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Retention Dynamics of Amphiphilic Polymers PEG-Lipids and PVA-Alkyl on the Cell Surface

Osamu Inui,[†] Yuji Teramura,[‡] and Hiroo Iwata*[,†]

Department of Reparative Materials, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-Cho, Shogoin, Sakyo-Ku, Kyoto 606-8507, Japan, and Radioisotope Research Center, Kyoto University, Yoshida-Konoe-Cho, Sakyo-ku, Kyoto 606-8501, Japan

ABSTRACT We tested two kinds of amphiphilic polymers for cell surface modification: a poly(ethylene glycol)-conjugated phospholipid (PEG-lipid) and a poly(vinyl alcohol) that carried alkyl side chains (PVA-alkyl). Both polymers were expected to anchor to the lipid bilayer of the cell membrane through hydrophobic interactions. We followed the kinetics of these fluorescently labeled amphiphilic polymers (fPEG-lipid, fPVA-alkyl) over time on living cells with confocal scanning laser microscopy and flow cytometry. We found that fPEG-lipids and fPVA-alkyl polymers were not cytotoxic, and they were released from the cell surface without triggering endocytosis. The gradual release from the cell surface was influenced by the hydrophobicity of the alkyl chains, which affected their stability. The amphiphilic polymers tended to aggregate on the cell surface; in particular, the aggregation of PVA-alkyl was clearly identified. Although most of PEG-lipids and PVA-alkyl polymers did not appear to in the cytoplasm, the cells were able to endocytose lipid molecules, as expected. These results suggested that the retention time of modified amphiphilic polymers on the cell surface should be a consideration when modifying cell surfaces to enhance cell transplantation.

KEYWORDS: cell transplantation • surface modification • amphiphilic polymers • poly(ethylene glycol)-conjugated phospholipid (PEG-lipid) • poly(vinyl alcohol)

1. INTRODUCTION

The interactions of water-soluble synthetic polymers with living cells have been examined in conjunction with gene transfer, cell fusion, and cell immobilization. For example, cationic poly(ethylene imine) (PEI) and poly-L-lysine have been used to carry plasmid DNA into cells (1, 2). Poly(ethylene glycol) (PEG) has been used to induce cell fusion in preparing a hybridoma to produce monoclonal antibodies (3). Recently, polymers have been applied to modify the cell surface in order to improve graft survival in cell transplantation, especially transplantation of islets of Langerhans (islets) (4−17). A recent report showed that bioactive substances, like urokinase, thrombomodulin, and heparin, could be immobilized to the surface of islets (11−13), which improved the graft survival of donor islets (10−13). A thin polymer membrane could also be formed with a layer-by-layer method on the negatively charged surface of islets (4, 5, 12, 13); this served to isolate donor cells from the host immune response. Thus, understanding the interactions between soluble polymers and living cells is important for cell engineering endeavors in various biomedical fields.

The surface modification of living cells, or cell surface engineering, has been achieved primarily by chemical or enzymatic treatment or by metabolic introduction (23−25). In particular, enzymatic treatment and metabolic introduction have been applied to cell lines to affix various molecules, like biotin, azide, and ketone groups, to the living cell surface. The introduction of these molecules enabled cells to gain new biological functions. However, there are some difficulties in applying these methods to primary cells, like islets of Langerhans, because metabolic introduction could perturb cell physiology and cell function (18, 19). In addition, these technologies are limited to the introduction of small molecules with specific functional groups and to specific cells. Furthermore, the chemical method that uses an *N*-hydroxyl-succinimidyl ester (NHS) group to covalently immobilize molecules on membrane proteins (7, 10, 11) is nonspecific chemical modifications that could perturb membrane proteins and cause the deterioration of cell functions. In addition, most of the molecules introduced would disappear from the cell surface with time (7, 10, 17), even though molecules covalently immobilized on the cell membrane were expected to be stable and remain on the cell surface.

Recently, we synthesized amphiphilic polymers, including PEG-phospholipid conjugates (PEG-lipid) and poly(vinyl alcohol) polymers that carried alkyl side chains (PVA-alkyl) for cell surface modifications (4, 5, 12−17). Other groups have also developed noncovalent cell surface modification techniques (18, 19). When amphiphilic polymers were applied to a cell suspension, they spontaneously attached to the cell surface, anchoring through hydrophobic interactions between their alkyl chains and the lipid bilayer of the cell membrane, without reducing cell viability (17). The introduced polymers, however, gradually disappeared from the cell surface. To improve the efficacy of cell surface modifica-

* Corresponding author. Tel/Fax: +81 75 751 4119. E-mail: iwata@frontier.kyoto-u.ac.jp.

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[†] Department of Reparative Materials, Institute for Frontier Medical Sciences, Kyoto University.

[‡] Radioisotope Research Center, Kyoto University.

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tions in various biomedical scenarios, it is necessary to understand the dynamic features of amphiphilic polymers on the cell surface, including uptake and exclusion.

