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Utilizing temperature-sensitive association of Pluronic F-127 with lipid bilayers to control liposome–cell adhesion

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Abstract

The temperature sensitive properties of Pluronic F-127 (MW ~12 600, PEO_{98}-PPO_{67}-PEO_{98}), a block co-polymer or poloxamer, was used to control liposome–cell adhesion. When associated with liposomes, the PEO moiety of the block co-polymer is expected to inhibit liposome–cell adhesion. Liposomes were made using egg phosphatidylcholine and different mole% of Pluronic F-127. Size measurement of the liposomes at different temperatures, in the presence and absence of Pluronic F-127, shows significant reduction in the size of multilamellar vesicles, at higher temperatures, by the Pluronic molecules. Negative stain electron microscopy study showed the presence of individual molecules and micelles of Pluronic, respectively at temperatures below and above the critical micellar temperature (CMT). Measurement of the surface associated Pluronics indicated that they associated with liposomes when the sample was heated above the Pluronic CMT, and dissociated from liposomes when cooled below the CMT. Attachment of the Pluronic containing liposomes to CHO cells was inhibited at temperatures above the CMT, but not at temperatures below CMT, indicating that temperature-sensitive control of liposome–cell adhesion is achieved.

Keywords: Polyethylene glycol; Stealth liposome; Light scattering; CHO cell; Electron microscopy; Micelle; Poloxamer

1. Introduction

'Stealth' liposomes [1] or 'cryptosomes' [2] have been used to improve the efficiency of drug delivery by liposomes. Conventional liposomes are intercepted at an early stage of circulation by the mononuclear phagocyte system (MPS) [3]. Unlike conventional liposomes, 'stealth' or sterically stabilized liposomes show reduced uptake by the MPS, thus prolonging their circulation half-life considerably [4–6]. Stealthing of conventional liposomes is done primarily by using polyethylene glycol (PEG) conjugated lipids at different concentrations and compositions [7,8]. In recent years poloxamers, a group of tri-block co-polymers, have also been used to sterically stabilize liposomes [9–11].

Poloxamers, or Pluronics, are polyethylene oxide (PEO)-polypropylene oxide (PPO)-polyethylene oxide tri-block co-polymers of different molecular weights. The hydrophobic PPO group in the middle links the two hydrophilic PEO groups. The amphiphilic nature of the poloxamers makes them extremely useful in various applications as emulsifiers and stabilizers [12]. In an aqueous environment, po-

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loxamers at a given concentration would remain as individual (non-associated) co-polymers, from here on termed as 'monomers', at temperatures below their critical micellar temperature (CMT). Above the CMT the molecules become more lipophilic, and form micelles with hydrophobic PPO groups at the core of the micelle. Poloxamers of different molecular weights and with different hydrophil–lipophil balance (HLB) have different CMTs [13]. This monomer-to-micellar transition process is extremely temperature-sensitive. With a small change of temperature, the corresponding critical micellar concentration (CMC) may change by several orders of magnitude [14].

Several studies have been done regarding the interaction of poloxamers with liposomes [15–17]. Poloxamers causing moderate to severe structural changes in liposomes were observed using cryo-transmission electron microscopy [18]. As observed by techniques like NMR [17], dynamic light scattering [15–17] and differential scanning calorimetry [19], poloxamers can either be incorporated in or adsorbed on the liposome surface to cause steric stabilization.

Although poloxamers have been used for stabilizing liposomes, no report has been published on utilizing the thermal properties of poloxamers to manipulate liposome–cell adhesion resulting in better adhesion of sterically protected liposomes to the targeted site of delivery. Our approach in this paper is to utilize the temperature sensitive transitions of poloxamers to first sterically protect and subsequently de-protect liposomes in vitro. We used Pluronic F-127 (MW $\sim$12 600, PEO_{98}-PPO_{67}-PEO_{98}), a poloxamer, in this study for its high molecular weight, desired HLB and a CMT around the physiological temperature. Pluronic F-127 has versatile use including use in the pharmaceutical industry. Here we report our characterization of temperature dependent size change of the liposomes in the presence of Pluronic F-127. Negative stain electron microscopy study shows the presence of individual Pluronic F-127 molecules and micelles at temperatures below and above the CMT, respectively. We measured the amount of liposome-associated Pluronics through the stealthing and de-stealthing processes. Lastly, a cell attachment study of the Pluronic-containing liposomes shows the effectiveness of the control of liposome–cell adhesion processes.

