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Interaction between Cells and Poly(Ethylene Glycol)-Lipid Conjugates

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ABSTRACT

Eight types of PEG-lipids carrying different lipid tails were synthesized. These PEG-lipids were labeled with fluorescein isothiocyanate (FITC-PEG-lipids) to examine their interaction with cells and to quantitatively determine amounts of PEG-lipids bound on the cell surface. FITC-PEG-lipids spontaneously anchored to the cell membrane within 15 min without loss of cell viability. The type of lipid had very little effect on the anchoring rates, while an increase in the hydrophobicity of the lipid portion of the PEG-lipids slowed their dissociation rates. Densities of FITC-PEG-lipids on the cell surface ranged from 1×10^{-3} to 1×10^{-2} molecules/nm², depending on the kinds of lipids employed. The relationship between the stability of the lipids on the cell membrane and the hydrophobicity of the lipid moieties will give a basis for the selection of a hydrophobic moiety in PEG-lipid conjugates for use in specific applications.

KEYWORDS

Cell surface modification, amphiphilic polymer, poly(ethylene glycol)-phospholipid conjugates, hydrophobic interaction,

INTRODUCTION

The cell surface is complex in structure and composed of various molecules, including lipids, proteins, and carbohydrates. Cell-cell and cell-extracellular matrices interactions through these molecules play important roles in embryo development and tissue morphogenesis, and also in cell and tissue functions in adults. Previous investigations have modified the cell surface in an effort to control these interactions with biomolecules and to examine their potential in biomedical applications, such as nucleic acid or protein delivery to cells [1, 2], cell delivery to a particular organ [3], and tissue engineering to promote the regeneration of tissues [4]. The methods to modify the cell surface can be grouped into three categories: covalent conjugation; electrostatic interaction; and hydrophobic interaction between amphiphilic molecules with the lipid bilayer of the cell membrane [5, 6]. Each of these methods has merits and demerits. The covalent bond formation with membrane proteins or with sugar moieties of the membrane proteins should be stable [7, 8], but this bond might deteriorate or modify the functions of membrane proteins. In electrostatic interactions [9], polycations can be immobilized on the cell surface by simply adding a solution of the polycation to a cell suspension, because most of the cell surface is negatively charged due to the presence of sialic acids. Most polycations, however, are highly cytotoxic, resulting in cell death. Amphiphilic conjugates have also been examined for cell surface modification [10-12]. The hydrophobic part of the amphiphilic conjugate anchors into the lipid bilayer of the cell membrane through hydrophobic interaction. The hydrophobic interaction between the hydrophobic region of amphiphilic conjugate and the lipid bilayer of the membrane is relatively weak. Although amphiphilic conjugates are not predicted to disturb cell functions, they are released from the cell surface easily.

We have previously employed amphiphilic conjugates, single-stranded DNA-poly(ethylene glycol)-phospholipids (ssDNA-PEG-lipids), to modify the cell surface [6, 13, 14]. The lipid moiety is efficiently inserted into the lipid bilayer of the cell membrane through the hydrophobic interaction and thus the ssDNA-PEG-lipid, which is immobilized on the cell membrane, can be used for various biomedical applications. Proteins, urokinase, and liposomes containing anticoagulant have been immobilized on islets of Langerhans (islets) to increase their blood compatibility [15, 16], and the islets have been micro-encapsulated with other cells [17, 18]. Additionally, cells have been immobilized in a site-specific way on two-dimensional patterns using the conjugates [14, 19]. We observed that the stability of the ssDNA-PEG-lipid on the cell membrane

was highly dependent on the hydrophobicity of lipid moieties. We proposed that the properties of ssDNA-PEG-lipids could be adjusted for specific purposes by changing the hydrophobicity of the lipids.

In this study, we synthesized eight kinds of PEG-lipid conjugates carrying fluorescein isothiocyanate (FITC-PEG-lipids) and analyzed their interactions with cells. Our goal was to elucidate the relationship between the hydrophobicity of the lipids and their stability on the cell membrane. Clearer knowledge of this lipid/cell membrane interaction will provide a basis to select the best hydrophobic moiety in ssDNA-PEG-lipids to optimize the use of these conjugates in specific applications.

MATERIAL AND METHODS

Materials.