In this study, we studied four different polymers to determine the effects of alkyl chain length and the number of alkyl chains per molecule on the dynamics and stability of these polymers on the cell surface. We prepared three kinds of PEG-lipids that carried alkyl chains of different lengths, and a PVA-alkyl that carried approximately 28 alkyl side chains. These amphiphilic polymers were fluorescently labeled, and we examined their dynamics on the surfaces of living cells in detail with a confocal scanning laser microscope and flow cytometry.

2. EXPERIMENTAL PROCEDURE

2.1. Materials. 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylethanolamine (DMPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine (DSPE) were purchased from NOF Corporation (Tokyo, Japan). α-*N*-Hydroxysuccinimidyl-ω-*tert*-butoxycarbonyl poly(ethylene glycol) (NHS-PEG-Boc, molecular weight 5000) was purchased from Nektar Therapeutics (San Carlos, CA). PVA (molecular weight 7.4×10^4) was purchased from Unitika, Ltd. (Osaka, Japan). Pyridinium chlorochromate and cytochalasin D were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Trifluoroacetic acid, sodium monochloroacetate, 1-hexadecanol, sodium hydroxide, florisil, and Celite were purchased from Wako Pure Chemical (Osaka, Japan). Chloroform, dichloromethane, dimethylsulfoxide, triethylamine, and diethyl ether were purchased from Nacalai Tesque (Kyoto, Japan). Fluorescein isothiocyanate (FITC) was purchased from Dojindo Laboratories (Kumamoto, Japan). Hanks' balanced salt solution (HBSS), RPMI-1640 medium, tetramethyl rhodamine-labeled transferrin, and tetramethyl rhodamine-labeled dextran (molecular weight 1.0×10^4) were purchased from Invitrogen Co. (Carlsbad, CA). Fetal bovine serum was purchased from BioWest (Miami, FL, USA). Liquid chromatography PD-10 columns (Sephadex G-25) were purchased from Amersham Biosciences (Uppsala, Sweden).

2.2. Cell Culture. The CCRF-CEM cell line, established from acute lymphoblastic leukemia T-cells, was obtained from the Health Science Research Resources Bank (Tokyo, Japan). Suspended CCRF-CEM cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen Co.), at 37 °C and 5% CO_2.

2.3. Synthesis of PEG-Lipids. Synthesis of PEG-lipids (PEG-DMPE, PEG-DPPE, and PEG-DSPE) was performed with methods described previously (17). Briefly, DPPE (21 mg), DMPE (18 mg), or DSPE (24 mg) was dissolved in 3 mL of dichloromethane; NHS-PEG-Boc (185 mg) and triethylamine (3 μL) were added to the reaction solution and then stirred for 4 days at room temperature (RT). The NHS group of NHS-PEG-Boc readily reacted with an amino group of the phospholipids. The protective group, Boc, an amino group, was removed after the addition of 2 mL of 99% trifluoroacetic acid and an additional incubation at 4 °C for 30 min. The crude product was purified by reprecipitation in diethyl ether. After extraction with chloroform and evaporation, the PEG lipids were obtained as white solids. The PEG-DPPE, PEG-DMPE, and PEG-DSPE yields were 74, 80, and 75%, respectively (3). Their nuclear magnetic resonance (^{1}H NMR) profiles ($CDCl_3$, 400 MHz, δ ppm) were PEG-DMPE 0.88 (t, 6H, $-CH_3$), 1.25 (br, 40H, $-CH_2-$), 2.29 (dd, 4H, $-O-C(=O)-CH_2-$), 3.64 (br, 500H, PEG); PEG-DPPE 0.88 (t, 6H, $-CH_3$), 1.25 (br, 48H, $-CH_2-$), 2.29 (dd, 4H, $-O-C(=O)-CH_2-$), 3.64 (br, 480H, PEG); PEG-DSPE 0.88 (t, 6H, $-CH_3$), 1.25 (br, 56H, $-CH_2-$), 2.30 (dd, 4H, $-O-C(=O)-CH_2-$), 3.71 (br, 580H, PEG).

2.4. FITC-Labeling of Synthetic Polymers and DSPE. For fluorescent labeling, Boc was removed and the resulting PEG-lipids (20 mg) were allowed to react with FITC (3.1 mg) in acetone for 12 h (fPEG-lipids). fPEG-lipids were purified by gel permeation chromatography (Sephadex G-25). Synthesis of PVA-alkyl was performed with methods described previously (17). For labeling, PVA-alkyl (12.6 mg) was allowed to react with FITC (1.2 mg) in DMSO for 24 h (fPVA-alkyl), followed by dialysis against deionized water for 3 days and PBS for 1 day. DSPE (10 mg) was allowed to react with FITC (27 mg) (fDSPE) in chloroform for 24 h at RT. After evaporation of the chloroform, the crude product was washed with pure water. fDSPE (7 mg) was obtained after extraction with methanol and evaporation.