2. Materials and methods

2.1. Materials

All the lipids – egg phosphatidylcholine (EPC), dioleoyloxytrimethylammonium propane methyl sulfate (DOTAP), dipalmitoyl rhodamine phosphatidylethanolamine (DPRhPE) and distearoyl(polyethylene glycol 5000) phosphatidylethanolamine (PEG5000-DSPE) – were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The lipids were stored at −80°C. Pluronic F-127 was provided by BASF (Mount Olive, NJ, USA) as a free sample. Cobalt nitrate and ammonium thiocyanate were obtained from Aldrich (Milwaukee, WI, USA). Ethyl acetate was obtained from EM Sciences (Gibbstown, NJ, USA). Absolute (100%) ethanol was from Pharmco Products (Brookfield, CT, USA). Cell culture media, including F-10, minimum essential medium (MEM), newborn calf serum (NCS), RPMI 1640 and penicillin-streptomycin-neomycin, were purchased from Gibco BRL (Grand Island, NY, USA). Twelve-well tissue culture dishes were purchased from Corning (Corning, NY, USA). Chinese hamster ovarian (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Triton X-100 was obtained from Kodak (Rochester, NY, USA). Copper specimen grids, and other negative stain experiment reagents were purchased from Electron Microscopy Sciences (Fort Washington, PA, USA). All reagents used were of analytical grade.

2.2. Liposome preparation

All liposomes were made with EPC and different mole (or weight) % of Pluronic F-127. Multilamellar vesicles (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) were made for different experiments. Pluronics were either co-solubilized with the lipid during preparation of liposomes or added afterwards to the already formed liposomes. For cell adhesion experiments, DOTAP and DORhPE were added to make the liposomes.

Lipids, in chloroform, were mixed in a round-bottomed flask and dried under a gentle stream of nitrogen gas to form a thin layer on the flask wall. The film was dried further in a vacuum desiccator, for

3–4 h, to remove any remaining solvent. MLVs were formed by first resuspending the dry lipid film with deionized water, or buffer, followed by vortexing. SUVs were formed by sonicating the MLV solution in a bath type sonicator (Laboratory Supplies, Hicksville, NY, USA). Sonication was done under a nitrogen atmosphere, for 10 min or more, until the solution turned clear. LUVs were formed by extruding the MLV solution through a 0.4 μm polycarbonate filter (Millipore, Bedford, MA, USA), for 15 times or more. All liposomes were prepared and kept inside a cold room (4°C), before use in the experiment.

2.3. Temperature dependent liposome size measurement

Liposome sizes were measured by quasi-elastic light scattering (QLS), using a model 370 submicron particle sizer (Nicomp Particle Sizing Systems, Santa Barbara, CA, USA). The particle sizer was calibrated with polystyrene latex spheres (Interfacial Dynamics, Portland, OR, USA) of sizes between 30 and 2980 nm and was found accurate for all diameters. An argon ion laser, with a maximum CW output of 2 W, was used as the light source. The photon counts were always adjusted to about 300 kHz. The size of the liposomes was analyzed by the multimodal NICOMP vesicle analysis program. The volume-weighted mean vesicle diameter was used for all our experimental purposes. Viscosity and index of refraction values of the medium were adjusted to allow for the change with changing temperature. The lipid concentration of each sample was 1.1 mg/ml and final Pluronic F-127 concentration in the liposome solution was 0.032% (w/v). All the samples were made at 4°C. During the experiment, the sample to be measured was kept at the desired temperature by a Peltier unit of the QLS machine. Each sample was kept in the sample chamber for at least 15 min, to achieve thermal equilibrium, before starting measurement. All the other samples, used in the same experiment, were kept at their respective desired temperatures in water baths. QLS was also used to determine the micellar size of the Pluronic in an aqueous environment.

Because we used MLVs made of EPC, DOTAP and DPRhPE for the experiment involving liposome adhesion to CHO cells, we must also measure the thermally induced change in the size of EPC/DOTAP MLVs. To be consistent with the liposome adhesion experiment, we performed this experiment through the same temperature range of 4–41°C. All samples were made, at 4°C, with EPC and 8 mole% DOTAP. The 'control' MLVs did not have any Pluronic F-127 while the 'F-127' MLVs had 0.03% (w/v) Pluronic F-127 in the sample environment. The temperature was gradually cycled from 4°C to 41°C to 4°C during measurement.

Sample turbidity provided an estimation of vesicle size changes. We used static (90°) light scattering, at 600 nm, to measure the turbidity, i.e. relative scattering intensity (which corresponds to the size), of the liposomes at different temperature points with a SLM 8000 (SLM Instruments, Urbana, IL, USA) fluorimeter.

2.4. Negative stain electron microscopy of liposomes

The negative stain electron microscopy of the Pluronic-containing liposomes was used to examine the association of the Pluronic with liposomes at different temperatures. EPC liposomes, with or without 1 mole% (0.03% w/v) Pluronic F-127, were prepared and then temperature treated, as described in Section 2.5. The temperature treated liposomes were used as samples for negative staining.

