Stabilization of Liposomes by Perfluorinated Compounds

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ABSTRACT: Perfluorinated compounds (PFCs) are emerging persistent environmental contaminants that may be toxic to animals and humans. To gain fundamental insights into the mechanism of their toxicity, the interactions of phosphocholine (PC) liposomes as model membranes were studied with three types of PFCs, including perfluorooctanoic acid, perfluorooctane sulfonate, and perfluorohexanesulfonic acid potassium salt, together with three common surfactants: sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), and sodium 1-heptanesulfonate (SHS). The interactions were systematically characterized by zeta potential measurement, dynamic light scattering, negative-stain transmission electron microscopy, and fluorescence spectroscopy. Unmodified liposomes, calcine-loaded liposomes, and Laurdan dye-embedded liposomes were all tested. By gradually increasing the temperature, the three PFCs and SHS decreased the leakage of calcine-loaded 1,2-dipalmitoyl-sn-glycero-3-phosphocholine liposomes, whereas SDS and CTAB increased the leakage. The PFCs that affected the lipid membranes stronger than SHS were attributable to their perfluorooalkyl carbon chains. Packing of the lipids was further studied using Laurdan dye as a probe. Calcine leakage tests also indicated that PFCs inhibited lipid membrane leakage induced by inorganic nanoparticles such as silica and gold nanoparticles. This study confirmed the similar effect of the PFCs as cholesterol in affecting membrane properties and would be helpful for understanding the interaction mechanism of PFCs and cell membranes.

INTRODUCTION

Perfluorinated compounds (PFCs) or perfluoroalkyl substances are a class of molecules with each hydrogen atom on alkyl chains replaced by fluorine. They encompass a diverse range of chemicals that are produced in large quantities for various technical applications such as fire extinguishing media and electroplating baths, preparation of fluoropolymers, and consumer products. Because of their widespread use and nonbiodegradability, PFCs have been detected in both the aquatic environment and biological tissues. These emerging pollutants in turn result in adverse effects to the environment and human health.

The most commonly seen PFCs contain six to ten saturated carbons with full fluorine substitution and terminated with a carboxyl or sulfonate headgroup, which make them interesting surfactants. Compared to typical surfactants with hydrocarbon tails, PFCs have even more hydrophobic perfluoroalkyl carbon chains. The fluorine atom on the carbon chain has a partial negative charge because of its strong electron-withdrawing inductive effect. Therefore, in addition to the anionic headgroups, these carbon chains also contribute to its overall negative charges. Furthermore, PFCs have a high chemical stability because of the high bond energy (∼533 kJ mol⁻¹) of the C–F bond, which makes them persistent, bioaccumulative, and widely distributed in the environment.

Studying the interaction of PFCs with liposomes, a model cell membrane, may enable fundamental insights into the mechanism of cytotoxicity. Some previous work has studied the effects of PFCs on cellular lipids and some showed that PFCs increased the permeability of cell membranes and altered membrane fluidity. PFCs also partitioned into membranes and PFCs with a longer chain showed a higher tendency to penetrate into the bilayer. The phase transition of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) liposomes in the presence of PFCs was also studied. PFCs were found to lower and broaden the phase transition of DPPC membranes, in a way similar to cholesterol. Most previous works used advanced spectroscopy methods such as NMR and thermal methods such as differential scanning calorimetry (DSC) and studied only a single type of lipid with one PFC. In this work, we systematically studied the interactions of three PC liposomes with three PFCs, together with three common surfactants and thus is a more systematic work. In addition, we relied on common optical spectroscopy such as dynamic light scattering (DLS), fluorescence spectroscopy, and transmission electron microscopy (TEM) and such techniques are available to more researchers. In complementary to previous studies, this work highlights higher efficacy of PFCs than normal surfactants.

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the same carbon chain length in affecting the property of lipid membranes.