2.5. Cell Surface Attachment and Release of Synthetic Polymers. FITC-labeled synthetic polymers were used in this study unless otherwise specified. CCRF-CEM cells (2×10^6) were collected by centrifugation at RT. After removal of the supernatant, 50 μL of fPEG-DMPE (0.5 mg/mL), PEG-DPPE (0.5 mg/mL), fPEG-DSPE (0.1 mg/mL), or fPVA-alkyl (0.5 mg/mL) dissolved in PBS or 20 μL of fDSPE (7 mg/mL in methanol) was added to the cell suspension and incubated for 30 min with gentle agitation at 4 °C. Then, 10 mL of HBSS was added to the mixture and the cells were collected by centrifugation (180*g*, 5 min, RT, twice). These procedures were repeated twice to remove unreacted FITC-labeled synthetic polymers. Cell viabilities were determined by the trypan blue exclusion method.

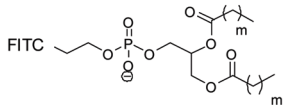
After treatment with synthetic polymers, cells were incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. Cell surface fluorescent intensities emanating from the polymers were followed at appropriate time intervals with a confocal laser scanning microscope (FLUOVIEW FV500, Olympus, Tokyo, Japan). Fluorescent intensities of the culture media were also determined with a fluorophotometer (F-2500, Hitachi, Co., Tokyo) as a function of time. The cells were also analyzed with a fluorescence-activated cell sorter (FCM, FACSCalibur, Becton Dickinson and Company, Franklin Lakes, NJ).

2.6. Effects of Cell Membrane Dynamics on Amphiphilic Polymers. The following experiments were carried out to determine whether the polymers were internalized through clathrin mediated endocytosis. A cell pellet (2×10^6 cells) was obtained after centrifugation at RT. After adding100 μL of rhodamine-labeled transferrin (0.10 mg/mL) to the cell suspension, the suspension was incubated for 30 min with gentle agitation at 4 °C. The cells were suspended in HBSS and then centrifuged at 180*g* for 5 min at 4 °C. After adding 50 μL of fPEG-DPPE or fPVA-alkyl (each at 0.50 mg/mL) or 20 μL of fDSPE (7 mg/mL in methanol) to the cell suspension, the suspension was incubated for 30 min with gentle agitation at 4 °C. After washing with HBSS, the modified cells were cultured for 3 h at 37 °C. The modified cells were then observed with a confocal laser scanning microscope.

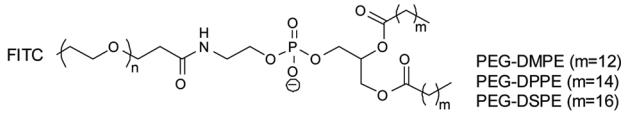
Cytochalasin D is known to specifically inhibit actin polymerization. Cells were treated with cytochalasin D to determine whether actin polymerization played a role in the behavior of amphiphilic polymers on the cell membrane. Cells (2×10^6) were collected by centrifugation at RT. After removal of the supernatant, 10 μL of cytochalasin D (10 μM) was added to the cell suspension. The suspension was incubated for 30 min with gentle agitation at RT and washed with HBSS to obtain cytochalasin D treated cells. Then, 50 μL of PVA-alkyl (0.5 mg/mL) was added to the cell suspension and incubated for 30 min with gentle agitation at 4 °C. The modified cells were washed with HBSS by centrifugation (180*g*, 5 min, 4 °C, 3 times) and then incubated in RPMI-1640 medium supplemented with 10% fetal bovine serum for 3 h at 37 °C under 5% CO_2. The modified cells were observed with a confocal laser scanning microscope.

Scheme 1. Chemical Structures of Lipid and Alkyl Polymers and Their Interactions with the Cell Surface[a]

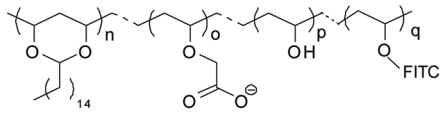
(a) Phospholipid (fDSPE, m=16)



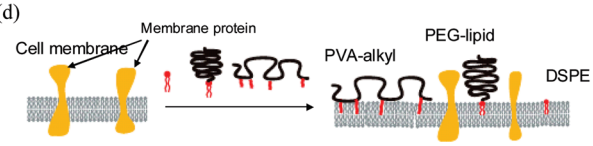
(b) fPEG-lipid (PEGMw:5000) (m=12,14,16)



(c) fPVA-alkyl (Mw 95000)



(d)



[a] m = the number of methyl groups on a polymer. (a) DSPE = 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine, (b) polyethyleneglycol (PEG)-conjugated lipids labeled with fluorescein isothiocyanate (FITC), $n \approx 114$: fPEG-DMPE (PEG with 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylethanolamine); fPEG-DPPE (PEG with 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine), and fPEG-DSPE; (c) fPVA-alkyl=poly(vinyl alcohol) with an alkyl side chain (n ≈ 55, o ≈ 117, p ≈ 1500, q, not detemined) Side groups were randomly introduced. (d) Schematic illustration of the interactions of fPEG-lipids, fPVA-alkyl, and fDSPE with the lipid bilayer of a living cell membrane.