A drop of the liposome suspension was added to the surface of a carbon/Formvar coated 400 mesh grid, and after a few seconds the excess sample was removed. This was immediately followed by the addition of a drop of 2% ammonium molybdate solution. After removing the excess stain, the dried grids were kept in grid holders for future observation. All the preparations were done in a dust-free environment. The cold and hot samples were prepared inside a temperature-controlled room.

A Siemens 101 electron microscope was used for the transmission electron microscopic (TEM) observation of the sample grids. Usually a 60 000–100 000 magnification was used to take all the pictures.

2.5. Pluronic assay

The assay was designed to detect the amount of surface associated Pluronic F-127 present in the lipo-

somes. EPC MLVs were made at 4°C and a concentrated (60 mg/ml) aqueous Pluronic solution was added to the MLVs to make the F-127 concentration in the solution about 0.75% (w/v). Pluronic was added after the formation of the liposomes to ensure that no Pluronic was present inside the liposomes. Eppendorf tubes were used to hold the samples. One third of the samples were kept at 4°C ('cold' samples), while the remaining were kept at 41°C. After 30 min, half of the samples at 41°C were brought back to 4°C ('thermally cycled' samples). The samples kept at high temperature were used as the 'hot' samples. Thermally cycled samples were kept in cold condition for 30 min to attain thermal equilibrium. Each sample was then diluted 10 times (i.e. 1/10), using distilled water, and centrifuged for 20 min at $15\,700 \times g$. A pellet formed at the bottom of the centrifuge tube. The supernatant was carefully removed, using a micropipet, so as not to disturb the pellet, and stored for further analysis. Dilution, centrifugation and supernatant removal was done in cold conditions (4°C), for the 'cold' and 'thermally cycled' samples, or hot conditions (41°C), for the 'hot' samples. 40 μl of 10% Triton X-100 and 160 μl distilled water were added to each pellet and vortexed to thoroughly dissolve the pellet into a solution. These pellet and supernatant solutions were used for the assay of Pluronics.

A simple assay for Pluronics was originally developed by Greff et al. [20] and later modified by Tercyak and Felker [21]. Further simplification involved the formation of a complex of Pluronic with cobalt thiocyanate. The absorbance of the solubilized complex was measured spectrophotometrically and used as a quantitative measure of Pluronic F-68 [22].

We used the same assay procedure [22] for Pluronic F-127. Briefly, 100 μl cobalt thiocyanate solution, 200 μl ethyl acetate and 80 μl of absolute ethanol were added to 200 μl Pluronic F-127 standard (or a sample containing an unknown amount of Pluronic). The resulting suspension was mixed well, in a 1.5 ml Eppendorf centrifuge tube, by mild vortexing and then centrifuged for 2 min at $15\,700 \times g$. After centrifugation, the supernatant was carefully removed without disturbing the pellet. The pellet and tube walls were carefully washed with 200 μl of ethyl acetate, several times, until the aspirated ethyl acetate became colorless. The pellet was thoroughly dissolved in 2 ml acetone by vortexing and the absorbance was measured using a Zeiss spectrophotometer (Brinkmann Instruments, NY, USA) at 328 nm. The concentration of Pluronic in the unknown samples was determined using a calibration curve. Absorbance measurements were carried out in triplicate for all the standard dilutions and unknown samples.

2.6. Adhesion of liposomes to CHO cells

CHO cells were grown in F-10 medium plus 13% NCS at 37°C and 5% CO_2. All cells were passaged twice weekly. Cells were seeded at a concentration of 1×10^6 cells per well in 12-well plates. The cells were allowed to grow for 24 h, reaching approximately 80% confluence, before experimentation. Cells were washed twice with F-10 medium and incubated at cold (4°C) or hot (41°C) condition for 20 min before adding 100 μl liposome solution to each well. MLVs of EPC with 8 mole% DOTAP, 1 mole% DPRhPE and 1 mole% Pluronic F-127 were used. Control samples did not have any Pluronic in them. Since 0.75 mole% PEG5000 conjugated lipid is generally sufficient to inhibit cell adhesion to a bilayer surface [23], liposomes were made with 1 mole% PEG5000-DSPE, instead of 1 mole% Pluronic, to serve as positive control. The liposome solutions were treated with cold and/or hot conditions (similar to the treatment described in Section 2.5) for 20 min or more. After adding liposome solution to the wells, they were incubated at respective temperatures. The cells were washed twice with PBS to remove any non-adhering liposomes. Cells from each well were scraped into a cuvette. PBS solution was added to the cells to make a total volume of 3 ml. Fluorescence intensity of the resulting suspension was measured with an excitation wavelength of 550 nm and an emission wavelength of 590 nm using a SLM 8000 fluorimeter.