**RESULTS AND DISCUSSION**

**Insertion of the PFCs into Lipid Membranes.** In this study, we studied three types of PFCs (Figure 1a–c), which had carbon chains ranged from six to eight. Two contained a sulfonate group and one contained a carboxyl group, thus making them all negatively charged. In addition, three commonly used small molecular surfactants, sodium dodecyl sulfate (SDS), sodium 1-heptanesulfonate (SHS), and cetyltrimethylammonium bromide (CTAB) (Figure 1d–f), were also included in this study. We chose to study three types of liposomes: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and DPPC (Figure 1g–i). PC is the most abundant headgroup in the outer membrane of eukaryotic cells with an overall neutral charge. DOPC has a phase transition temperature \(T_c\) of \(-20^\circ C\), and thus it is in the fluid phase at room temperature. DMPC has a \(T_c\) of 23 \(^\circ C\), which is around room temperature allowing us to probe phase transition-related properties with high sensitivity. Finally, DPPC has a \(T_c\) of 41 \(^\circ C\), displaying a gel phase behavior at room temperature. These three lipids and six surfactants allowed us to systematically understand the interactions and set this study apart from most previous works that used only one lipid and one PFC.\(^{22,24}\)

Most previous works prepared PFCs containing liposomes by mixing lipids and PFCs before forming the liposomes. We herein prepared the single-component PC liposomes first and added the PFCs later, which better mimicked the real situation of cell membranes encountering PFCs in the environment. It needs to be noted though, model liposomes and real cell membranes are still quite different. The observations are mainly from the standpoint of a reduced physical system, while their biological implications need further studies to confirm. Because DPPC, DMPC, and DOPC liposomes are charge neutral, whereas the PFCs are negatively charged, we first monitored the zeta potential of the liposomes upon gradually titrating the PFCs (Figure 2a). The zeta potential quickly dropped to more negative upon addition of perfluorohexanesulfonic acid potassium salt (PFHxS) (see Figure 1c for structure), whereas the same concentrations of PFHxS alone (without liposomes) cannot produce light-scattering signals because of its very low concentration (e.g., scattering intensity only \(\sim 10\) kcps, whereas typically over 100 kcps is needed for the measurement). The quick drop is an indication of insertion of the PFCs into the membrane.\(^{25}\) The critical micelle concentration (CMC) of PFHxS, perfluorooctanoic acid (PFOA), and perfluorooctanesulfonic acid potassium salt (PFOS) are \(18-86\), \(25-30\), and \(6.3-8\) mM, respectively.\(^{21,26,27}\) In order to avoid formation of micelles, the final concentrations of these surfactants employed in this work (<0.5 mM) were much lower than the reported CMC concentrations. Because we have only titrated submM concentrations of the PFCs, the lack of the light-scattering signal on their own is expected. Apparent partition coefficient (APC) describes partitioning of a surfactant into lipid bilayers versus those remained in the bulk aqueous phase. The APCs of some PFCs and surfactants have been previously reported in the literature (e.g., \(6.3 \times 10^4\) for PFOS;\(^{10}\) \(1.0 \times 10^4\) for PFOA;\(^{20}\) \(1.0 \times 10^4\) for SDS;\(^{26}\) and \(4.7 \times 10^2\) for SHS).
Therefore, we can approximate that the added surfactants were all in the liposome membranes at low surfactant concentrations.

The lipid concentration in the above measurement was 100 μg mL⁻¹ (~130 μM), and PFHxS saturated the zeta potential at around 50 μM. This saturating PFHxS concentration was comparable to the lipid concentration in the outer leaflet. This is consistent with the fact that PFHxS can spontaneously insert themselves to the liposome membrane and can reach a high loading. After 50 μM, whether more PFHxS can be incorporated into the membrane was not clear from this experiment. Similar observations of insertion of the PFCs into lipid membranes were made by using other techniques such as fluorescence spectroscopy, DSC measurements, infrared spectroscopy, and nuclear magnetic resonance techniques. Our surface charge measurement represented a simpler and label-free method.