Boc-Protected-amino-PEG-carbonate-NHS (Boc-PEG-NHS, Mw 5000), methoxy-PEG-carbonate-NHS (MeO-PEG-NHS, Mw 5000), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from NOF Corporation (Tokyo, Japan). 1-Tetradecanoyl-*sn*-glycero-3-phosphoethanolamine (lysoPE (m=12)), 1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine (lysoPE (m=14)), and 1-octadecanoyl-*sn*-glycero-3-phosphoethanolamine (lysoPE (m=16)) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Fluorescein isothiocyanate (FITC) was purchased from Dojindo Laboratories (Kumamoto, Japan). Dichloromethane, chloroform, diethyl ether, toluene, *N,N'*-dimethylformamide (DMF), ethanol, triethylamine (TEA), and tetrahydrofuran (THF) were purchased from Nacalai Tesuque (Kyoto, Japan). 1,6-Diphenyl-1,3,5-hexatriene (DPH), trifluoroacetic acid (TFA), ethylenediamine, phospholipid C-test wako were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's phosphate buffered saline (PBS) was purchased from Nissui Pharmaceutical, Co., Ltd. (Tokyo, Japan). Cholesteryl chloroformate was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All of these reagents were used as obtained.

Synthesis of PEG-lipids and labeling with FITC.

NH₂-PEG-lipids were synthesized as previously reported [20]. Briefly, Boc-PEG-NHS (180 mg, 36 μmol) and each phosphatidylethanolamine (molar ratio = 1.2:1) were dissolved into 3 mL of dehydrated

dichloromethane, and TEA (5 μ L) was added to the solution. The reaction mixture was stirred overnight at room temperature (RT). Then, TFA (2 mL) was added and stirred for 30 min at 4 $^{\circ}$ C to remove the Boc group. The reaction mixture was poured into iced diethyl ether to precipitate the crude product. After filtration, the precipitate was dried under reduced pressure for 1 h and then the precipitate was extracted into a round bottom flask with chloroform. After evaporation, the solid was dissolved with 3 mL of benzene and freeze-dried.

For FITC labeling, each NH_2 -PEG-lipids (80 mg, 13 μ mol) and FITC (molar ratio = 1:4) were dissolved into dehydrated DMF (3 mL). The reaction mixture was shielded from light and stirred overnight at RT. The reaction product was collected as a precipitate and purified as described above. The FITC-PEG-lipid was obtained as a yellow powder. The FITC-PEG-lipids were dissolved in PBS and the solution was further fractionated into solutions containing polymeric product or low molecular weight substances by gel filtration using a Sephadex G-25 column. A series of reaction was checked by ^1H NMR and MALDI-TOF-MS (Figure S1-S4).

MeO-PEG-NHS (180 mg, 36 μ mol) and each phosphatidylethanolamine (molar ratio = 1.2:1) were dissolved into 3 mL of dehydrated dichloromethane solution and stirred overnight at RT. After precipitation and purification as described above, MeO-PEG-lipid was obtained as a white powder.

Synthesis of FITC-PEG-Cholesterol.

N^1 -Cholesteryloxycarbonyl-1,2-diaminoethane (NH_2 -Cholesterol) was synthesized according to the method reported previously [21]. Briefly, cholesteryl chloroformate (200 mg, 445 μ mol) in dichloromethane (20 mL) was added dropwise to 15 mL of ethylenediamine with vigorous stirring at 4 $^{\circ}$ C. The reaction mixture was stirred at RT for 18 h. After the addition of 100 mL of water, the mixed solution was extracted three times with dichloromethane. The combined organic layers were washed with saturated sodium bicarbonate solution, followed by drying over Na_2SO_4 . After evaporation, the resultant residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$ 4:1, with a small amount of TEA). NH_2 -Cholesterol was obtained as a white solid (155 mg, 78%).

FITC-PEG-Cholesterol was synthesized under similar conditions as the synthesis of FITC-PEG-lipids. Boc-PEG-NHS (100 mg, 20 μ mol), NH_2 -Cholesterol (20 mg, 42 μ mol) and TEA (5 μ L) were dissolved in dehydrated dichloromethane (5 mL) and stirred for 2 days at RT. After evaporation, TFA (0.5 mL) was

added and stirred for 30 min at RT to remove the Boc group. The resulting crude products were reprecipitated in chilled diethylether. The precipitate, NH₂-PEG-Cholesterol, was added to the solution of FITC (5 mg, 13 μ mol) in DMF (20 mL) with TEA (5 μ L) and stirred for 12 h at RT. After removal of solvent, the residue was purified by gel permeation chromatography (Sephadex G-25) to isolate FITC-PEG-Cholesterol from other small molecules including FITC.

Cell culture.

CCRF-CEM cell line (human acute lymphoblastic leukemia, T-cell), HEK293 cell line (human embryonic kidney), HeLa cell line (human cervical carcinoma), were obtained from the Health Science Research Resources Bank (Osaka, Japan). P3X63-Ag8.653 cell line (mouse myeloma) was obtained from Riken Cell Bank (Tsukuba, Japan). CCRF-CEM cells and P3X63-Ag8.653 cells were suspended and cultured in RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Nacalai Tesque), at 37 °C and 5% CO₂. HEK293 and HeLa cells were maintained in Eagle's minimum essential medium (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, at 37 °C and 5% CO₂. HEK293 and HeLa Cells were harvested by the addition of a 0.25% trypsin/1mM EDTA solution (Nacalai Tesque).

