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Fusion between Jurkat cell and PEO-lipid modified liposome

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Abstract

Direct fusion between Jurkat cell and a liposome modified with poly(ethylene oxide)-bearing lipid (PEO-lipid) was examined using diphtheria toxin fragment A (DTA) as the probe. Only the DTA-loaded liposome modified with PEO-lipid($n = 32$) (n is the number of ethylene oxide units) exerted significant cytotoxicity against Jurkat cells, while liposomes lacking either the PEO-lipid or DTA did not. Liposomes modified by the PEO-lipid with shorter PEO chain($n = 5$ or 15) did not show any cytotoxicity, irrespective of their DTA-loading. The cytotoxicity was observed even in the presence of cytochalasin B, an inhibitor of endocytosis. Judging from these results, we concluded that the PEO-lipid($n = 32$)-modified liposome directly fused with plasma membrane of Jurkat cell.

Keywords: Liposome; Fusion; Jurkat cell; Diphtheria toxin fragment A; Poly(ethylene oxide)-bearing lipid; Cytochalasin B

1. Introduction

It is of importance to establish a method for efficient and direct introduction of biologically active and labile substances, e.g. plasmid, DNA, RNA, protein, or enzyme. Because, if such substances are introduced into cells via endocytosis, they are often digested by lysosomal enzymes in endosome, which results in complete disappearance of their biological activity. From this point of view, several methods for introduction other than endocytosis have been established; e.g. micro-injection [1], electroporation [2], utilization of erythrocyte ghost [3], lipofectin [4], and DEAE-dextran [5]. However, some of them require severe conditions for the survival of the target cells.

Compared with these methods, liposomal fusion demonstrates several merits. Firstly, the conditions required for liposomal fusion are not severe to target cells. Secondly, liposomal fusion does not induce a homophilic cell-cell fusion. Okada and his co-workers have developed a fusogenic liposome, which was modified by a fusion-inducing protein derived from

Abbreviations: BSA, bovine serum albumin; BSS, balanced-salt solution; DTA, diphtheria toxin A fragment; FCS, fetal calf serum; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PEO-lipid, poly(ethylene oxide)-bearing lipid.

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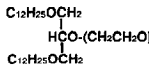

PEO-lipid (n = 5, 15, and 32)

Fig. 1. Chemical structure of PEO-lipids used in this work.

Sendai virus. This liposome successfully introduced biologically active materials into a target cell, e.g. diphtheria toxin A fragment (DTA), plasmids encoding DTA gene, thymidine kinase gene, or hepatitis B virus surface antigen gene [6–9]. However, this procedure requires much precaution for the handling of the Sendai virus.

On the way for searching a better method of liposome-cell fusion, we have recently found a fusogenic lipid, PEO-lipid($n = x$) (x is the number of ethylene oxide units, 5, 15, or 32; Fig. 1) [10]. A liposome, whose outer surface was modified with the PEO-lipid, was fusogenic to carrot protoplast [11,12] and HeLa cell [13]. In the present work, we attempt to adopt this fusogenic liposome for Jurkat cells (human T-lymphocyte origin) for the following reasons. Firstly, Jurkat cells grow in a suspension culture taking a way different from that of HeLa cells. Secondly, we expect that cells of lymphoid origin scarcely undergo endocytosis [14], which will be suitable for distinction between fusion and endocytosis. For genetic therapy of the immune system, this technique, introduction of bioactive materials into lymphocytes, must be of much importance. However, we have unexpectedly found that Jurkat cells showed uptake of PEO-lipid($n = 15$)-modified liposome by endocytosis [15].

DTA, a marker for fusion in this report, fully keeps the original activity of native diphtheria toxin. It shows strong cytotoxic activity by inactivating elongation factor 2 in cytosol of target cells [16]. One molecule of DTA kills a target cell, if it is introduced properly into the cytosol of a target cell. Lack of the receptor-binding domain of native diphtheria toxin makes DTA itself impotent to penetrate into cytosol, and subsequently nontoxic even at high concentration [17]. Therefore, the cytotoxicity of DTA-loaded liposome shows a good evidence of direct fusion between liposome and cytoplasmic membrane [18]. On the other hand, loss of DTA-dependent cytotoxicity proves that the toxin was internalized by endocytosis [15–21]. Using this excellent marker, we investigated the liposome-cell fusion between a PEO-lipid modified liposome and a Jurkat cell. In this article, we demonstrate that egg PC liposome modified with PEO-lipid($n = 32$), but not with PEO-lipid($n = 5$ or 15), fuses with a Jurkat cell.