3. RESULTS

3.1. Properties of Materials and Cells. The amphiphilic polymers used in this study are shown in Scheme 1. These polymers possessed long hydrophobic alkyl chains that interacted with the lipid bilayer of a cell membrane and thus, they anchored the polymer to the cell. PEG-lipids were prepared by conjugating a PEG chain with a phospholipid through an amide bond. Three PEG-lipids were prepared, including PEG-DMPE, PEG-DPPE, and PEG-DSPE, with chain lengths of 14, 16, and 18 alkyl groups, respectively. Each had two hydrophobic alkyl chains per PEG molecule that interacted with the cell membrane, as shown schematically in Figure 1d. PVA-alkyl was prepared by grafting an average of 28 alkyl chains onto a PVA molecule with acetal bonds. Thus, the PVA-alkyl polymer interacted with the cell membrane at multiple points.

3.2. Interaction of Materials with CCRF-CEM. CCRF-CEM cells float in culture and do not adhere to the cell culture dish. Treatment of cells with PEG-lipids and PVA-alkyls did not reduce cell viability. The cells proliferated during culture after treatment with those polymers. FITC fluorescence was observed at the periphery of all cells just after treatment (Figure 1a). This indicated that all of the polymers were anchored by their alkyl chains to the lipid bilayers of the cell membranes. However, different polymers exhibited different time courses and routes of disappearance from the cell membrane. fDSPE fluorescence was observed inside the cells 1 h after treatment (Figure 1). This implied that the lipid molecules, fDSPE, which were inserted into the lipid bilayer membrane, was rapidly taken up into the cell and then gradually disappeared from the inside of the cell. For the cases of the amphiphilic polymers, fPEG-lipids were also effectively incorporated into the cell membranes and then a small fraction of fPEG-lipids were observed inside of the cells. Most of fPEG-DSPE disappeared from the cell membrane over the 3 h of observation. fPVA-alkyl formed aggregates on parts of the cell membrane and/or internalized into a small region closed to the cell membrane (arrowheads, Figure 1) and then slowly disappeared from the cells.

3.3. Influence of the Alkyl Chain Length of fPEG-Lipids. The three PEG-lipids, PEG-DMPE, PEG-DPPE, and PEG-DSPE possessed 12, 14, and 16 methylene units, respectively, in their alkyl chains. These PEG-lipids were employed to examine the influence of alkyl chain length on the behavior of the polymer on the cell membrane (Figure 2). The fluorescent signals of all these fPEG-lipids were observed at the periphery of cells just after treatment. fPEG-DMPE, with the shortest alkyl chain length, rapidly disappeared from the cell membranes during the 3 h incubation. The fluorescence intensity of fPEG-DPPE decreased with time, but remained on the cell surface at 3 h. fPEG-DSPE remained on the cell surface even after incubation for 24 h. Thus, the stability of fPEG-lipids on the cell surface increased with increasing alkyl chain length. The release rates of fPEG-lipids also depended on the temperature of the cell culture medium (Figure 2). Strong fluorescence was observed on the periphery of cells in all three cases at 4 °C after 3 h of incubation. Thus, dissociation of fPEG-lipids from the cell membrane was highly dependent on temperature.

The decay of fluorescence intensities from cells was quantitatively followed by FCM analyses. Figure 3a shows the FCM profiles of fPEG-DPPE modified cells cultured at 37 °C for 0, 1, 3, 6, and 24 h. The cells had strong fluorescence intensities just after treatment with fPEG-DPPE, but the fluorescence intensity was decreased with time. The number of cells with low fluorescence intensity increased with time. This indicated that the number of fPEG-DPPE molecules on the cells decreased with time. The average fluorescence intensities at each time point were determined from FCM profiles and plotted in Figure 3b for cells treated with fDSPE, fPEG-DPPE, fPEG-DMPE, fPEG-DSPE, and fPVA-alkyl. Reductions in fluorescence intensity were caused by the release of polymers from the cells into the culture medium or by cell division. The fluorescence intensity of each cell would be expected to be halved with cell division. Thus, the relative fluorescence intensities per cell were calculated from the FCM analyses by adjusting for the increase in cell number due to cell division.

As shown in Figures 2 and 3, as the alkyl chain became longer, the fPEG-lipid remained attached longer on cells. These results suggested that hydrophobic interactions between alkyl chains and the lipid bilayers of cells were crucial for the sustained retention of fPEG-lipids on the cell surface.