Cell surface modification by FITC-PEG-lipids.

Cell suspension (10⁶ cells) and FITC-PEG-lipids (100-500 ng) were mixed in PBS (Total volume: 1 mL) and incubated for predetermined times at 37 °C for cell surface modification with FITC-PEG-lipids. The cells were collected by centrifugation at 180 $\times$ g for 5 min at 25 °C. The cells were resuspended in 10 mL of PBS and were collected by centrifugation. This washing procedure was repeated twice. After modification with FITC-PEG-lipids, the cells were observed by confocal laser scanning microscopy (FLUOVIEW FV500, Olympus, Tokyo, Japan). The fluorescence intensities of modified cells were examined by Flow cytometry (Guava EasyCyte mini, Merck Millipore, Billerica, Massachusetts, USA).

Cell viability assay.

Cell viabilities were determined using a Guava Viacount[®] assay kit following the manufacturer's instruction. Briefly, modified cells were cultured for predetermined hours in medium and then collected by

centrifugation at $180 \times g$ for 5 min at 25 °C. The cell suspension was diluted 10 times with Guava Viacount Reagent® and incubated for 5 min at RT. Cells were analyzed by the flow cytometer, Guava EasyCyte mini.

Determination of densities of FITC-PEG-lipids on cell surface.

Modified cells were collected as a cell pellet in a centrifugal tube by centrifugation. Then, 1 mL of ethanol solution was added to the tube and the solution was agitated to suspend the cells and the left for 1 hour at 25 °C in water bath. The suspension was centrifuged ($180 \times g$, 5 min, 25 °C) to collect the supernatant. The fluorescence intensity of the supernatant was determined by a fluorescence spectrophotometer (F-2500, Hitachi, Co., Tokyo, Japan). The relation between fluorescence intensities and the amounts of FITC-PEG-lipid in ethanol solution were determined using each FITC-PEG-lipid ethanol solutions with different concentrations. The surface area of cells were calculated from the diameter of cells determined by microscopy. The surface density of FITC-PEG-lipids were determined by dividing the amount of FITC-PEG-lipids by the cell surface area.

Determination of critical micelle concentration (CMC).

The CMC values of PEG-lipids were determined by the fluorescence method using MeO-PEG-lipids and 1,6-diphenyl-1,3,5-hexatriene (DPH) [22]. MeO-PEG-lipids were dissolved with PBS and diluted to several concentrations by PBS. Total volume was 2 mL. Then, DPH/THF solution (1 mM, 20 μ L) was added and then mixed well. The solutions were incubated for 30 min at 25 °C in a water bath. The fluorescent intensities of the mixtures were determined using a fluorescence spectrophotometer. The CMC values were calculated from the concentration at which the fluorescence intensity underwent a sharp linear increase.

RESULTS

The synthesized FITC-PEG-lipids are listed in Scheme 1. The molecular weight of PEG was 5,000 in all FITC-PEG-lipids. Three kinds of FITC-PEG-lipids with a single saturated alkyl chain with different chain lengths and four kinds of FITC-PEG-lipids with two saturated alkyl chains with differing chain lengths and with two unsaturated alkyl chains, DOPE, were prepared. A FITC-PEG-Cholesterol was also prepared. Interactions of these amphiphilic molecules with living cells were examined.

A CCRF-CEM cell suspension and a FITC-PEG-DPPE solution were mixed and incubated for 30 min at 37 °C. After the cells were washed with PBS, the cells were observed under a confocal laser scanning microscope. We observed cells modified with FITC-PEG-DPPE by a phase contrast image (Figure 1 (a-1)) and a confocal fluorescent image (Figure 1 (a-2)). The fluorescence derived from FITC was clearly observed at the periphery of the cells. In a control study, we treated cells with FITC-PEG without lipid and observed a phase contrast image (Figure 1 (b-1)) and a confocal fluorescent image (Figure 1 (b-2)). No fluorescence was observed at the periphery of the cells. These data was consistent with previous studies [13, 20]. We concluded that the hydrophobic lipid portion of FITC-PEG-DPPE became anchored to the lipid bilayer of the cell membrane through hydrophobic interaction, and the hydrophilic part of PEG could not permeate through the hydrophobic part of the lipid bilayer. Thus, FITC-PEG of the FITC-PEG-DPPE was located outside of the cell membrane (Figure 1 (c)). In some cases the fluorescence of FITC was observed inside a cell. It is possible that FITC-PEG-lipids on the cell membrane were taken into cell by endocytic pathway.