2. Materials and methods

2.1. Materials

PEO-lipids, synthesized and fractionated as described elsewhere [10,11], were exactly the same samples as we have used in our previous works [11–13,15]. Egg yolk lecithin (egg PC) was purchased from Nippon Oil and Fats (Tokyo), and the purity was carefully checked before use. The concentration of phosphatidylcholine in liposomal suspension was determined by using a Phospholipid C-Test Wako (Wako Pure Chemicals, Osaka), based on choline content released from phosphatidylcholine by phospholipase D treatment. The following reagents and media were commercially available: cytochalasin B (C-6762) and bovine serum albumin (BSA, fatty acid free, A-6003) from Sigma (St. Louis, MO), RPMI1640 medium from Biowhittaker (Walkersville, MD), fetal calf serum (FCS) from HyClone Laboratories, (Logan, UT) and kanamycin sulfate (Meiji Seika, Tokyo). DTA was prepared as previously reported [6].

2.2. Preparation of liposome

Large unilamellar liposome (LUV) was prepared by reverse evaporation method [22] with minor revision. Briefly, 10 mg of egg PC was dissolved in 600 μl of a mixture of diethyl ether and dichloromethane (1: 2, by vol.). DTA (0.1 mg) was dissolved in 200 μl of a balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, and 10 mM Tris-HCl (pH 7.6)) and mixed with an egg PC ethyl ether solution. The resulting cocktail was thoroughly mixed on a Vortex mixer for 30 s and filtered through a PTFE membrane (pore size 0.2 μm, Advantec, Tokyo) to prepare a homoge-

neous emulsion. Most of organic solvent was removed by evaporation under reduced pressure (340 mmHg) without heating. After the mixture became gel, an additional Vortex mixing was carried out. Then 800 μl of BSS was added to the mixture, and the resulting suspension was evaporated under 700 mmHg for 2 h, followed by ten times extrusion through a polycarbonate membrane filter (pore size 0.2 μm, Costar, Cambridge, MA). After removal of unencapsulated DTA by gel filtration column chromatography (∅1.6 × 8 cm, Sepharose 2B, Pharmacia, Uppsala), the liposomal phospholipid content was determined by Phospholipid-C Test Wako and exactly adjusted to 1 mM. Dextran (Sigma D-9260; molecular weight, 9300) was employed as a model macromolecule to estimate encapsulation efficiency of DTA [23]. The encapsulation efficiency was determined to be 1.3 ± 0.4% by four independent experiments. We estimated that one vesicle was carrying approximately one DTA molecule under the conditions employed.

2.3. Preparation of PEO-lipid-modified liposome

For the PEO-lipid modification, 500 μl of a liposomal suspension was incubated with an ethanolic solution of a given amount of PEO-lipid for 60 min at 37°C. The final ethanol concentration was always kept below 1%. Unless otherwise stated, egg PC liposome was modified with 20 mol% of PEO-lipid(n = 32).

Amount of PEO-lipid reconstituted in the egg PC liposome was determined as follows. A given amount of PEO-lipid was added to a liposomal suspension (1 mM egg PC) and incubated for 1 h at 37°C. For removing non-reconstituted PEO-lipid, the mixture so obtained was ultracentrifuged at 35 000 rpm for 4 h on a Beckman XL-90. The liposomal pellet was collected and dissolved in ethanol for complete removal of salts. The amounts of phosphatidylcholine and PEO-lipid in pellet were determined by IATROSCAN (HK5, Iatron, Tokyo), a system for quantitative determination of lipids on silica sticks almost similarly to the case of TLC. The two lipids were clearly separated with the separation solvent mixture, chloroform/methanol/water (65:25:4, by vol.): R_f was 0.13 for PEO-lipid and 0.55 for phosphatidylcholines.