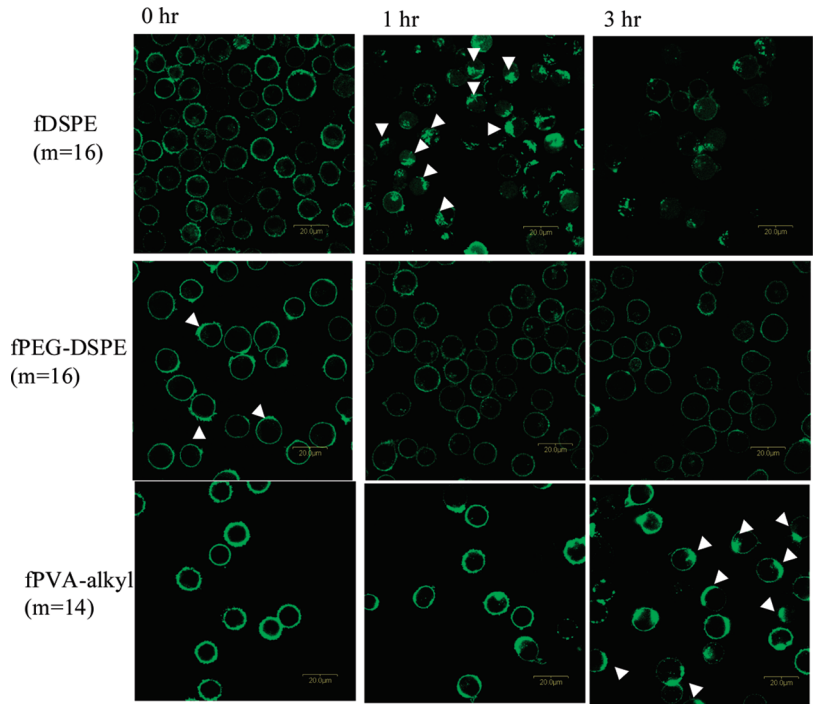


FIGURE 1. Representative confocal microscopic images of CCRF-CEM cells modified with FITC-labeled lipid and alkyl polymers. Images were taken just after (left, 0 h) the addition of the indicated polymers and 1 h (middle) and 3 h (right) later; *m* = the number of methyl groups on the polymer. (Top row) fDSPE on the cell membrane (0 h) was rapidly taken up into the cell (1 h; arrowheads), and gradually disappeared within the cell (3 h); (middle and bottom rows) fPEG-DSPE and fPVA-alkyl remained on the cell membrane and formed aggregates (arrowheads) before dissociating from the membrane.

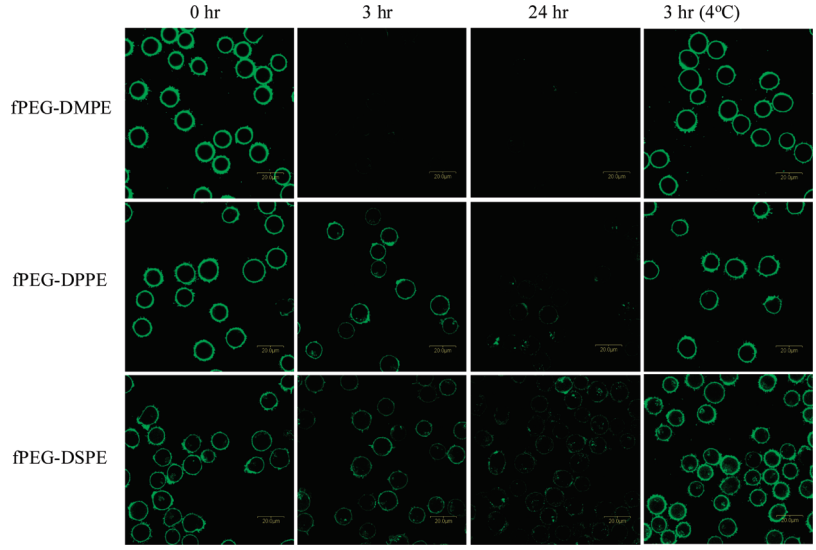


FIGURE 2. Representative confocal microscopic images of cells modified with FITC-labeled PEG-lipids. Images were taken just after (0 h, left) the addition of the indicated polymers, and 3 and 24 h later (middle left, middle right, respectively); for comparison, the behavior of attached polymers maintained at 4 °C for 3 h is shown in the right column. (Top row) fPEG-DMPE on the cell membrane (0 h) rapidly disappeared from the membrane (1 h, 3 h); (middle row) fPEG-DPPE and (bottom row) fPEG-DSPE remained on the cell membrane longer (3 and 24 h, respectively) and formed aggregates (bright spots).

Although fPEG-DPPE and fPVA-alkyl had the same number of methylene units in their alkyl chains, PVA-alkyl remained attached longer than fPEG-DPPE. This might be due to the multiple points of attachments by hydrophobic interaction between the PVA-alkyl polymer and the lipid bilayer. fDSPE in the lipid membrane was rapidly internalized into the cell, indicating that the mechanism of fluorescence intensity decay is different from fPEG-lipids and fPVA-alkyl. fDSPE that was internalized into the cell might be digested in lysosomes.