We then monitored the hydrodynamic diameter of the liposomes upon adding PFCs using DLS (Figure 2b). After adding PFHxS, the average size of our DPPC liposomes remained the same as those without PFHxS. Similar observations were also made with the DOPC and DMPC liposomes. Therefore, insertion of PFHxS did not change the size of the liposomes. The DLS size of our DOPC and DMPC liposomes in the presence of PFCs and other surfactants was also measured. Most surfactants did not significantly change the size of the liposomes, except that the size of the DMPC liposomes doubled after adding CTAB (Figure S1). This was attributed to the swelling of the DMPC liposomes in water in the presence of cationic CTAB. For the main topic of this study, the PFCs, they did not affect the liposome size. The shape of the DMPC and DOPC liposomes remained spherical after adding surfactants such as PFOS, SDS, and CTAB, as determined by negative-stain TEM (Figure S2).

**PFCs Increase the Integrity of DPPC Liposomes.** After confirming that the PFCs could spontaneously insert themselves into the liposomes, we then examined their effect on the integrity of the liposomes. We prepared DPPC liposomes with 100 mM calcine encapsulated inside. Such a high concentration of the dye induced self-quenching of...
fluorescence, and liposome leakage could be reflected from fluorescence enhancement. For the pure DPPC sample (Figure 3a), the fluorescence remained stable at low temperature until \( T_c \approx 50 \) °C. After that, the liposome started to leak and leakage continued until \( T_c \approx 45 \) °C. The gel phase DPPC liposomes are known to leak when the temperature is approaching its \( T_c \), whereas the liposome became stable again passing \( T_c \). We then took the first derivative of this fluorescence trace, and the fastest leaking temperature was found to be 40.5 °C (Figure 3b), consistent with its \( T_c \) of 41 °C. The area under this peak is proportional to the amount of calcein fluorophore leaked. Therefore, our observation was consistent with the literature, and our liposomes were of high quality for content encapsulation. In the end, Triton X-100 was added to fully rupture the liposomes and release all the encapsulated calceins. This allowed us to estimate the fraction of leakage at each temperature.

The background fluorescence in these experiments was due to free calcein molecules outside the liposomes. Our column purification method could not fully remove all the free calceins. Fortunately, the background fluorescence changed very little as a function of temperature. The first derivative of the melting curves is quite informative and can minimize the difference in background fluorescence of different samples. We then measured the same process in the presence of different PFCs, and the amount of fluorescence increase dropped in each case. For example, with 10 \( \mu \)M PFOS, the fastest leaking temperature remained at 40.5 °C (Figure 3c,d). Further increase of the PFOS concentration led to a shift to a lower peak temperature. We performed similar experiments with the other two PFCs, and a similar trend was also observed (Figures S3–S6). The shifting of DPPC’s \( T_c \) to lower temperature is reminiscent of cholesterol. Jbeily et al. discovered that fluorocarbon amphiphiles can affect DPPC bilayers similarly to cholesterol using solid-state nuclear magnetic resonance spectroscopy, DSC, and confocal microscopy. Brüning and Farago showed that PFOA had a similar effect on DMPC as cholesterol by combining DLS and neutron spin-echo. Our work reinforced the similarity between PFCs and cholesterol using different techniques.

To have a quantitative comparison, we also plotted the area under the peak in Figure 3d, which is a reflection of the amount of calcein dye leaked (Figure 3e). This suggests that PFOS can make the DPPC liposomes more resistant to temperature-induced leakage. We also plotted the peak position of these three PFCs (Figure 3f). Interestingly, it took even more PFOA to shift the peak temperature, whereas PFHxS did not show any shift in the concentration range we tested. PFOS and PFHxS have the same headgroup, but the former has eight fluorinated carbons while the latter has only six. They showed a drastic difference in their ability to shift the peak temperature. PFOA has seven fluorinated carbons and also a different headgroup, and it sits between PFOS and PFHxS in terms of peak shift and area. Therefore, the effect of