2.4. Interaction between liposome and Jurkat cell

Jurkat B6 (a clone of human lymphoblastoma of T-cell origin) cells were maintained in a RPMI1640 medium containing 10% fetal calf serum (FCS) and 60 μg/ml kanamycin sulfate. In all runs, Jurkat cells were incubated for 16 h at 37°C in RPMI1640 containing 1% FCS before exposure to the liposome. The Jurkat cells were then washed three times with PBS and re-suspended in PBS. The number of cell was adjusted to $6 \cdot 10^5$ cells/ml. Unless otherwise stated, this cell suspension (300 μl) was coincubated with 45 μl of a liposomal suspension (1 mM egg PC) at 37°C for 30 min with continuous shaking. The final concentration of DTA in the suspension was approximately 10 ng/ml. After the coincubation, the cell was separated from the liposome by washing twice with 0.5% FCS-containing PBS, re-suspended in 1 ml of a RPMI1640 medium containing 2% FCS, and cultured for one day on a 96-well flat-bottomed microtiter plate (MS-8096F, Sumitomo Bakelite, Osaka). Cell viability was determined by using the trypan blue staining test. All runs were tested in triplicate.

Of course, we have considered or tried not only the trypan blue assay but also methodologies using the MTT assay and the RI method. However, we could not obtain reasonable reliability by the latter methods in the present system, because (1) the cell

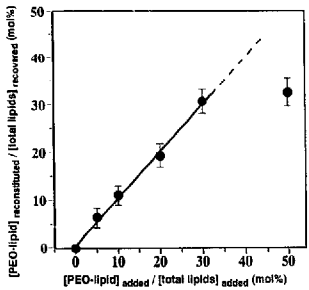

Fig. 2. Reconstitution of PEO-lipid(n = 32) into egg PC liposome.

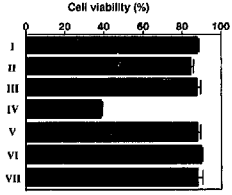

Fig. 3. Effect of DTA and PEO-lipid on cell viability. Vertical bars denote ±S.E. of the mean value for three separate determinations. I, Conventional liposome without DTA; II, 20 mol% PEO-lipid(n = 32)-modified liposome without DTA; III, conventional DTA-loaded liposome (DTA 10 ng/ml); IV, 20 mol% PEO-lipid(n = 32)-modified and DTA-loaded liposome (DTA 10 ng/ml); V, unencapsulated DTA (100 ng/ml) without liposome; VI, unencapsulated DTA (1000 ng/ml) without liposome; and VII, PBS as control.

growth was affected a little by PEO-lipid itself and (2) the repeated washing of the cell made it difficult to keep the number of spread cells constant. Therefore, to support and complement the results of the trypan blue assay, we carefully visualized the status of the cell by direct microscopic observation.

To completely inhibit endocytosis, Jurkat cells were treated with cytochalasin B, both before coincubation with liposomes as pretreatment and during coincubation with liposomes. First, Jurkat cells were incubated for 16 h at 37°C in RPMI1640 containing 1% FCS before exposure to liposome as described above. In Experiment A without cytochalasin B (control experiment of Experiment B), the cells were treated with 0.2% DMSO for the last 30 min of the incubation, washed four times with PBS, resuspended in PBS containing 0.2% DMSO, and coincubated with the liposomal suspension for another 30 min at 37°C. In Experiment B with cytochalasin B, the cells were treated with 2.5 μg/ml cytochalasin B dissolved in DMSO for the last 30 min of the incubation, where the final concentration of DMSO was 0.2% in the medium. Afterwards, the cells were washed four times, resuspended in PBS containing 2.5 μg/ml of cytochalasin B and 0.2% DMSO, and coincubated with a liposomal suspension for another 30 min at 37°C. In both experiments, the cells were washed enough after coincubation with the liposomes, and cultured on a 96-well microtiter plate for one day at 37°C. Under these conditions, the endocytosis of Jurkat cells was completely inhibited as previously reported [15].

3. Results

3.1. Modification of egg PC liposome with PEO-lipid

PEO-Lipid(n = 32) at varying concentrations was added to an egg PC liposomal suspension. At concentration below 30 mol%, PEO-lipid(n = 32) added to the liposome was totally recovered as pellet (Fig. 2), while the PEO-lipid(n = 32) itself was left in supernatant. This indicates that the added PEO-lipid(n = 32) was quantitatively reconstituted into the egg PC liposome under the conditions employed. Unless otherwise stated, egg PC liposomes were modified by 20 mol% of PEO-lipid(n = 32).