3. Results

3.1. Temperature dependent liposome size measurement

This experiment is designed to see the effect of Pluronic F-127, if any, on the size of the EPC

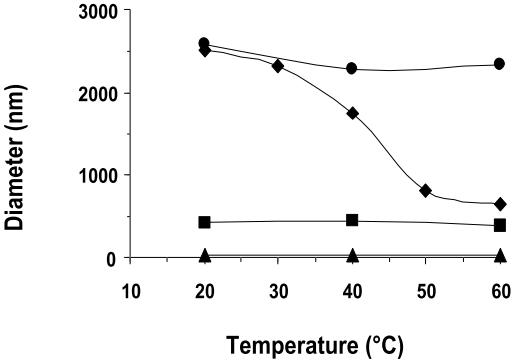

Fig. 1. Change in vesicle size with temperature, as determined using QLS. All vesicles are made of EPC and 0.032% (w/v) Pluronic F-127 except the control sample which has no F-127. ♦, MLV; ■, LUV; ▲, SUV; ●, control MLV.

vesicles during temperature change. Fig. 1 shows the result of the dynamic light scattering experiment. The control sample contains MLVs made of EPC only. The other three samples, MLV, LUV and SUV, are all made of EPC and 1 mole% (0.03% w/v) Pluronic F-127. The initial diameter of the 'control', MLV, LUV and SUV samples was 2.58 μm, 2.52 μm, 0.43 μm and 34 nm, respectively. From Fig. 1, we can see that 'control' sample diameter does not decrease much over the whole temperature range of 20–60°C, ending at 2.34 μm, a decrease to 91% of the initial size. The MLV sample diameter does not decrease significantly at 30°C. But at 40°C it decreases to 1.75 μm, which is 70% of the initial diameter. From 40°C to 50°C, the size reduces even further to a diameter of 0.81 μm (32%). At 60°C the diameter is 0.65 μm. The LUV diameter reduces slightly to 0.39 μm, about 90% of the low temperature value. SUV sample size remains more or less the same throughout the temperature range. Thus, the presence of 1 mole% Pluronic F-127 seems to affect the MLV sample most, decreasing its diameter to 26% of the initial value over this temperature range.

The average diameter of a Pluronic F-127 micelle, as determined by QLS, is 23 nm. The size of MLV

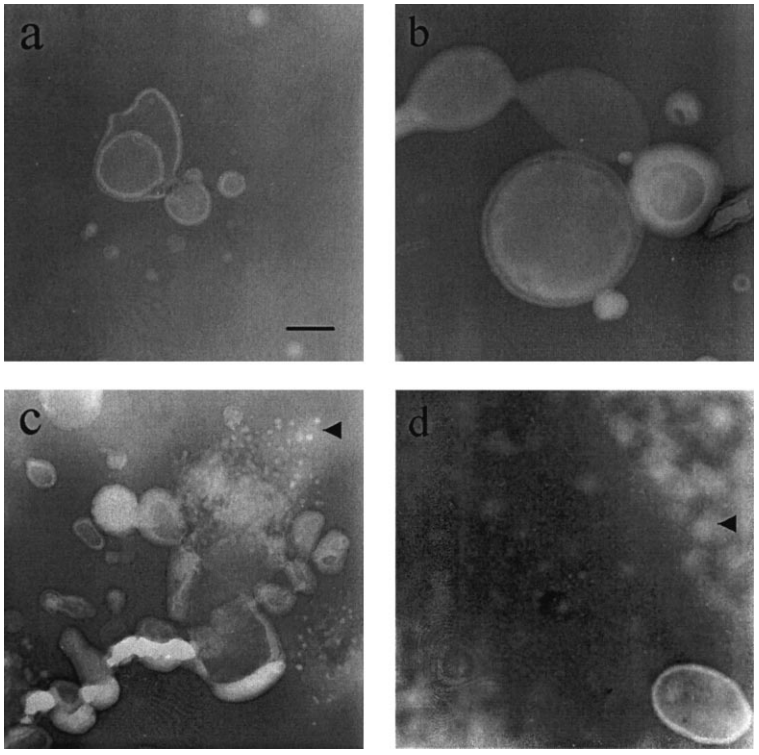

Fig. 2. Negative stain electron micrographs. The control MLV samples (a,b) are made of EPC only, while the F-127 MLV samples (c,d) are made of EPC with 0.032% (w/v) Pluronic F-127. Micrographs a and c are of samples made at 4°C and micrographs b and d are of samples made at 41°C. The presence of individual Pluronic molecules (small white dots) in panel c and micelles (fluffy white structures) in panel d is indicated by arrowheads. Scale bar is 100 nm.

containing 8 mole% DOTAP, with or without Pluronic F-127, was also measured. The result of the size measurement experiment of EPC/DOTAP MLVs is presented in Table 1. The experiment was performed at two temperatures: 4°C and 41°C. For both temperatures, the size of the 'F-127' DOTAP MLVs is larger than that of the 'control' DOTAP MLVs. There is an average increase of 23% and 15% in the diameter of the 'F-127' samples over those of the 'control' samples, respectively at 4°C and 41°C. All DOTAP-containing MLVs are smaller than their counterpart without DOTAP (Fig. 1).