Figure 4. Effect of SDS, CTAB, and SHS on the stability of calcein-loaded DPPC liposomes. Fluorescence intensity plotted against temperature of calcein-loaded DPPC liposomes with different concentrations of (a) SDS, (d) CTAB, and (g) SHS. The first derivatives of the releasing data with different concentrations of (b) SDS, (e) CTAB, and (h) SHS. Peak area indicative of total released calcein plotted against the concentration of (c) SDS, (f) CTAB, and (i) SHS.
the headgroup chemistry appeared to be less important, whereas the fluorinated carbon chain length governed the interaction.

For comparison, we also tested SDS, which has an even longer carbon chain (Figure 4a). In this case, adding more SDS released more calcein, instead of stabilizing the membrane (Figure 4b). The peak position also shifted to lower temperatures and this is similar to the effect of the PFCs (Figure 4c). Similar observations were also made with cationic CTAB (Figure 4d–f). We suspected that the main difference was the length of the tails. Therefore, SHS, a seven carbon chain surfactant was tested (see Figure 1e for structure), which has the same carbon chain length as PFOA. Interestingly, SHS can also stabilize the lipid membrane as PFCs (Figure 4g–i). Therefore, the inserted short chain PFCs (or SHS) did not make pores on the membrane. The longer chains might affect lipid packing and thus cause leakages. In addition, SDS and CTAB can form micelles at lower surfactant concentrations, and these micelles might dissolve some lipids as a way to induce leakage.

**Laurdan Dye as a Probe.** The above calcein leakage assays clearly indicated that the PFCs can affect the phase behavior of liposomes. To further study on this front, Laurdan was also used as a probe. Laurdan is a dye commonly used to diagnose a membrane’s phase behavior. Different from hydrophilic calcein loaded in the aqueous compartment of liposomes, Laurdan is embedded within the hydrophobic bilayer region. To conform the successful incorporation of Laurdan dye to our DMPC liposomes ($T_c = 23 ^\circ C$), we measured its fluorescence as a function of temperature (Figure S7). At 30 $^\circ C$, DMPC was in its fluid phase, the Laurdan emission red shifted to a peak wavelength at 480 nm with respect to that in the gel phase at 20 $^\circ C$ of 450 nm. This was consistent with the literature report. After confirming the quality of the Laurdan-loaded liposomes, the PFCs were, respectively, added, and the emission shifted from 450 to 480 nm (Figure 5a–c). It indicated that the PFCs have made the membranes to a more fluid phase.

When SHS was added to the DMPC liposomes, the Laurdan emission did not shift (Figure 5d). This means that the perfluoroalkyl carbon chains in the PFCs were the main factor influencing the DMPC membrane. Similar experiments were performed with SDS and CTAB. SDS was accompanied by the shift of emission from 450 to 480 nm (Figure 5e), whereas for...
liposomes, and we conﬁrm that this was also consistent with the calcein leakage data, where perﬂuorinated chains were more potent in this regard than the hydrocarbon chain. The increased ﬂuidity corresponded to a lower Tc, and this was also consistent with the calcein leakage data, where peak leakage occurred at lower temperatures with the surfactants. Laurdan ﬂuorescence experiment for the DOPC system has been carried out. The emission of Laurdan-loaded DOPC is 480 nm and cannot be changed by PFCs and other surfactants (Figure S8).

To quantitatively evaluate the Laurdan emission spectra data, the membrane ﬂuidity at different concentrations of the PFCs or other surfactants was expressed by generalized polarization GP = (I440 − I490)/(I440 + I490), where I440 and I490 are the emission intensities at these two wavelengths, respectively. A larger GP value corresponds to lower membrane ﬂuidity. As shown in Figure 6, the surfactants with a larger GP value change are more potent in ﬂuidizing the membrane. Because we now have two ways of analyzing the surfactant effect from calcein leakage and from the shift of the Laurdan emission, we wanted to see whether these two effects were correlated or not. For this purpose, we plotted the fastest leaking temperature and GP at the same concentration of surfactants. Indeed, the fastest leaking temperature increased with the increase of GP.