Cell surface modification using FITC-PEG-DPPE was also tested for various cell types including HEK293 (human epithelial cell line), HeLa (human epithelial cell line), P3X63-Ag8.653 (mouse myeloma cell line). The densities of FITC-PEG-DPPE on cell surfaces were determined by measuring fluorescence intensities of ethanol solutions in which FITC-PEG-DPPE was extracted from cells. Surface density of FITC-PEG-DPPE increased with increasing FITC-PEG-DPPE concentration in solution (Figure 2). No significant differences in the density of FITC-PEG-DPPE were observed among all cell types. This result clearly demonstrates that PEG-DPPE efficiently modify cell surfaces for various cell types. In the following experiments, CCRF-CEM cells were employed to examine interaction between cells and FITC-PEG-lipids having different lipid anchors because of the ease of handling. In confocal fluorescent images of cells modified with eight different types of FITC-PEG-lipids and their fluorescence intensity profiles obtained by flow cytometry analyses (Figures 3(a) and 3(b), Figure S5), we observed that although fluorescence appeared at the periphery of the cells in all cases, their brightness was highly dependent on the kinds of lipids employed. When FITC-PEG-lysolipids which carry a single alkyl chain were examined, the fluorescence intensities on the cells increased as the alkyl chains became longer. These results indicate that the amount of anchored PEG-lipids increased as the hydrophobicity of the lipid parts increased. In contrast, the fluorescence intensities did not consistently increase with increasing alkyl chain lengths when the cells were treated with FITC-PEG-lipids having lipids with two acyl chains. The fluorescence intensity of cells modified with FITC-

PEG-DPPE (m=14) was stronger than that of cells modified with FITC-PEG-DMPE (m=12), but that of cells modified with FITC-PEG-DSPE (m=16) was weaker than that of cells modified with FITC-PEG-DPPE (m=14). FITC-PEG-DOPE has an alkyl chain length the same as that of FITC-PEG-DSPE, but it carries unsaturated lipid. The fluorescence intensity of cells modified with FITC-PEG-DOPE was stronger than that of cells modified with FITC-PEG-DSPE. FITC-PEG-Cholesterol was also effectively immobilized on the cell surface as shown in Figure 3(a) and 3(b).

Animal cells are easily damaged when they are exposed to a non-physiological environment. Viabilities of cells in which the surfaces were modified by exposure to FITC-PEG-lipids were examined. We compared cell viabilities just after modification and after the cell culture for 24 h in the presence of 10% FBS (Table 1). Cell viabilities were well maintained. More than 90% of the cells were viable when FITC-PEG-lipids with two saturated alkyl chains were applied to cells. When FITC-PEG-lipids with single saturated alkyl chains were applied, cell viabilities tended to be slightly less than 90%. This outcome might reflect stronger cytolytic activity of lysolipids than lipids with two saturated alkyl chains [23]. Overall, we observed no serious toxic effects regardless of the types of lipids employed. Around 90% of cells were viable in all cases.

We also determined the densities of the various FITC-PEG-lipids on cell surfaces. FITC-PEG-lipids were extracted into ethanol from the cell membrane by exposing modified cells to ethanol. Densities of FITC-PEG-lipids were calculated from the fluorescence intensities of the ethanol supernatant. Figure 4 shows the variations of surface densities of FITC-PEG-lipids as a function of incubation time. Although the surface densities tended to scatter slightly with time, they were nearly constant over an incubation time of 15 to 60 min in all of FITC-PEG-lipids examined. This result indicated that FITC-PEG-lipids rapidly anchored to a lipid bilayer of the cell membrane within 15 min regardless of the types of lipids employed. Densities of FITC-PEG-lipids on cell surface ranged from 1×10^{-3} to 1×10^{-2} molecules/nm², depending on the nature of the lipids employed. The greatest densities, about 1×10^{-2} molecules/nm², occurred when FITC-PEG-DPPE, FITC-PEG-DOPE or FITC-PEG-Cholesterol solutions were used. This value was about one tenth of the value, 10^{-1} molecules/nm², which is typical for polymer chain density of a polymer brush formed on a solid substrate [24]. The cell surface is complex in structure and composed of lipids, proteins and carbohydrates. Taking the complexity of the cell membrane into consideration, the densities of FITC-PEG-lipids seem considerably high. When cells were added to a solution of FITC-PEG-lipids and left for a while, the equilibrium between FITC-PEG-lipid in solution and that anchored into the cell membrane will be

established. The surface density of FITC-PEG-lipid on the cell membrane should increase as the concentration of the FITC-PEG-lipid in solution increases. Confocal fluorescent images of cells modified at different concentrations of FITC-PEG-DPPE clearly showed that the ring-shaped fluorescence became brighter as the FITC-PEG-DPPE concentrations increased (Figure 5(a)). In a quantitative evaluation, we determined that the densities of FITC-PEG-lipids on the cell surfaces increased as the concentrations of the labeled PEG-lipids in the treatment solutions increased (Figure 5(b)). For all of the labeled PEG-lipid conjugates, the cell surface densities increased as their concentrations in the treatment solutions increased.

