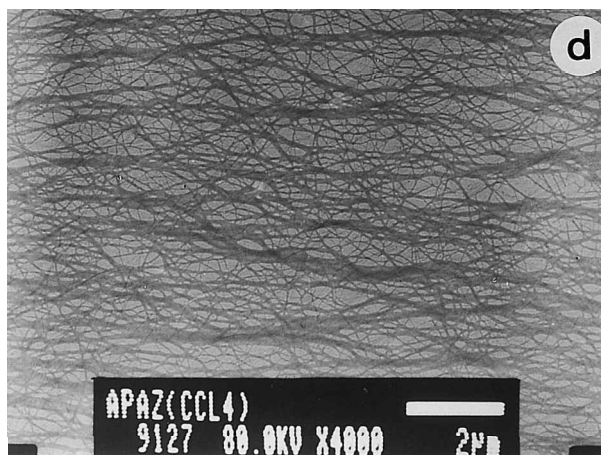
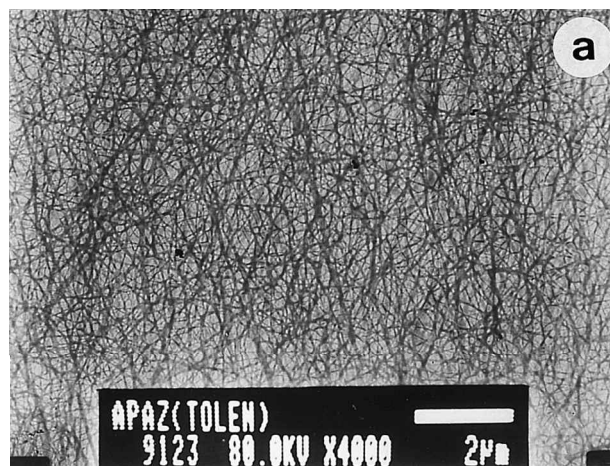


Figure 6. TEM images of **1a** aggregates in the cast films from (a) 1mM toluene, (b) *p*-xylene, (c) cyclohexane, (d) tetrachloromethane, (e) tetrahydrofuran and (f) ethanol solutions.

To estimate the *cac*, we focused on a cyanine dye, especially NK-77. The cyanine dyes have large extinction coefficients and they are very sensitive to micro-environment [21, 22]. However, we detected no significant spectral change for the **1a** and NK-77 mixed system. This can probably be explained by the fact that **1a** is non-ionic while NK-77 is cationic. Therefore, lipid **2** was used for the evaluation of *cac* instead of **1a**. Lipid **2** has a carboxylic acid head group and can produce gels in several organic solvents.

Figure 7 shows the concentration effect of **2** on the visible spectra of NK-77 in benzene. As shown in figure 8, the extinction coefficient at 570 nm decreased remarkably for concentrations above 0.03 mM. At these concentrations, **2** did not produce a gel state. Therefore, we

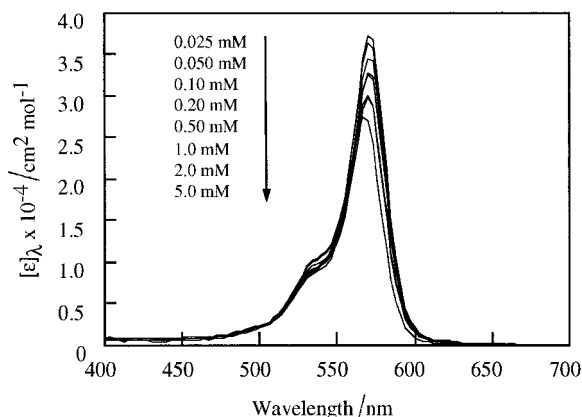


Figure 7. Concentration effect of lipid **2** on the visible spectra of NK-77 (0.025mM) at 25°C.

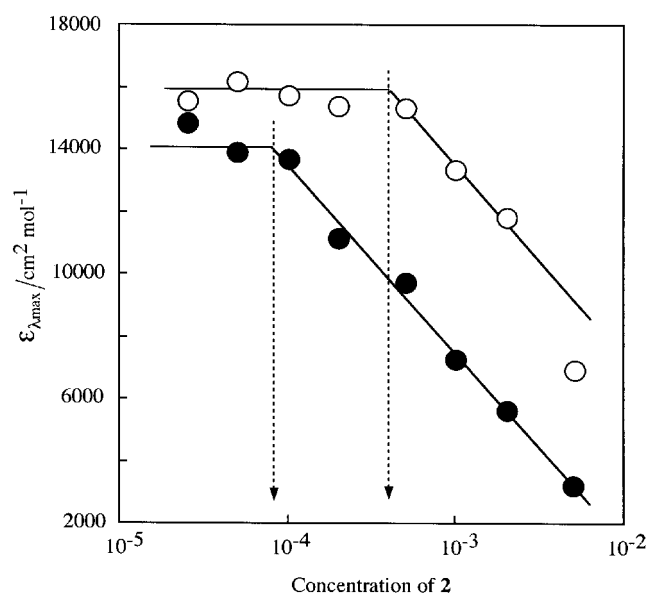


Figure 8. Concentration effects of lipid **2** on the extension coefficient (ϵ) at λ_{\max} of NK-77 (0.025mM) at 25°C. Solvent: toluene (●) and tetrahydrofuran (○).

Table. Critical aggregation concentration (*cac*) of **2** in various organic solvents at 25°C, $[\text{NK-77}] = 2.5 \times 10^{-5} \text{ M}$.

Solvent	$cac \times 10^3 \text{ mol l}^{-1}$
Benzene	0.03
Toluene	0.08
Tetrahydrofuran	0.6
Chloroform	0.9
Ethanol	> 2

consider that the change of the extinction coefficient is not related to the gel formation, but is brought about by the micro-environmental change effected by the concentration on the aggregates of **2**. According to the plots, we evaluated the *cac* using several organic solvents. As shown in the table, all the *cac* values are much smaller than their *cgc* values. And it should be noted that *cac* is much dependent on the solvent. The solvent order (benzene, toluene \ll tetrahydrofuran, chloroform \ll ethanol) of the *cac* can be explained by the order of polarity of the solvents. These results prove that lipid **2** can form highly oriented aggregates even at the sol-forming concentration.

4. Conclusion

This study has clarified the aggregation structures of the xerogel and the organic gel from L-glutamic acid-derived lipids by means of electron microscopy and X-ray analyses. ^1H NMR and IR spectroscopic studies have shown that hydrogen bonding interactions are included in the driving force for the molecular aggregation. We have also shown, by using electron microscopy and a dye-complexation method, that the critical aggregation concentration exists at a concentration below the critical gel concentration.

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