Effect of Nanoparticles on Membrane Integrity. The above assays mainly focused on the phase transition of the liposomes, and we conﬁrmed that the PFCs could be inserted into the membrane. In the environment, the cell membranes may also encounter various nanomaterials and their adsorption might also cause leakage of liposomes. Thus, we were also interested in understanding how such PFC-inserted liposomes react with nanoparticles. AuNPs and SiO2 NPs were used as representative nanomaterials for the test. AuNPs represent a metal, whereas SiO2 represents a nonmetallic material. AuNPs can leak DOPC liposomes as we have reported before, and this is also reﬂected in Figure 7a. When the PFCs or SDS was added to the liposome ﬁrst followed by adding the AuNPs, the DOPC liposomes did not leak. SHS and CTAB alone can induce partial leakage of DOPC liposomes (Figure S9). For SiO2 NPs, a high salt concentration was needed to promote its interaction with PC liposomes. Similar to the AuNPs, PFCs and SDS inhibited membrane leakage induced by SiO2 NPs (Figure 7b). However, CTAB boosted DOPC leakage with SiO2 NPs. It may be related to the positive charge of CTAB that attracted negatively charged SiO2 NPs. It is worth mentioning that SHS did not have any effect on SiO2-induced DOPC leakage. Overall, all of the PFCs stabilized the liposomes against nanoparticle-induced leakage.

CONCLUSIONS

In summary, the interaction of three common PFCs with a few representative PC liposomes was systematically studied using various techniques including DLS, Laurdan ﬂuorescence, TEM, and liposome leakage assays. Three types of PC liposomes with the same headgroup chemistry but with different Tc were investigated. The DLS data showed that the PFCs can all be inserted into lipid membranes, while they had little effect on the hydrodynamic diameter of liposomes. The melting curves indicated that PFCs increased the thermal stability of liposomes because the amount of dye leaked decreased, although Tc were shifted to lower temperatures. At room temperature, the PFCs failed to induce any leakage of the liposomes and they even inhibited lipidome leakage induced by two representative inorganic nanoparticles. Compared with common surfactants such as SHS, SDS, and CTAB, the perﬂuoroalkyl carbon chains in the PFCs are the primary factor of inﬂuencing the lipid membranes. With different types of surfactants and lipids, this work provides a comprehensive fundamental understanding of the interaction between lipid bilayers and PFCs, which may offer insights into the interaction with cell membranes and toxicity of PFCs.

MATERIALS AND METHODS

Chemicals. All of the phospholipids used in this study, including DOPC, DMPC, and DPPC were purchased from Avanti Polar Lipids (Alabaster, AL). PFOA, PFOS, PFHxS, SHS, disodium calcein, 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan), and Triton X-100 were purchased from Sigma-Aldrich. SDS, CTAB, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium phosphate, and sodium chloride were purchased from Mandel Scientiﬁc (Guelph, ON, Canada). Milli-Q water was used to prepare all buffers and suspensions.

Preparation of Liposomes. Our liposomes were prepared using the standard extrusion method, as described previously. Briefly, DOPC, DMPC, or DPPC (2.5 mg) was dissolved in chloroform. After evaporating chloroform by N2, the samples were dried in a vacuum oven at room temperature overnight to