Turbidity measurement (data not shown) supports the QLS size determination, showing similar trend in size reduction of the vesicles upon heating. These results also show that liposome sizes remain the same even when the samples are cooled down, indicating that there is no significant aggregation during the cooling process.

3.2. Negative stain electron microscopy

This experiment is designed to visualize the effect of Pluronic F-127 on liposomes. Pluronic F-127, at a concentration of 0.03%(w/v), has a CMT of about 35°C. Negative staining samples were prepared below CMT (4°C) and above CMT (41°C) to observe the behavior of Pluronic F-127 molecules in the presence of lipid vesicles at two significantly different temperature conditions. Fig. 2 consists of micrographs showing images of negative stained EPC MLVs at different temperatures. Fig. 2a and b are control samples consisting of EPC MLVs only while Fig. 2c and d are F-127 samples consisting of EPC MLVs with 0.03% (w/v) Pluronic F-127. Fig. 2a and c show samples made at 4°C, while the samples seen in Fig. 2b,d are made at 41°C. Fig. 2a shows vesicles with an even background. Fig. 2c, in contrast, shows vesicles with tiny white dots in the background. The sizes of these dots on average are about 10 nm diameter and roughly correspond to the size of the individual Pluronic F-127 molecules in aqueous solution. The diameter of individual Pluronic F-127 molecules, based on the calculation of their radius of gyration [24], was 7 nm. Fig. 2b again shows vesicles with smooth background. Fig. 2d shows one vesicle at the lower right hand corner, and fluffy white structures over the background area. These fluffy structures are larger than the white dots seen in Fig. 2c. Again, the average size of these structures (44 nm diameter), though somewhat higher, corresponds to the size of the Pluronic F-127 micelles (23 nm) as measured by the quasi-elastic light scattering technique.

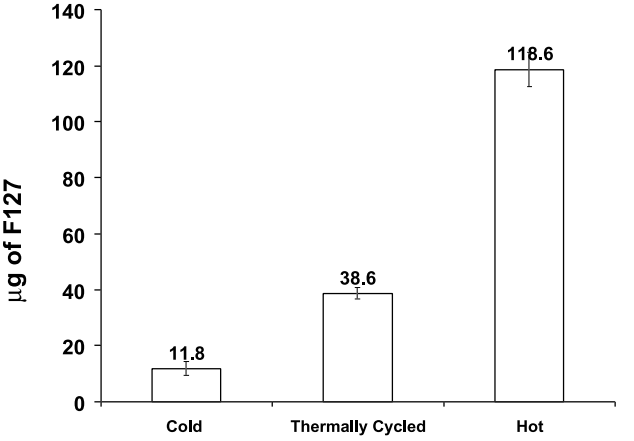

Fig. 3. Amount of Pluronic F-127 associated with liposomes in a sample, as determined by the Pluronic assay. The three different samples are: cold, thermally cycled and hot. The number above each sample point represents μg of Pluronic F-127 for the corresponding sample. Error bar represents variations among at least three repeating samples.

3.3. Pluronic assay

In order to understand the association/dissociation of Pluronic F-127 with the liposome bilayers during the temperature change, we measured the amount of Pluronic F-127 present in each sample. Fig. 3 shows

Table 1
Change in diameter of EPC (with 8 mole% DOTAP) MLVs with temperature

Temperature (°C)	Diameter (nm) 'F-127' sample	Diameter (nm) 'control' sample
4	1051	852
41	991	864

'F-127' and 'control' samples are liposomes with and without Pluronic F-127, respectively.

the amount of Pluronic F-127 associated with the liposomes of different samples. The three samples shown are pellets obtained from the 'cold', 'thermally cycled' and 'hot' samples (see Section 2.5 for details). The primary data obtained from the experiment were OD (optical density) values, which were converted to μg of F-127, using a calibration curve. The assayed amount of Pluronic in all the different samples fell in the linear portion of the calibration curve. The amount of F-127 present in the 'cold', 'thermally cycled' and 'hot' sample is 11.8, 38.6 and 118.6 μg, respectively. Apparently, the 'hot' sample retains 10-fold more Pluronic than the 'cold' sample, and 3-fold more than the 'thermally cycled' one.