FITC-PEG-lipids were anchored to the lipid bilayer of the cell membrane through hydrophobic interaction. FITC-PEG-lipids were expected to be released from the cell membrane into a medium during culturing of surface modified cells, because the hydrophobic interaction is not as stable as a covalent bond. Indeed, the FITC-PEG-lipid density on the cell surface decreased during culturing (Figure 6). Most of FITC-PEG-lipids were released from the cell membrane within one day culture. In FITC-PEG-DSPE, the lipid DSPE consists of two long saturated alkyl chains ($m=16$) and this conjugate remained longer on the cell surface than the other FITC-PEG-lipids. Half of FITC-PEG-DSPE still remained on the cell membrane after 24 h.

DISCUSSION

The aim of this study is to gain kinetic parameters on interaction between cell surface and PEG-lipids carrying different lipid anchors. The densities of FITC-PEG-lipids were determined by measuring fluorescence intensities of ethanol solutions in which FITC-PEG-lipids were extracted from cells. This method using ethanol extraction cannot distinguish between internalized and membrane-bound PEG-lipids. However, confocal fluorescence images showed that most of fluorescence was observed at cell periphery (Figures 1, 3, 5), suggesting localization of FITC-PEG-lipids at cell membrane. Therefore, measured fluorescence intensity of ethanol solution after extraction is considered to be mostly attributed to FITC-PEG-lipids in the cell membrane.

The association of a FITC-PEG-lipid with the lipid bilayer of the cell membrane may be expressed by the following equation:

where A represents the FITC-PEG-lipid, B represents the anchoring sites on the cell membrane, AB is the complex formed by FITC-PEG-lipid anchored to the cell membrane, k_{on} is the rate constant of the anchoring process and k_{off} is the rate constant of the dissociation process. The kinetic equation of the above process can be expressed by

$$\frac{d[AB]}{dt} = k_{on}[A][B] - k_{off}[AB] \quad (1)$$

Under our experimental conditions with the FITC-PEG-lipid anchored to the cell membrane, about 90% of the initial FITC-PEG-lipid was still in the solution when the reaction reached at equilibrium. So, we assumed that [A] is constant in the anchoring reaction for the ease of analysis. The initial concentration of anchoring sites in the lipid bilayer is described as $[B]_0$. The B concentration at time t, [B], is expressed by

$$[B] = [B]_0 - [AB]$$

The initial concentration of AB is

$$[AB]_0 = 0$$

Under these conditions, the concentration of membrane-anchored FITC-PEG-lipid, [AB], at time t can be described as below.

$$[AB] = \frac{k_{on}[A]_0[B]_0}{k_{on}[A]_0 + k_{off}} \{1 - \exp[-(k_{on}[A]_0 + k_{off})t]\} \quad (2)$$

The concentration of anchored FITC-PEG-lipid, [AB], at equilibrium, $t = \infty$, is written as follows,

$$[AB]_{\infty} = \frac{k_{on}[A]_0[B]_0}{k_{on}[A]_0 + k_{off}} \quad (3)$$

The Eq. (3) can be modified as

$$\frac{[AB]_{\infty}}{[A]_0} = K_a[B]_0 - K_a[AB]_{\infty} \quad (4)$$

where

$$K_a = \frac{k_{on}}{k_{off}} \quad (5)$$

As shown in Figure 5(b), $[AB]_{\infty}$ increased as $[A]_0$ increased. Plotting $[AB]_{\infty}/[A]_0$ against $[AB]_{\infty}$, the values of $K_a[B]_0$ and K_a may be obtained from the y-intercept and the slope, respectively. Values of K_a obtained are listed in Table 2. The K_a values of FITC-PEG-lysolipids (m=12, 14) and FITC-PEG-DMPE were not shown because their dependence of $[AB]_{\infty}$ on $[A]_0$ was so small (Figure 5(b)) and thus their experimental results were not included for the analysis. The K_a values of other FITC-PEG-lipids ranged from

3.4 to $8.4 \times 10^6 \text{ M}^{-1}$ except for FITC-PEG-DSPE. K_a values of FITC-PEG-DSPE were several times larger than those of FITC-PEG-lipids.