Most of fPEG-lipids and fPVA-alkyl were released into the culture medium of the cells from the cell surface. Fluorescence intensities of the culture media were determined with a fluorophotometer and plotted against incubation time in

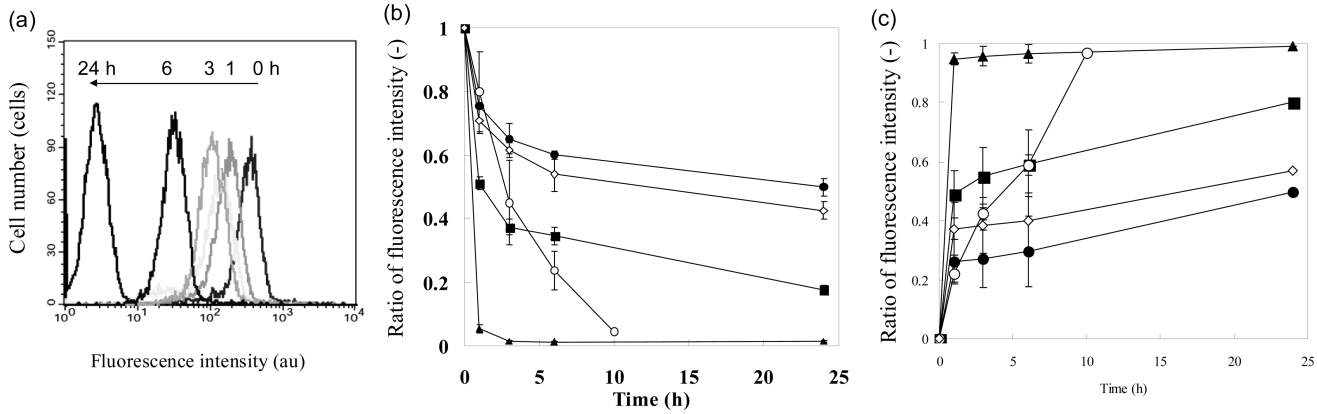


FIGURE 3. Retention of FITC-labeled DSPE and amphiphilic polymers on the cell surface. (a) Polymer attachment to the cell membrane was analyzed by flow cytometry at 0, 1, 3, 6, and 24 h after addition of fPEG-DPPE. Each curve represents the cell number plotted against the fluorescence intensity of fPEG-DPPE at each time point (time points increase from right to left). (b) Relative changes in averaged fluorescence intensities of cells modified with fDSPE (open circles), fPEG-DPPE (filled squares), fPEG-DMPE (filled triangles), fPEG-DSPE (filled circles), and fPVA-alkyl (open diamonds) over the indicated time periods. The values were normalized to the mean fluorescence intensity at 0 h. Those cells were analyzed by flow cytometry after wash with HBSS. (c) The release of FITC-labeled DSPE and amphiphilic polymers from living cells into the culture medium. The relative fluorescent intensities of polymers (fPEG-lipids, fPVA-alkyl) in the medium at each time point were calculated under assuming the total fluorescence intensities of cells and media to be 1 at 24 h. For fDSPE, total fluorescence intensity was normalized at 10 h. Symbols are the same as those in b.

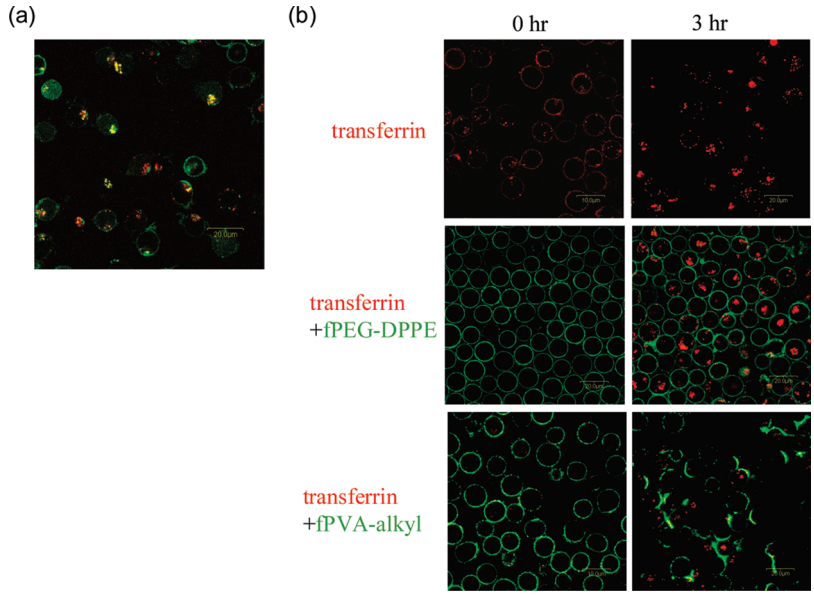


FIGURE 4. Effect of clathrin-mediated endocytosis on the behavior of fDSPE and amphiphilic polymers on the cell membrane. (a) Confocal microscopic images of cells modified with fDSPE (green) and rhodamine-transferrin (red) after incubation at 37 °C for 3 h. Yellow areas indicate the colocalization of fDSPE and rhodamine-transferrin. (b) Confocal microscopic images of cells treated with rhodamine-transferrin (red) and amphiphilic polymers fPEG-DPPE and fPVA-alkyl (green) after incubation at 37 °C for 3 h.