3.4. Liposome–cell adhesion experiment

After we characterized the interaction of Pluronic F-127 with liposomes, we proceeded to study the effect of Pluronic F-127 in controlling the adhesion of the liposomes to cell surfaces at different temperature conditions. Fig. 4 shows the result of association (adhesion plus internalization) of fluorescently labeled liposomes with CHO cells. The lipids used for this experiment were EPC with 8 mole% DOTAP and 1 mole% fluorescent lipid DPRhPE, with or without 1 mole% Pluronic F-127. DOTAP at 8 mole% was the optimum amount to facilitate adhesion without suppressing the inhibitive effect of liposome–cell adhesion by Pluronic F-127. The control sample was without F-127. The samples marked 'cold', 'thermally cycled' and 'hot' were with F-127, as described in Section 3.3, while the sample marked 'PEG5000' was made with 1 mole% of PEG5000-DSPE, instead of the F-127. This sample serves as a positive control. All the fluorescence intensity values were normalized against the respective (hot or cold) 'control' values. After normalization, the 'thermally cycled' sample, with a fluorescence reading of 1.59, is as fluorescent as the 'cold' sample (1.61). The 'hot' sample, on the other hand, has as low fluorescence intensity as that of the 'PEG5000' sample. The normalized fluorescence intensity values of 0.29 and 0.34 for 'hot' and 'PEG5000' samples, respectively, are significantly lower than the fluorescence intensity value of the 'control' sample, showing inhibition to the adhesion of liposomes to the cell surfaces.

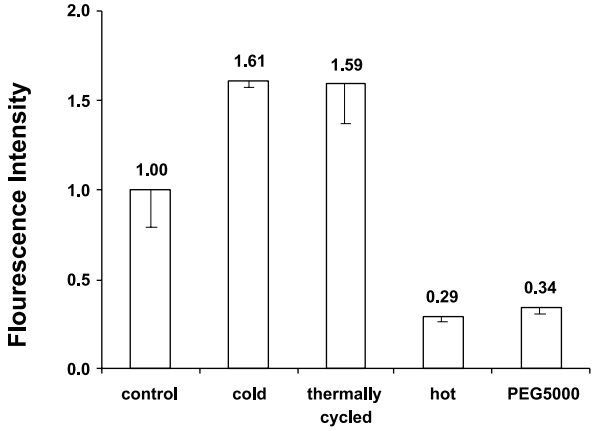

Fig. 4. Association of fluorescently labeled MLVs to CHO cells. All samples are made of EPC, 8 mole% DOTAP and 1 mole% DPRhPE. Additionally, the cold, thermally cycled and hot samples have 0.03% (w/v) Pluronic F-127, while the PEG5000 sample has 1 mole% PEG5000-DSPE. The number above each sample point represents the fluorescence intensity value for the corresponding sample. Error bar represents variations among at least three repeating samples. All samples are normalized against the control sample.

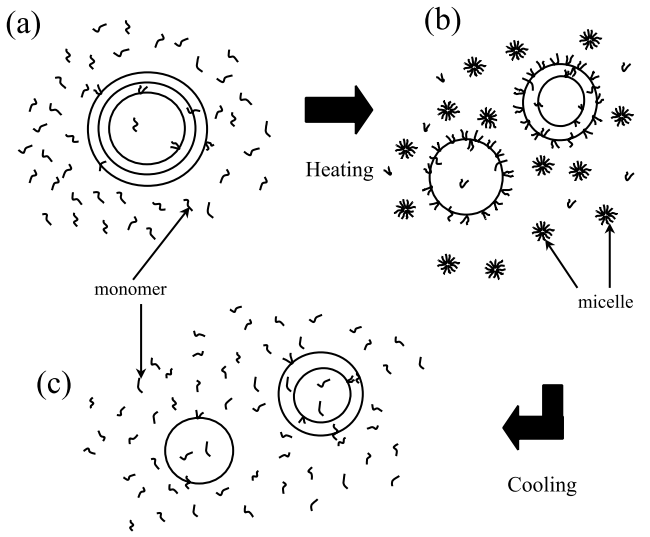

Fig. 5. Schematic diagram of the fate of EPC, in the presence of Pluronic F-127, during thermal cycling: (a) at 4°C, larger MLVs with few individual Pluronic F-127 molecules associated with the liposomes and most individual molecules floating outside the liposomes; (b) at a temperature above the CMT (e.g. 41°C), smaller size liposomes with many surface associated Pluronic F-127 and presence of Pluronic F-127 micelles outside the liposomes; (c) when cooled down below the CMT (e.g. 4°C), liposome size remains the same with individual Pluronic F-127 molecules present all around.

4. Discussion

The use of PEG conjugated lipids to form stealth liposomes is a well-accepted process. The predecessors of PEG-lipids, namely GM1 ganglioside [5,25] and phosphatidylinositol [5,26], though effective in increasing $t_{1/2}$ of the conventional liposomes, could not match the superior shielding ability of PEG-lipids. Moreover, being a synthetic lipid, PEG has the advantage of alterable chain length to accommodate specific needs. Our previous study [23] as well as other studies [27,28] show that about 1 mole% of PEG-lipids of adequate polymer size can completely cover a lipid bilayer surface to provide total inhibition of liposome–cell adhesion. This percentage of PEG-lipids is generally much lower than that commonly used to make stealth liposomes (5–15 mole%) in in vivo applications.