When modified cells were incubated in the absence of FITC-PEG-lipid, the FITC-PEG-lipid was released from the cell surface into the culture medium, and defines the dissociation process. The free FITC-PEG-lipid concentration, $[A]$, at any time, is assumed to be 0 due to its very small concentration in the culture medium compared with the concentration of the lipid anchored to the cell membrane. The Eq.(1) can be simply expressed by

$$\frac{d[AB]}{dt} = -k_{\text{off}}[AB]$$

This differential equation is solved as,

$$[AB] = [AB]_0 \exp(-k_{\text{off}}t) \quad (6)$$

At the beginning of dissociation ($t = 0$), the concentration of FITC-PEG-lipid anchored on the membrane is expressed by $[AB]_0$. The values of k_{off} were estimated by fitting the graph of the dissociation curves (Figure 6) with Eq. (6). Table 2 lists these kinetic constants for each FITC-PEG-lipid. In the case of double alkyl chains, the values became smaller with the increase of the length of the alkyl chains. These data indicate that the interaction between FITC-PEG-lipids and cell membrane becomes stronger with an increase of the hydrophobicity of the lipid moiety.

Values of k_{on} could hardly be estimated from anchoring process of FITC-PEG-lipids, because the process is so rapid for kinetic analysis as shown in Figure 4. Values of k_{on} were calculated from the values of k_{off} and K_a using Eq. (5) and are also listed in Table 2. They range between 4.3 to $5.8 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$. NMR and MALDI-TOF-MS studies suggest the presence of unreacted NH_2 -PEG-lipids or FITC-PEG (Figure S1-S4, Supplementary materials). The modification rate of FITC and DPPE was calculated from the NMR study to be 83% and 65%, respectively. These byproducts decrease actual FITC-PEG-lipids concentrations and therefore increase values of K_a and k_{on} . We observed no clear dependence of k_{on} values on the length of the alkyl chain, although the k_{on} value of FITC-PEG-DSPE was slightly larger than those of other FITC-PEG-lipids. These results suggest that the anchoring process of FITC-PEG-lipids onto the cell membrane is mainly determined by the diffusion of FITC-PEG-lipids toward the cell surface. Vermaas *et al.* showed that association of free phospholipid to biological membrane surface is the rate-limiting step in the insertion of free lipid into membrane by computational models [25]. The molecular weight of PEG is 5000, much larger

than the mass of the lipids. The hydrodynamic size of the FITC-PEG-lipids is mainly determined by PEG and thus their diffusion constants are expected to be the same among all the FITC-PEG-lipids, resulting in no differences between the k_{on} values of FITC-PEG-lipids.

FITC-PEG-lipids, having a long alkyl hydrophobic chain and a hydrophilic PEG moiety, were amphiphilic molecules. Consequently, we expected the FITC-PEG-lipids to form micelles at concentrations higher than their CMCs. In the above kinetic analyses, all of the FITC-PEG-lipids were assumed to exist as single molecules and to directly interact with the lipid bilayer of the cell membrane. We questioned if FITC-PEG-lipids could form micelles at the concentration we tested. Since the fluorescent FITC moiety of FITC-PEG-lipids interferes with the CMC determination by the usual fluorescence method [22], we employed methoxy-terminated PEG-lipids (MeO-PEG-lipids) for determination of CMC values of PEG-lipids. For MeO-PEG-lysolipids, fluorescence intensities showed clear increases above the baseline values as shown in Figure 7(a) and this inflection point was determined as the CMC value. The CMC values were 600, 190 and 65 μM for MeO-PEG-lysoPE ($m=12$), MeO-PEG-lysoPE ($m=14$) and MeO-PEG-lysoPE ($m=16$), respectively. The values of CMC decreased with the hydrocarbon chain length of the lipid moieties. Figure 7(b) shows the results of MeO-PEG-lipids with two saturated alkyl chains. CMC values were determined to be 17, 14 and 9 μM for MeO-PEG-DMPE, MeO-PEG-DPPE and MeO-PEG-DSPE, respectively. There are little differences in the CMC values between the three PEG-lipids. In this study, the concentration of FITC-PEG-lipids, $[A]$, were much lower than the values of CMCs for all of MeO-PEG-lipids. This suggests that the PEG-lipids exist as single molecules at the concentrations we tested. Therefore, the assumptions in the kinetic analyses are acceptable.

K_a and k_{on} values of FITC-PEG-DSPE were significantly larger than those of other FITC-PEG-lipids. It has been reported that amphiphilic molecules with long alkyl chains form premicelle-like aggregates at concentrations lower than its CMC [26]. In Figure 7(b), fluorescence intensities showed slow increases in low FITC-PEG-lipids concentrations. These facts suggest that MeO-PEG-DSPE formed premicelle-like aggregates. Formation of the premicelle-like aggregates might result in a decrease in the concentration of FITC-PEG-DSPE present as single molecules. The above kinetic analyses could not be applied to the interaction of FITC-PEG-DSPE with the cell membrane.

We also examined the behavior of FITC-PEG-Cholesterol in this study. No specific differences between FITC-PEG-Cholesterol and other FITC-PEG-lipids with long hydrocarbon chains were observed.

Cholesterol is known to form lipid-rafts with sphingolipids. We did not examine the location of the FITC-PEG-cholesterol on the cell membrane. FITC-PEG-Cholesterol may be useful for specific modification of lipid-rafts.