3.2. Exposure of DTA-loaded and PEO-lipid modified liposome to Jurkat cell

The DTA-loaded liposome was tested for cytotoxicity. Only when the DTA-loaded and PEO-lipid(n =

Table 1
Cytotoxicity of DTA-loaded and PEO-lipid(n = 5, 15 or 32) modified liposome

Expt. 1. Effect of PEO-chain length

PEO chain length (n) of PEO-lipid	Modification (mol%)	Cell viability (%)	
		without DTA	with DTA
	None	90.4 ± 0.9	90.0 ± 1.4
5	20	92.1 ± 0.9	91.3 ± 0.8
15	20	82.0 ± 0.8	84.4 ± 1.9
15	33	78.4 ± 2.1	79.6 ± 1.1
15	50	66.6 ± 1.6	60.6 ± 0.4
32	20	76.3 ± 0.5	37.1 ± 6.3

Expt. 2. Effect of density of PEO-lipid(n = 32) in liposome

PEO-lipid(n = 32) modified (mol%)	Cell viability (%)	
	without DTA	with DTA
None	88.8 ± 1.2	87.5 ± 0.2
2	84.8 ± 1.7	85.8 ± 2.3
7	85.0 ± 2.6	81.4 ± 3.5
20	82.1 ± 1.3	54.9 ± 11.1

Data represent means ± S.E. from three determinations.

32)-modified liposome was employed, approximately 50% of Jurkat cells were killed (Fig. 3). This phenomenon indicates that DTA was properly introduced into cell cytosol via a pathway different from endocytosis. Neither unencapsulated DTA itself nor DTA-loaded liposomes modified with PEO-lipid($n = 5$ or 15) did show DTA-dependent cytotoxicity (Fig. 3 and Table 1). None of the liposomes without DTA showed significant toxicity, irrespective of modification with PEO-lipid.

Images of phase contrast microscopy (Fig. 4) well agreed with these results. A large number of cells were killed only when the DTA-loaded and PEO-lipid-modified liposome was employed (Fig. 4D). Contrary to this, the conventional liposome without PEO-lipid showed no toxicity (Fig. 4A and B), irrespective of the existence of DTA. The cell treated with the PEO-lipid-modified liposome showed minor damage even without DTA (Fig. 4C).

The appearance of the cytotoxicity was rather slow; the cytotoxicity gradually appeared after 15 min coincubation and became significant after 30 min incuba-

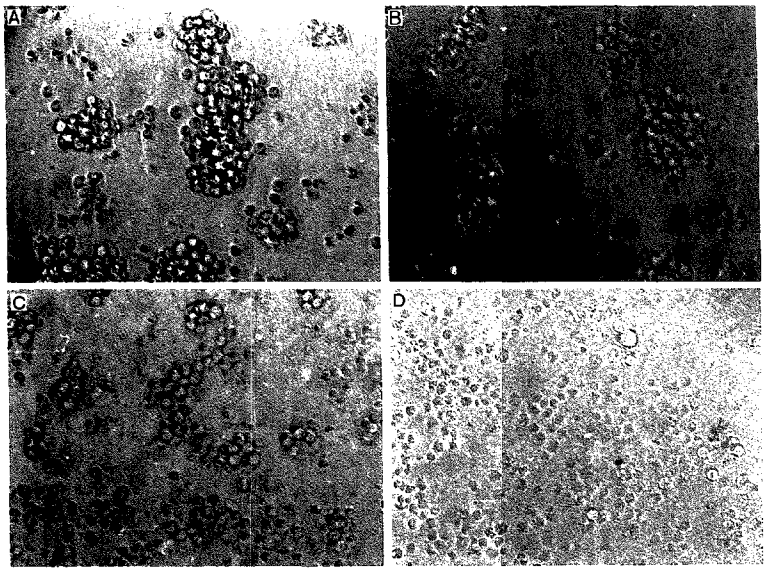

Fig. 4. Phase contrast image of microscopic observation of the cells treated with liposome. The photograph was taken at the end of one day incubation in a Lab-Tek chamber (Nunc), the trypan blue dye exclusion test was performed. (A) The cell coincubated with the conventional liposome without DTA; (B) the cell coincubated with the conventional DTA-loaded liposome; (C) the cell coincubated with the 20 mol% PEO-lipid($n = 32$)-modified liposome without DTA; and (D) the cell coincubated with the 20 mol% PEO-lipid($n = 32$)-modified and DTA-loaded liposome.

