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MODEL AND CELL MEMBRANE PARTITIONING OF PERFLUOROOCTANESULFONATE IS INDEPENDENT OF THE LIPID CHAIN LENGTH

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Abstract

Perfluorooctanesulfonic acid (PFOS) is a persistent environmental pollutant that may cause adverse health effects in humans and animals by interacting with and disturbing of the normal properties of biological lipid assemblies. To gain further insights into these interactions, we investigated the effect of PFOS potassium salt on dimyristoyl- (DMPC), dipalmitoyl- (DPPC) and distearoylphosphatidylcholine (DSPC) model membranes using fluorescence anisotropy measurements and differential scanning calorimetry (DSC) and on the cell membrane of HL-60 human leukemia cells and freshly isolated rat alveolar macrophages using fluorescence anisotropy measurements. PFOS caused a concentration-dependent decrease of the main phase transition temperature (T_m) and an increased peak width (ΔT_w) in both the fluorescence anisotropy and the DSC experiments, with a rank order DMPC > DPPC > DSPC. PFOS caused a fluidization of the gel phase of all phosphatidylcholines investigated, but had the opposite effect on the liquid crystalline phase. The apparent partition coefficients of PFOS between the phosphatidylcholine bilayer and the bulk aqueous phase were largely independent of the phosphatidylcholine chain length and ranged from 4.4×10^4 to 8.8×10^4 . PFOS also significantly increased the fluidity of membranes of cells. These findings suggest that PFOS readily partitions into lipid assemblies, independent of their composition, and may cause adverse biological effects by altering their fluidity in a manner that depends on the membrane cooperativity and state (e.g., gel versus liquid crystalline phase) of the lipid assembly.

Keywords

Fluorescence anisotropy; differential scanning calorimetry; cooperativity; phosphatidylcholines; DPH; TMA-DPH; HL-60 human leukemia cells; alveolar macrophages

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1. Introduction

Perfluorooctanesulfonate and related chemicals belong to a class of organic fluorochemicals in which all carbon-hydrogen bonds are replaced by carbon-fluorine bonds. PFOS-based chemicals, in particular perfluorooctanesulfonamides, have been used extensively in a range of industrial and consumer application, including water repellants on assorted fabrics, surfactants, waxes and gloss finish enhancers, anticorrosion agents and lubricants [1,2]. Like other perfluorinated compounds, the perfluorinated tail of PFOS-derived compounds is stable towards biological, chemical and thermal degradation because of the extreme stability of the carbon-fluorine bond. At the same time, PFOS-derived compounds are highly lipophobic and hydrophobic. These properties make PFOS-derived compounds uniquely useful for the above mentioned commercial applications. PFOS itself is the ultimate degradation product of PFOS-based chemicals [3–7] and has been detected worldwide in the environment, wildlife and humans [2,8]. It is readily absorbed but poorly eliminated in wildlife and humans, with an estimated half-life of 4 to 5 years in humans [9]. Its prevalence and persistence in the environment raises environmental and public health concerns. As a result, the 3M Company as the worldwide major manufacturer of PFOS-based chemicals has phased out the production of PFOS-based chemicals from 2000 to 2002 [8]. However, PFOS and related chemicals will remain an environmental and public health problem for many years to come due to the large scale commercial use and persistence of these chemicals.

A particular concern associated with exposure to PFOS-derived compounds is their developmental toxicity [10,11]. In humans, low level *in utero* exposure to PFOS correlates with low birth weight [12]. PFOS has also been reported to induce developmental toxicity and other adverse effects in rats, mice and Cynomolgus monkeys, which included decreased neonatal survival following *in utero* and postnatal exposure, reduction in mean post natal body weight, and a significant delay in sexual maturation [11,13]. *In vitro* studies have shown that PFOS interferes with mitochondrial bioenergetics [14], gap junctional intercellular communication [15], and fatty acid protein binding in the liver [16]. In addition, PFOS causes peroxisome proliferator activated receptor alpha (PPAR α)-mediated hepatic peroxisome proliferation in rats and mice [17,18]. Studies with a structurally related chemical, perfluorooctanoic acid (PFOA), in PPAR α null versus wild type mice demonstrate that knockout of PPAR α abolishes the neonatal toxicity of PFOA [19]. However, a similar study with PFOS showed no differences in the neonatal mortality between PPAR α null and wild type mice, thus suggesting that the developmental toxicity of PFOS is PPAR α -independent [13].