Our result showed that more than the half of PEG-lipids was released on the cell surface after 24 h (Figure 6). This indicates that cell surface can be transiently modified with PEG-lipids used in this study. The transient cell surface modification will allow for control of short-term biological reactions occurring at cell surface. Actually, we have demonstrated that surface modification of islets with PEG-DPPE derivatives and additional conjugation with biomolecules effectively prevent blood-mediated reactions such as blood coagulation and activation of the complement system, both of which cause death of islets [27]. In order to employ our technique for controlling long-term biological events such as cell-cell communication, further materials design need to be considered.

CONCLUSION

FITC-PEG-lipids can effectively modify the cell membrane without cytotoxicity. Densities of FITC-PEG-lipids on cell surface ranged from 1×10^{-3} to 1×10^{-2} molecules/nm² depending on the types of lipids employed. This study gives a basis for selection of a hydrophobic moiety in the preparation of ssDNA-PEG-lipids that can be used in specific applications.

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FIGURE CAPTIONS

Scheme 1. Chemical structures of eight different FITC-PEG-lipids.

Figure 1. Microscopic images of cells exposed to FITC-PEG-DPPE or FITC-PEG solutions. (a-1); Phase contrast image of cells exposed to a 83 nM FITC-PEG-DPPE solution for 30min, (a-2); Confocal fluorescence image of cells exposed to a FITC-PEG-DPPE solution, (b-1); Phase contrast image of cells exposed to a 83 nM FITC-PEG solution, (b-2); Confocal fluorescence image of cells exposed to a FITC-PEG solution, (c); Schematic illustration of cell membrane modified with FITC-PEG-lipids. Scale bar is 20 μm .

Figure 2. The densities of FITC-PEG-DPPE on cell surface for various cell types. Cells were incubated in various concentrations of FITC-PEG-DPPE for 30 min at 37 °C. ●, CCRF-CEM ▲, HEK293; ■, HeLa; ◆, P3X63-Ag8.653, respectively.

Figure 3. Cell surface modification using various FITC-PEG-lipids. (a); Confocal fluorescence images of cells modified with eight different FITC-PEG-lipids (83 nM for 30 min) and non-treated cells. Scale bar is 50 μm . (b); Fluorescence intensity profiles of cells modified with eight different FITC-PEG-lipids determined by flow cytometry. (A) Non-treated, (B) FITC-PEG-lysoPE (m=12), (C) FITC-PEG-lysoPE (m=14), (D) FITC-PEG-lysoPE (m=16), (E) FITC-PEG-DMPE (m=12), (F) FITC-PEG-DPPE (m=14), (G) FITC-PEG-DSPE (m=16), (H) FITC-PEG-DOPE (m=16, unsaturated), (I) FITC-PEG-Cholesterol, respectively.

Figure 4. Relationship between the densities of FITC-PEG-lipids on the cell surface and the incubation time. Cells were incubated in 83 nM solution of FITC-PEG-lipids at 37 °C. ○, FITC-PEG-lysoPE (m=12); △, FITC-PEG-lysoPE (m=14); □, FITC-PEG-lysoPE (m=16); ●, FITC-PEG-DMPE (m=12); ▲, FITC-PEG-DPPE (m=14); ■, FITC-PEG-DSPE (m=16); ■, FITC-PEG-DOPE; ◆, FITC-PEG-cholesterol, respectively. Data shown are means $\pm$ SD ($n = 3$).

Figure 5. The effects of the concentration of FITC-PEG-lipids in solution on their densities on the cell surface. (a) Confocal fluorescence images of cells modified with FITC-PEG-DPPE at various concentrations. Scale bar is 20 μm . (b) Densities of FITC-PEG-lipids on cell surface at their various concentrations. $\bigcirc$, FITC-PEG-lysoPE (m=12); $\triangle$, FITC-PEG-lysoPE (m=14); $\square$, FITC-PEG-lysoPE (m=16); $\bullet$, FITC-PEG-DMPE (m=12); $\blacktriangle$, FITC-PEG-DPPE (m=14); $\blacksquare$, FITC-PEG-DSPE (m=16); $\blacksquare$, FITC-PEG-DOPE; $\blacklozenge$ (gray), FITC-PEG-Cholesterol, respectively. Data shown are means $\pm$ SD ($n = 3$).

Figure 6. Retention of FITC-PEG-lipids on cell surface. Cells modified with FITC-PEG-lipids were cultured in medium supplemented with 10% FBS. $\bigcirc$, FITC-PEG-lysoPE (m=12); $\triangle$, FITC-PEG-lysoPE (m=14); $\square$, FITC-PEG-lysoPE (m=16); $\bullet$, FITC-PEG-DMPE (m=12); $\blacktriangle$, FITC-PEG-DPPE (m=14); $\blacksquare$, FITC-PEG-DSPE (m=16); $\blacksquare$, FITC-PEG-DOPE; $\blacklozenge$ (gray), FITC-PEG-Cholesterol, respectively. Data shown are means $\pm$ SD ($n = 2$).