Figure 3c. These release profiles coincided well with the attachment profiles determined from the FCM analyses of cells.

3.4. Synthetic Polymer Surface Modification Influence on the Cell. Although fDSPE was rapidly internalized into the cytoplasm, most of fPEG-DSPE was not internalized, but released into the medium directly from the cell membrane. Most of the fPEG-lipids were uniformly distributed on the cell membrane during the observation period, but the PVA-alkyls formed aggregates on part of the cell membrane. The fate of the amphiphilic polymers was highly dependent on their molecular structures. We studied the dynamic features of the cell membranes to gain insight into the behaviors of amphiphilic polymers on the cell membrane.

It is known that transferrin is rapidly internalized through clathrin mediated endocytosis (20, 21). Cells were treated with a mixture of rhodamine-labeled transferrin and fDSPE to compare their dynamics. A confocal laser scanning microscopic image of the cells taken 3 h after treatment (Figure 4a) showed that some areas inside the cells were yellow; this indicated that some DSPE on the cell membrane had been drawn into endocytotic vehicles with transferrin. In separate experiments (Figure 4b), cells were sequentially treated with rhodamine-transferrin and either fPEG-lipid or fPVA-alkyl. These procedures were performed at 4 °C to

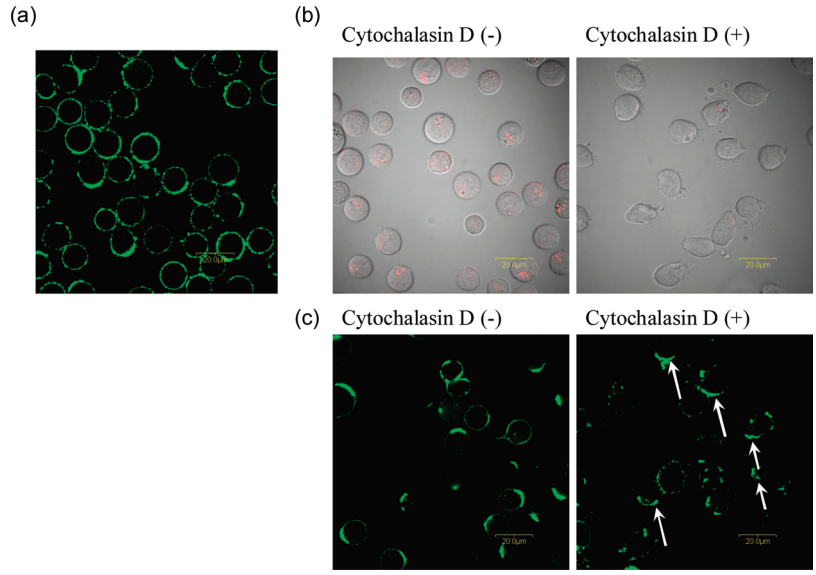


FIGURE 5. Effect of temperature and addition of cytochalasin D on the behavior of fPVA-alkyl on the cell membrane. (a) Confocal microscopic images of cells modified with fPVA-alkyl after incubation for 3 h at 4 °C. (b) Uptake of rhodamine-dextran (red) during incubation of cells treated with or without cytochalasin D. The pictures were merged images of fluorescence image which shows location of rhodamine-dextran (red) and differential interference image. (c) Behavior of fPVA-alkyl on the membranes of cells treated with or without cytochalasin D. Note that fPVA-alkyl aggregates formed in both conditions (arrows).

suppress endocytosis. Both green and red fluorescent signals were observed at the periphery of the cells just after treatment although the red fluorescence was weak for observation. The cells were then incubated for 3 h at 37 °C. At that time, most of the transferrin had been internalized into the cytoplasm and could be observed inside cells; however, the fPEG-DPPE remained on the cell surface. Moreover, fPVA-alkyl had aggregated on part of the cell membrane. The transferrin was selectively taken up through clathrin mediated endocytosis, even though transferrin coexisted on the cell membrane with either the fPEG-lipids or fPVA-alkyls. The disappearance of these amphiphilic polymers from the cell surface was independent of transferrin induced endocytosis.

The temperature of the culture medium highly affected the dynamics of fPEG-lipids on the cell membrane (Figure 2). The temperature dependence of fPVA-alkyl aggregation on the cell membrane was also examined (Figure 5a). Although fPVA-alkyl aggregated on part of the cell membrane during the 3 h incubation at 37 °C (Figure 1a), its aggregation could not be observed on cells incubated at 4 °C for 3 h (Figure 5a).

Cytochalasin D is a specific inhibitor of actin polymerization (22). Dextran is known to be taken up via a nonclathrin mediated process (21) that depends on actin polymerization. Figure 5b shows that dextran was taken up into the cytoplasm without cytochalasin D treatment, but not with cytochalasin D treatment. Furthermore, cytochalasin D treatment caused cell deformation. We also examined the effects of cytochalasin D on the dynamics of fPVA-alkyl on the cell membrane. We observed that fPVA-alkyl formed aggregates on part of the cell membrane even after cytochalasin D treatment (Figure 5c). Therefore, the aggregation of fPVA-alkyl on the cell membrane did not correlate with a nonclathrin mediated endocytosis.