The ultimate goal of targeting liposomes is to deliver the encapsulated material to the predetermined site. One way of performing that operation efficiently is for the stealth liposomes to adhere to the target site on command. The stealth coating of the liposomes would prevent such adhesion. If there were a mechanism to de-stealth these liposomes at the target site, it would be possible for the liposomes to adhere to target cells and accomplish the delivery. It is very difficult to dislodge PEG conjugated lipids from the membrane to effect de-stealthing. Instead of using PEG conjugated lipids, we aim to use a polymer molecule that would dissociate from the liposome surface on command to make de-stealthing easier. Pluronics are our choice of polymer molecules. The hydrophilic PEO groups of a Pluronic, on either side of the central PPO unit, act as PEG molecules and can provide steric protection to a bilayer surface. The central PPO unit, being hydrophobic, would tend to push into the bilayer interior serving as an anchor. It is possible to dislodge the Pluronic molecule from the bilayer by reducing its hydrophobicity. A unique property of Pluronic is that it becomes increasingly hydrophobic with increasing temperature. In an aqueous medium, Pluronics stay as individual molecules at temperatures below their CMT, but at temperatures above the CMT, individual molecules are forced to form micelles to shield the lipophilic PPO units from the aqueous environment. In the presence of lipid bilayers, some Pluronics would partition into the bilayers in addition to forming micelles with other Pluronic units. If the temperature again goes below the CMT of the Pluronics, they would try to dislodge themselves from the bilayers or micelles to become individual molecules again. This property could be utilized to protect or de-protect liposomes. Moreover, due to the availability of various synthetic Pluronics, it is possible to choose one that will have: (a) a large enough PEO unit to cause complete stealthing; (b) an optimum size PPO unit which would provide enough anchoring to attach to the membrane; and, most importantly, (c) a CMT value around the physiological temperature (i.e. 37°C) corresponding to a relatively small concentration of Pluronic to make it useful in the in vivo conditions. Pluronic F-127 is one of the desirable candidates for our work because it has all the requisite qualities.

4.1. Liposome size studies

The temperature dependent liposome size changes show that at the concentration of 0.032% (w/v), used in our experiment, Pluronic F-127 causes significant size reduction of the MLVs at temperatures above the CMT. Moreover, the turbidity measurement shows that upon cooling, the sizes of the vesicles remain the same. This suggests that cooling does not have any further detrimental effect such as aggregation or destabilization. Johnsson et al. reported a reduction in size of the MLVs by Pluronics, at temperatures above the CMT, interpreting this as a disruptive effect by the polymer at those temperatures [18]. We, using different methods, have also observed such effects. We have used a much lower concentration of Pluronic in our experiment in comparison to that used by the Johnsson group. The disruption is not complete at the Pluronic concentration we used, and the polymer has no significant effect on LUV and SUV (Fig. 1).

Fig. 5 is a cartoon depiction of the polymer–bilayer association/dissociation mechanism in terms of the morphological observation. Fig. 5a shows the initial stage of the process when MLVs are formed. At 4°C, Pluronic F-127 molecules are individual molecules. Few of the Pluronic F-127 molecules are present in the liposomes – inside the aqueous core or in the lamellae. The individual molecules

associated with the lipid bilayers of the liposomes can be either translamellar or anchored to the bilayer from one side. Most of the individual Pluronic F-127 molecules are floating outside the liposomes. When these MLVs are heated to a temperature (say 41°C) above the Pluronic CMT, Pluronic F-127 molecules would form micelles (Fig. 5b). Also, there will be an increasing number of Pluronic F-127 molecules associated with the liposome now, since the hydrophobicity of the molecules has increased. MLV size would go down considerably due to breaking up of the outer wall of MLV, in accordance with the results of the liposome size measurement. Micelles could also be present in the liposome core. The size of the micelles observed by electron microscopy to be 'fluffy structures' of 44 nm in diameter is likely to be an overestimate due to the uncertain and limited penetration of stain into the loose PEG shield. When these hot liposomes are cooled below the Pluronic CMT, Pluronic F-127 micelles become individual molecules again and leave the liposomes with low polymer shielding (Fig. 5c). Also notable is that the sizes of the liposomes remain the same after cooling.