Figure 7. Effect of the PFCs and surfactants on the leakage of calcein-loaded DOPC liposomes with (a) AuNPs and (b) SiO2 NPs. At 3 min, various surfactants were, respectively, added to the liposomes. At 6 min, AuNPs or SiO2 NPs were added, and at 9 min, Triton X-100 was added. For AuNPs, the reaction was in buffer A, whereas for SiO2, the buffer was 10 mM HEPES, 500 mM NaCl, and pH 7.6.
fully remove chloroform. The dried lipid films were stored at −20 °C in a N2 atmosphere prior to use. To prepare DOPC liposomes, the lipid films were hydrated with 0.5 mL of buffer A (10 mM HEPES, pH 7.6 with 100 mM NaCl) yielding a lipid concentration of 5 mg mL−1. The resulting cloudy suspension was extruded 21 times through two stacked polycarbonate membranes with a pore size of 100 nm. Because DMPC has a phase transition temperature (Tc) of 23 °C, its lipid films were hydrated at 35 °C for 2 h and were extruded at 35 °C, whereas DPPC (Tc of 41 °C) was hydrated and extruded at 60 °C. To encapsulate calcein, the lipid films (DOPC or DPPC) were hydrated with 100 mM calcein overnight followed by extrusion. Free calcein was removed by passing 35 μL of the sample through a PD-10 column using buffer A for elution. The first 600 μL of the fluorescent fraction was collected. Laurdan-loaded DMPC liposomes were prepared by mixing 10 μL of Laurdan (1 mM) with 500 μL of DMPC lipid (5 mg mL−1) in chloroform. Then, similar procedures were carried out for hydration and extrusion at 35 °C.

Zeta Potential and DLS. The zeta potential and hydrodynamic diameter of the liposomes with different concentrations of PFCs were measured on a Zetasizer Nano ZS 90 instrument (Malvern) with a He–Ne laser (633 nm) at 90° optics at 25 °C. A PFHxS solution was gradually titrated to DOPC, DMPC, and DPPC liposome suspensions (100 μg mL−1 in 10 mM HEPES for pH 7.6), respectively. The data were analyzed by Malvern Dispersion Technology software 4.20.

Melting Curves. To study the effect of the PFCs and surfactants on the thermal stability of the liposomes, calcine-loaded DPPC liposomes were used. 5 μL of calcine-loaded DPPC liposomes was first added to 595 μL of buffer A. Then, different concentrations of PFCs, SDS, SHS, and CTAB solutions were, respectively, added to the above DPPC liposomes solution. Finally, 50 μL of each mixed sample was loaded into a real-time PCR tube and the temperature was increased by 1 °C from 4 to 80 °C with a 2 min equilibrate time at each designated temperature. Then, the fluorescence intensity of the sample was measured at the end of each 2 min incubation using the FAM channel. The first derivatives of the fluorescence increase curves were also plotted.

Laurdan-Loaded Liposome Assays. Laurdan-loaded DMPC liposomes (5 mg mL−1) were first added to 490 μL of buffer A in a quartz cuvette at room temperature. Then, different concentrations of PFCs, SDS, SHS, and CTAB were, respectively, titrated into the DMPC sample. The Laurdan fluorescence was monitored by a Varian Eclipse fluorometer with an excitation wavelength at 340 nm. Note that the Laurdan dye was loaded in the hydrophobic region of the bilayer, whereas calcein was loaded in the internal aqueous compartment of the liposomes.

Liposome Leakage by Nanoparticles. To monitor liposome leakage, 5 μL of the above purified calcine-loaded DPPC liposomes was added to 595 μL of buffer A in a quartz cuvette at room temperature. The background fluorescence was monitored for 3 min before adding the PFCs (or SDS, or SHS, or CTAB). The fluorescence was monitored for another 3 min followed by adding 10 μL of 13 nm citrate-capped gold nanoparticles (AuNPs, 10 nM) or silica nanoparticles (SiO2 NPs, 1 mg mL−1). At 9 min, 10 μL of 5% Triton X-100 was added to fully rupture the liposomes. Calcein was excited at 485 nm, and its emission was monitored at 525 nm.©

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02448.

- Zeta potential, DLS, negative-stain TEM micrographs, temperature-dependent calcein leakage, and control experiments with the Laurdan dye (PDF)

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Notes

The authors declare no competing financial interest.

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