Based on *in vitro* studies, we and others have hypothesized that interactions of PFOS with the lipid components of pulmonary surfactant may be a mechanism by which PFOS causes perinatal mortality in animal studies [20,21]. Despite the lipophobic character of PFOS-derived compounds, PFOS itself can partition into model mono- and bilayers [20,22], with an apparent partition coefficient of PFOS between DPPC model membranes and the bulk aqueous phase of approximately 6×10^4 [20,22]. At the air-water interface, PFOS more readily partitions into DPPC monolayers in the liquid-expanded compared to the liquid-condensed state [23]. PFOS causes changes in the fluidity of model monolayers [23], bilayers [20,22] and cell membranes [24], and increases the area per molecule of DMPC and DPPC at the air-water interface [23]. At the same time, molecular dynamic calculations indicate that PFOS improves the packing of phosphatidylcholine bilayers in the liquid crystalline phase [23]. In addition, there are preliminary reports that PFOS alters the dynamic behavior of pulmonary surfactant [25] and probably is present in neonatal airways immediately after birth, as suggested by its presence in the amniotic fluid after gestational PFOS exposure [26].

Phosphatidylcholines with different hydrocarbon chain length (e.g., C₁₄, C₁₆ and C₁₈) have different surface properties and display an increase in their cooperativity in lipid assemblies

as a function of chain length. They are major components of biological membranes and other biological lipid-containing structures and fluids. For example, a saturated diacyl phosphatidylcholine, DPPC, is the major constituent of pulmonary surfactant, where it exists as a monolayer and undergoes rapid changes from liquid-expanded to liquid-condensed states with each respiratory cycle. The aim of this study is to contribute to our understanding of the effect of PFOS on the major component of pulmonary surfactant and other biological lipid assemblies by systematically investigating the interaction of PFOS with model lipid assemblies composed of phosphatidylcholine with different chain lengths and cell membranes of living cells using both steady-state fluorescent anisotropy and DSC

2. Experimental

2.1. Chemicals and reagents

Potassium perfluorooctanesulfonate (PFOS) was obtained from Fluka Chemie GmbH (Buchs, Switzerland, batch # 312421000). According to ^{19}F Nuclear Magnetic Resonance analysis, this batch of technical grade PFOS contained approximately 82% linear, 10% isopropyl, 10% internally (including 1.2% α -methyl) and 0.4% t-butyl branched isomers of perfluorooctanesulfonate [27]. The PFOA potassium was synthesized by neutralization of the corresponding acid with an equimolar amount of potassium hydroxide. 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar Lipid (Alabaster, AL, USA). The fluorescence probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4 trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), were obtained from Molecular Probes (Eugene, OR, USA). All cell culture supplies were purchased from GIBCO BRL (Grand Island, NY, USA). Sterile 0.9% sodium chloride solution, tetrahydrofuran (THF), ethanol, methanol and chloroform were purchased from Fisher Scientific (Pittsburgh, PA, USA) and were HPLC or ACS grade. All solvents and chemicals were used without further purification. Deionized water for the DSC studies was purified by distillation as described earlier [28,29], whereas deionized ultra-filtered water ($>18\text{ m}\Omega$) for the fluorescence studies was obtained by reverse osmosis and ion exchange using a Millipore water system (Milli-Q, Millipore, Billerica, MA, USA).