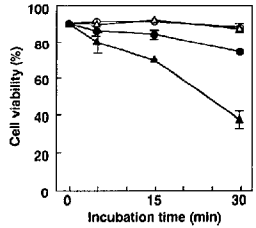

Fig. 5. Cell viability as a function of coincubation time between Jurkat cell and liposome. Open circles, the conventional liposome without DTA; closed circles, the 20 mol% PEO-lipid(n = 32)-modified liposome without DTA; open triangles, conventional DTA-loaded liposome; and closed triangles, the PEO-lipid(n = 32)-modified and DTA-loaded liposome. Vertical bars denote ±S.E. of the mean value for three separate determinations.

tion (Fig. 5). Even after longer coincubation periods, up to 60 min, empty liposome modified with PEO-lipid showed also more or less cytotoxicity (data not shown). Therefore the cell viability was affected also by the liposome concentration (Fig. 6).

To investigate the influence of serum on fusion

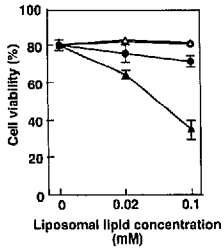

Fig. 6. Cell viability as a function of liposome concentration. Open circles, the conventional liposome without DTA; closed circles, the 20 mol% PEO-lipid(n = 32)-modified liposome without DTA; open triangles, the conventional DTA-loaded liposome; closed triangles, the PEO-lipid(n = 32)-modified and DTA-loaded liposom. Vertical bars denote ±S.E. of the mean value for three separate determinations.

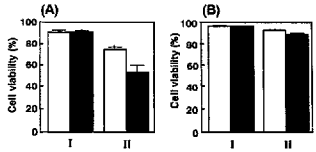

Fig. 7. Effect of FCS on cell viability. Before coincubation with the 20 mol% PEO-lipid(n = 32)-modified liposome, Jurkat cell was precultured in RPMI1640 with 1% FCS (A) or 10% FCS (B) for 16 h. Open columns show the viability of the cell treated with the liposome without DTA, while closed columns show the case treated with the DTA-loaded liposome. Vertical bars denote ±S.E. of the mean value for three separate determinations. I, The conventional liposome without the PEO-lipid modification; II, the PEO-lipid(n = 32)-modified liposome.

efficiency, Jurkat cells were cultured for 16 h in two different media, RPMI1640 medium with 1% and 10% FCS. Fig. 7 clearly shows that the cells were killed more significantly when the serum concentration in the medium was lower.

We examined also the effect of cytochalasin B on the DTA-dependent cytotoxicity. Cytochalasin B is a depolymerization reagent of actin and, therefore, behaves as an inhibitor of endocytosis [24,25]. As shown in Fig. 8, the DTA-dependent cytotoxicity was still significant even in the presence of cytochalasin B.

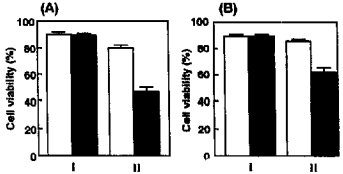

Fig. 8. Effect of cytochalasin B on cell viability. Experiment A, without cytochalasin B; and Experiment B, with cytochalasin B (see text). Open columns show the case for the liposome without DTA, while closed columns show that for DTA-loaded liposome. I, The conventional liposome without the PEO-lipid(n = 32) modification; II, the 20 mol% PEO-lipid(n = 32)-modified liposome. Vertical bars denote ±S.E. of the mean value for three separate determinations.

4. Discussion

The DTA-loaded and PEO-lipid($n = 32$)-modified liposome showed significant cytotoxicity against Jurkat cells. This proves a direct introduction of DTA from the liposomal interior into the cytosol of the cell via a pathway different from endocytosis [15–21]. The fact that DTA-dependent cytotoxicity was still significant even in the presence of cytochalasin B again excluded the possibility that DTA was introduced by endocytosis. A simple mixture of unencapsulated DTA and the empty liposome modified with PEO-lipid($n = 32$) did not show any cytotoxicity. This result surely eliminates the possibility that free DTA is introduced through a defect of the cell membrane.

Taking all the results into account, we can conclude that fusion certainly occurs between the PEO-lipid($n = 32$)-modified liposome and the Jurkat cell. To our knowledge, this is the first demonstration of direct fusion between liposomal membrane and cytoplasmic membrane induced by an artificial fusogen. However, at present, the introduction efficiency is not so high as that observed with the virus protein-modified liposome.