Figure 7. Determination of the CMC values. For these studies, we used MeO-PEG-lipids, employing the methodology described in the Experimental section. The fluorescent intensities of DPH are presented as a function of the concentration of the MeO-PEG-lipids in the solutions. The CMC values were calculated from the concentration at which the fluorescence intensity underwent a sharp, linear increase. (a) MeO-PEG-lysolipids and (b) MeO-PEG-lipids. Solid line shows the clear increase of the fluorescence intensities. Dashed line shows slow increase of the fluorescence intensities.

Table 1. Viabilities of cells modified with FITC-PEG-lipids

	Just after modification [%]	After 24 h culture* [%]
Control	94.6 ± 4.7	94.7 ± 4.6
FITC-PEG-lysoPE (m=12)	88.7 ± 3.2	88.7 ± 3.2
FITC-PEG-lysoPE (m=14)	85.6 ± 2.2	91.3 ± 1.6
FITC-PEG-lysoPE (m=16)	88.8 ± 1.8	92.0 ± 2.3
FITC-PEG-DMPE (m=12)	92.0 ± 6.4	92.2 ± 5.9
FITC-PEG-DPPE (m=14)	91.8 ± 6.4	92.4 ± 1.7
FITC-PEG-DSPE (m=16)	93.3 ± 4.7	92.3 ± 6.4
FITC-PEG-DOPE (m=16, unsaturated)	85.0 ± 4.9	96.6 ± 0.6
FITC-PEG-Cholesterol	90.7 ± 6.4	96.0 ± 1.8

*; 24 h culture in the presence of 10% FBS

Table 2. Rate constants and association constants of FITC-PEG-lipids.

	$k_{\text{off}} \times 10^5 \text{ [s}^{-1}\text{]}$	$K_{\text{a}} \times 10^{-6} \text{ [M}^{-1}\text{]}$	$k_{\text{on}} \times 10^{-2} \text{ [M}^{-1}\text{s}^{-1}\text{]}$
FITC-PEG-lysoPE (m=12)	14.3	-	-
FITC-PEG-lysoPE (m=14)	12.1	-	-
FITC-PEG-lysoPE (m=16)	16.9	3.44	5.81
FITC-PEG-DMPE (m=12)	7.90	-	-
FITC-PEG-DPPE (m=14)	7.54	5.76	4.34
FITC-PEG-DSPE (m=16)	2.64	25.1	6.62
FITC-PEG-DOPE (m=16, unsaturated)	6.93	8.40	5.82
FITC-PEG-Cholesterol	4.78	8.02	3.83

FITC-PEG-lipids

R

lysoPE (m=12, 14, 16)

DMPE (m=12), DPPE (m=14), DSPE (m=16)

DOPE (unsaturated)

NH₂-Cholesterol

Scheme 1

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Supplementary materials

Interaction between Cells and Poly(Ethylene Glycol)-Lipid Conjugates

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Experimental

General

^1H NMR was recorded on a Bruker DPX-400 spectrometer (400 MHz). MALDI-TOF-MS spectra were recorded using a Bruker ultraflex III-KE with Super-DHB as a matrix.

Figure S1. ^1H NMR spectrum of $\text{NH}_2\text{-PEG-DPPE}$ in CDCl_3 . Asterisk is assigned to protons of methyl and methylene in lipid moiety.

Figure S2. ^1H NMR spectrum of FITC-PEG-DPPE in CDCl_3 . Asterisk are assigned to protons in FITC.

Figure S3. MALDI-TOF-MS spectrum of NH₂-PEG-DPPE. Calculated for C₂₆₆H₅₃₁N₃O₁₂₂P [M - H]⁻ 5740.49; found 5740.07. The averaged degree of polymerization for PEG is calculated to be 113.

Figure S4. MALDI-TOF-MS spectrum of FITC-PEG-DPPE. Calculated for C₂₈₂H₅₃₃N₃O₁₂₆PS [M - H]⁻ 6129.42; found 6129.47. The averaged degree of polymerization for PEG is calculated to be 111.

Figure S5. The flow cytometry data of FITC-PEG-lipids modified cells. (a) FITC-PEG-lysoPE (m=12), (b) FITC-PEG-lysoPE (m=14), (c) FITC-PEG-lysoPE (m=16), (d) FITC-PEG-DMPE (m=12), (e) FITC-PEG-DPPE (m=14), (f) FITC-PEG-DSPE (m=16), (g) FITC-PEG-DOPE (m=16, unsaturated), (h) FITC-PEG-Cholesterol, (i) Non-treated, respectively.