2.2. Fluidity measurements using fluorescence anisotropy with model membranes

DPH and TMA-DPH-labeled multilamellar vesicles (MLV) suspensions of all three phosphatidylcholines were prepared as described by Xie et al. [22]. Subsequently, large unilamellar vesicle (LUV) suspensions were obtained by extruding the MLV suspensions ~ 15 times through a double-stacked polycarbonate membrane filter (pore size: 200 nm) using a LiposoFast extruder (Avestin Inc., British Columbia, Canada) above the melting temperature of the respective phospholipid [30]. Suspensions with PFOS concentrations ranging from 0 to 372 $\mu\text{mol/L}$ (0 to 200 mg/L) were prepared by diluting the LUV suspensions 100-fold with an aqueous solution of PFOS. Fluorescence anisotropy in the lipids bilayer was measured using a LS55 Luminescence Spectrometer from PerkinElmer (Shelton, CT, USA). The temperature of the samples was controlled using a PerkinElmer PTP-1 Peltier System (Shelton). Steady-state DPH and TMA-DPH anisotropy were determined at $\lambda_{\text{ex}} = 350\text{ nm}$ and $\lambda_{\text{em}} = 452\text{ nm}$ [31] and $\lambda_{\text{ex}} = 360\text{ nm}$ and $\lambda_{\text{em}} = 430\text{ nm}$ [32], respectively. An excitation slit width of 10 nm, an emission slit width of 10 nm and a one second average sampling time were employed. Phosphatidylcholine suspensions were equilibrated at 40°C (DMPC), 52.5°C (DPPC) or 67.5°C (DSPC) for 15 minutes and the DPH and TMA-DPH anisotropy values were recorded while cooling the sample to 10°C (DMPC), 22.5°C (DPPC) or 37.5°C (DSPC) at a rate of 0.2°/minute. The samples were stirred continuously at low speed. The temperature of the main fluid-gel phase transition (T_m), the transition width (ΔT_w) and the onset and offset of the transition were

determined upon cooling from plots of the fluorescence anisotropy value as a function of temperature. All fluorescence anisotropy experiments were carried out at least in triplicates.

The apparent partition coefficient K of PFOS between the lipids bilayers and the bulk aqueous phase was estimated using the following equation [33]:

$$-\Delta T_m = \frac{RT_{m,o}^2}{\Delta H_m} \left(\frac{C_{PFOS,o} K}{55.5 + C_{Lipids} K} \right) \quad \text{Equation 1}$$

where ΔT_m is the change in melting temperature, R is the gas constant, $T_{m,o}$ is the melting temperature of hydrated lipids, ΔH_m is the phase transition enthalpy (31.4 KJ/mol [34]), $C_{PFOS,o}$ is the initial aqueous concentration of PFOS, and C_{lipids} is the lipid concentration.

2.3. Differential scanning calorimetry

Calculated amounts of PFOS were dissolved in chloroform-methanol (3:1, v/v) at the appropriate mole fractions, the solvent was removed and the PFOS-lipid mixture was hydrated using an excess of water (three times by weight) [28,29,35,36]. Samples were heated above the lipid transition temperature for 5 minutes and vortexed for 2 minutes. This process was repeated approximately eight times. Samples were stored at 4°C overnight before collecting the DSC scans. Thermograms were recorded using a Thermal Analysis 2920 differential scanning instrument. The DSC cell was purged with 60 mL/min and the refrigerated cooling system (RSC) with 120 mL/min dry nitrogen, respectively. The hydrated samples were weighed into DSC aluminum pans, cooled to 4 °C at a rate of 10°/min and then heated from 4 °C to 80 °C with a rate of 5°/min [28,36,37]. All samples were subjected to two subsequent heating cycles. Onset, maximum and offset temperatures as well as peak width of the pre-transition and the main phase transition were determined upon heating for the second DSC run using the Universal Analysis NT software [28,29,35,38,39]. All DSC experiments were carried out in triplicates.