4. DISCUSSION

Our group has previously examined amphiphilic polymers, including PEG-lipids and PVA-alkyls for surface modification and engineering of living cells for transplantation (4, 5, 12−17). However, amphiphilic polymers also tended to disappear from the cell surface with time (17). Therefore, it was necessary to understand the dynamic behaviors of amphiphilic polymers introduced onto the cell surface for applications of surface modification in various biomedical scenarios.

For this study, we used phospholipids and amphiphilic polymers, like PEG-lipids and PVA-alkyls, with hydrophobic sections that could form an anchor to the lipid bilayer of cell membranes via hydrophobic interactions. The phospholipid alone, DSPE, was quickly internalized into the cytoplasm (Figure 1), reflecting rapid cycling of lipid molecules between the cell membrane and the cytoplasmic compartment (20). It is well-known that macrophages take up all lipid molecules into the cytoplasm every 30 min (21). We found that the addition of a large hydrophilic residue, like PEG or PVA, to a hydrophobic lipid could form amphiphilic polymers, fPEG-lipids and fPVA-alkyls, which were rarely taken up into the cytoplasm. Interestingly, we found the bright area on the cell membrane several hours after the cells treated with amphiphilic polymers with multipoint anchoring to the lipid bilayer membrane, particularly in the case of fPVA-alkyl. The bright area may be formed by aggregation of the polymers or zones of slower desorption of the polymers. Photos of cells treated with fPVA-alkyl shown in Figure 1 were taken under the same condition. The area indicated by arrowheads became brighter with time during initial few hours. This fact indicates that fPVA-alkyl tended to form aggregates on the cell surface. The intramolecular aggregation between alkyl chains of fPVA-alkyl appeared to compromise its stability,

and its maintenance on the membrane was shorter than expected. Moreover, PVA-alkyl aggregated on the cell membrane even after cells were treated with cytochalasin D. Therefore, fPVA-alkyl was not endocytosed nor inhibit nonclathrin-mediated endocytosis of fluorescence-labeled dextran. There are many cell activities involving membrane trafficking, lipid raft formation and addition of newly synthesized lipids and proteins to the cell membrane which may cause the aggregation of fPVA-alkyl. Additional studies are required to identify the mechanism of fPVA-alkyl aggregate formation.

Cell surface modifications with amphiphilic polymers are applied for different purposes in various biomedical scenarios as follows: (i) To suppress the early loss of grafted islets, caused by instant blood-mediated inflammatory reactions in intraportal transplantation. Cell surface modifications of islets include the attachment of biomolecules, like heparin, thrombomodulin, urokinase, and complement receptor, to confer antithrombogenic and anti-inflammatory properties; this function is required for only a few days after infusion of the donor islets into the recipient liver. (ii) To immuno-isolate transplanted islets. The modified membrane covers the donor islets to isolate them from the recipient's immune system. In this case, the modified membrane should be stable for a long time, to allow the islets to function. As mentioned above, molecules attached with hydrophobic interactions and even with covalent bonding tend to disappear after several hours to several days. Thus, the cell surface modifications described in the present study are expected to be applicable to the suppression of early loss of islets, but would not be stable enough for immuno-isolation. Future studies should focus on improving the conformal coatings necessary for long-term immuno-isolation. (iii) We have previously developed cell arrays for high throughput cell-based assays (26). In this application, cell surface modification was used to immobilize selected cells to a fixed spot. After the cells were attached to the spots, the amphiphilic polymers would detach from the cell surface with time, allowing the unmodified cell to function freely. The PEG-lipids examined in this study would be suitable for that application. (iv) Similarly, surface modifications for the purpose of cell−cell interaction analyses (14) introduced amphiphilic polymers on the cell surface that could detach after the induction of intercellular attachments; thus, the polymers would not disturb the intercellular interactions between cells. In this case, stable attachments to the cell surface might have adverse effects. Thus, the methods of cell surface modification should be specifically designed to meet the requirements necessary for each application.

5. CONCLUSION

The surface of a cell can be modified with amphiphilic polymers, like fPEG-lipids and fPVA-alkyls, which attach to the membrane through hydrophobic interactions. We showed that the stability of these polymers on the cell surface was influenced by the length and number of alkyl chains. fPEG-DMPE was rapidly released from the cell surface, but fPEG-DPPE and fPEG-DSPE were gradually released, perhaps because of stronger hydrophobic interaction with increasing alkyl chain length. Although the PVA-alkyl had multiple interactive points, the intramolecular aggregation between alkyl chains appeared to compromise its stability, and its maintenance on the membrane was shorter than expected. Thus, these types of amphiphilic polymers would not be useful for covering the whole cell surface for long periods of time. This study demonstrated that the stability of modified amphiphilic polymers on the cell surface was influenced by both hydrophobicity and molecular structure.

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