4.2. Pluronic assay

The amount of Pluronic associated with liposomes at each temperature is a critical issue. In order to establish the thermal association/dissociation mechanism as predicted in Fig. 5, we have to quantitate the amount of Pluronic associated with liposomes. There are two important considerations for our Pluronic assay. Since we are dealing with small unknown quantities of Pluronic in each sample, it is extremely important to collect not only all of the Pluronic molecules adhering to the liposomes, but also collect a sample of sufficient quantity which would result in an adequate signal. A major problem is to collect the surface attached Pluronics in the high temperature samples by centrifugation. Liposomes in the 'hot' sample, at a temperature of 41°C, become very fluffy and would trap a large quantity of free Pluronic during centrifugation. This is the reason for us to dilute the Pluronic anchored liposome suspension 10 times to reduce the amount of entrapped Pluronic in the pellet. Care was taken that the Pluronic concentration remained well above the CMC even for the dilute samples. The concentration of the Pluronic solution was chosen carefully so that the solution CMT, before as well as after dilution, was always below 41°C.

The assay depends on the formation of a colored complex between the Pluronic and cobalt thiocyanate. The complex forms a precipitate that sediments upon centrifugation following the reaction of cobalt thiocyanate with Pluronic F-127. The precipitate, thus formed, dissolves in acetone and the intensity of color is proportional to the amount of Pluronic present in the sample. The structure of the cobalt thiocyanate–Pluronic complex is due to hydrogen, ammonium, or cobalt ion complexation with oxygen groups in ether to form oxonium ion, which reacts with a suitable anion like thiocyanate [20]. However, it was later found that many oxygen atoms in the ether also interact with inorganic ions such as heavy metal, alkali, or earth alkali ions and that the complex has a helical configuration to accommodate the ion in the center [29]. The color of the complex has an absorbance spectrum with two peaks, one with a sharp absorbance peak at 328 nm and a second broader peak at 624 nm. The peak ratio for this complex is $A_{328}:A_{624}=4.4$ [21]. Since the intensity of the measurement is considerably higher at 328 nm, we have measured all our Pluronic F-127 samples at that wavelength.

4.3. Liposome–CHO cell association

In this experiment, the Pluronic-containing liposomes were added to cells either hot (41°C) or cold (4°C). Liposomes are expected to be sterically protected at high temperature and not to adhere to the cell surface extensively. On the contrary, cold and thermally cycled liposomes, with low surface shielding, are expected to adhere and may subsequently be internalized into cells. The fluorescence intensity of the relative amount of fluorescent lipid DPRhPE present in the respective sample represents the amount of liposomes adhered and internalized in cells. Since the procedure was consistent throughout the experiment, we can expect the relative fluorescence intensity to be higher in samples with de-protected liposomes. The result does show that the presence of Pluronic F-127 causes this difference in fluorescence intensity values. It is somewhat puzzling that the intensity values of the 'cold' and 'thermally

cycled' samples are significantly higher than that of the 'control' sample. Our fluorescence microscopy observation of the washed cell samples showed uneven fluorescence including discrete fluorescent spots for all the samples (data not shown), presumably resulting from the adhering and internalized fluorescently labeled liposomes. Thus, the cell surfaces were not completely covered by liposomes. The visual observation of fluorescence intensities of different samples was consistent with our fluorimetry results. One possible explanation for the above-mentioned difference in intensity could be that the fluorescent surface areas of liposomes are different. From Table 1, we know that MLVs formed in the presence of Pluronic F-127 are larger than those formed in the absence of Pluronic F-127. Assuming liposome adhesion on the CHO cell surface as a random process, i.e. under identical surface conditions, similar numbers of liposomes adhere to a unit cell surface area; an increased size would result in a larger fluorescently labeled surface area. From Table 1, we observe that at 4°C, the average diameter of 'F-127' samples is 23% larger than that of the 'control' samples. This translates into a 51% larger surface area, which would explain the apparent difference in fluorescence intensity between Pluronic and control samples in Fig. 4. It is noteworthy that the diameter of the MLVs made with DOTAP is much smaller than that of MLVs made without DOTAP, as seen in Fig. 1. This is mainly due to the presence of cationic DOTAP molecules, which cause formation of smaller vesicles due to charge repulsion among the DOTAP molecules of the same liposome.

In summary, we have utilized the temperature sensitive properties of Pluronic F-127 to control liposome–cell adhesion in vitro. At temperatures above their CMT, the Pluronic molecules can cause destabilization of the liposome membrane. This was evident from the drastic size reduction of MLVs. We have given visual evidence, using negative stain electron microscopy, of the presence of individual Pluronic molecules and micelles, respectively, at temperatures below and above the Pluronic CMT. The Pluronic assay showed, above CMT, the presence of a substantial amount of surface-associated Pluronic molecules, indicating possible steric protection of the liposome surface. Subsequent cooling below its CMT showed a significant reduction in surface-associated Pluronic pointing toward de-protection. A liposome–cell adhesion study, using Pluronic containing liposomes and CHO cells, also showed the control of liposome–cell adhesion capability of Pluronic F-127, the protection level being comparable to the level imparted by PEG5000 lipids.

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