We are able to see a perspective to improve the fusion efficiency from the results of Fig. 7, where fusion efficiency was improved by preculture of the cell in a medium with low serum concentration. In order to evoke the fusion, it is necessary that the liposome first adheres to the cell surface, as already certified by electron microscopic observation [13]. Serum-derived proteins may cover the cell surface and function as a blocker of the liposome-cell fusion. Therefore, preculture of the cell in a medium with low serum concentration might decrease the amount of proteins adhered onto the cell surface and result in enhanced fusion efficiency. When the cell was precultured in RPMI1640 medium containing 1% FCS plus 3 mg/ml of BSA (this concentration is comparable to that of a medium containing 10% FCS), the cell was not killed even by the DTA-loaded and PEO-lipid-modified liposome, just as in the case of 10% FCS-containing medium for preculture. Added BSA might adhere to the cell surface and inhibit the liposome-cell fusion. We have observed also that the addition of FCS into coincubation medium inhibits the DTA-dependent cytotoxicity. Optimization of the fusion efficiency would be also performed by removing serum proteins as much as possible.

It should be pointed out that HeLa cells fuse with the PEO-lipid($n = 15$)-modified liposome [13]. However, the PEO-lipid($n = 15$)-modified liposome did not fuse with Jurkat cells, but was incorporated by the cell via endocytosis [15]. As a result, behavior of the PEO-lipid-modified liposome seemed to be affected by at least two factors, the cell type and the structure of PEO-lipid (hydrophilic and hydrophobic balance (HLB) of the PEO-lipid molecule). The difference in fusogenicity between HeLa and Jurkat cells would be due to the culture system and the cell type as well: HeLa cells are cultured as monolayer while Jurkat cells are cultured as suspension culture. Mori and Sunamoto have revealed that the location of membrane proteins on the cell surface of murine melanoma B16 was altered by 'capping', when the cells were cultured as monolayer [26]. The behavior of cell surface proteins must largely affect the susceptibility of fusion with the PEO-lipid-modified liposome [27]. For the HLB effect of the PEO-lipid, molecular design is necessary to improve its fusogenicity. This will be pursued after more detailed physicochemical characterization of them such as lipid monolayer study [28,29]. PEO has been most commonly used for cell-cell fusion of plant protoplasts [30], mammalian cells [31,32], as well as for liposome-liposome fusion [33].

Therefore, it is of interest to compare two different methods of fusion, the PEO-lipid induced fusion in this work, and the conventional fusion induced by free PEO. There are at least two plausible explanations for the mechanism of the PEO-induced fusion. One explanation is that a decrease of the free water between the two lipid membrane surfaces leads to their close attachment [33,34]. The other is that naked cell surfaces are provided after PEO-induced clustering of membrane-associated proteins. This also causes close attachment of the two cell membrane surfaces [27,35]. To induce the fusion between two lipid membranes, in any event, the PEO-moiety has to be concentrated at the interface of the two fusing cell membranes. These explanations give us an idea about the fusogenecity of the PEO-lipid modified liposome; the condensing and the freezing of the PEO-moiety on the liposomal surface.

Nevertheless, we have to point out difference be-

tween the two fusion systems. First, compared with the free PEO-induced fusion, the PEO-lipid induced fusion proceeds rather slowly. The free PEO-induced fusion usually completes within 5 min, while the present PEO-lipid induced fusion requires more than 15 min (Fig. 5). Second, there is a large difference in the concentration of PEO moiety between the two cases to induce the fusion. With the free PEO (m.w. 6000) induce fusion, approximately 60% (by wt.) as the PEO concentration is required, while with the PEO-lipid($n = 32$) induced one, approximately 0.004% (by wt.) is good enough for inducing the fusion [11,36]. The two fusion mechanisms should be entirely different each other, although the real mechanism is still unclear.

In spite of large benefit of PEO in cell biology, medicine and biotechnology, the function of PEO is still controversial. Modification of liposomal surface with PEO prevents endocytic uptake by reticuloendothelial system and subsequently extends circulation time of the liposome in blood [37–42]. On the other hand, several liposomes modified with PEO-conjugated phosphatidylethanolamines accumulate in the spleen [43,44]. We have also found that the reconstitution of PEO-lipid into the liposome induces endocytic uptake of Jurkat cell [15]. Furthermore, PEO-bearing surfactants show a spermicidal activity [45,46] and hemolysis [47]. These findings tell us that it is still difficult to generalize the function of PEO and/or PEO-modified lipids. We need more careful investigations on this subject.

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