2.4. Fluidity measurements in cell membranes of mammalian cells in culture

2.4.1. HL-60 cell culture maintenance and cytotoxicity assays—A human promyelocytic leukemia cell line (HL-60, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) was used to assess the effect of PFOS on the fluidity of a mammalian cell membrane. In parallel, the effect of the structurally related PFOA was investigated. HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C and humidified air with 5% CO₂. To ensure that PFOS (and PFOA) were not cytotoxic in the concentration range investigated, HL-60 cells were seeded into 96-well plates at a density of 1×10^5 cells per well in 200 μ L medium and 30 μ L of a PFOS (or PFOA) solution in FBS-free medium were added into each well to give the desired PFOS (or PFOA) concentration (0 to 372 μ mol/L). The cytotoxicity of PFOS and PFOA was assessed 3 and 72 hours after exposure using the trypan blue exclusion assay [40] and the resazurin reduction assay [41].

2.4.2. Cellular phospholipid content—In order to employ phospholipid concentrations that are comparable to the ones used in the fluorescence anisotropy experiments, the phospholipid-to-cell number ratio of HL-60 cells was determined. In short, 1×10^7 cells were collected in the logarithmic growth phase and extracted with a chloroform: methanol solution (1:2, v/v) as described previously [42]. After vortexing the extract with 1.25 mL 1.5 M NaCl, the mixture was centrifuged at $500 \times g$ to separate the organic from the aqueous phase. The organic layer was collected and quantified using a modified Bartlett assay [43].

2.4.3. Isolation of alveolar macrophages—The animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Iowa. Female Sprague-Dawley rats (240–270 g; Harlan Bioproducts for Science, Indianapolis, IN, USA) were housed two per cage in an environmentally controlled room at 22°C, with a 12-hour dark-light cycle, and with food and water ad libitum. Rats were asphyxiated with CO₂, euthanized by cervical dislocation and the thoracic cavity was opened. Bronchoalveolar lavage (BAL) was performed with sterile 0.9% sodium chloride solution (pH 7.4; 5 mL per 100 g body weight). For each experiment, the BAL fluid from 4 rats was pooled and centrifuged at 4°C for 5 min at 300 g. The supernatant was removed the cell pellet, resuspended in 200 µL Hank's balanced salt solution by gentle vortexing, and the total cell number was determined by differential cell count. Approximately 97% of cells in the BAL fluid were alveolar macrophages.

2.4.4. Fluidity measurement in membranes of living cells—The fluidity of the plasma membrane of HL-60 cells and alveolar macrophages was measured as previously described [44]. In short, the cells were labeled for 2.5 h with 2×10^{-6} M TMA-DPH at 37 °C to allow the probe to incorporate into the cell membranes. The labeled cells were washed to remove excess TMA-DPH and resuspended in phosphate buffered saline (PBS). HL-60 cells or alveolar macrophages were diluted to approximately 1×10^5 cells/mL. PFOS (or PFOA) solutions were added to the cell suspension to give final concentrations ranging from 0 to 372 µmol/L (0 to 200 mg/L) and a final volume of 2.5 mL. The cells were incubated for 50 min at 37°C in a shaking water bath and subsequently equilibrated in the LS55 Luminescence Spectrometer for 10 minutes at 37°C. The steady-state anisotropy was then averaged over 10 minutes at 37°C. The TMA-DPH probe was excited at a wavelength of 340 nm, and the emission wavelength was set at 420 nm [44].

2.4.5. Statistics—All data are expressed as the mean $\pm$ standard deviations of at least three independent experiments. The data analysis was performed using the GLM procedure of SAS/STAT software, Version 9.2 of the SAS System for Windows. Responses at different concentrations were compared with the reference control. Since these comparisons involve a common control, Dunnett's test was used to adjust for their effect on hypothesis testing. Significance level 0.05 was used for the Dunnett's test.

3. Results

3.1. DPH and TMA-DPH fluorescence anisotropy: model membranes

Representative fluorescent anisotropy versus temperature curves of DPH and TMA-DPH-labeled DMPC bilayers in the presence of selected concentrations of PFOS are shown in Figure 1. The analogous figures for DPPC [20,22] and DSPC (Figure S1) display similar features. The DPH and TMA-DPH fluorescence anisotropy versus temperature curves of pure phosphatidylcholine bilayers displayed a sharp increase of the anisotropy value at the respective main phase transition temperature due to the phase transition from the liquid-crystalline to the gel phase upon cooling. Addition of increasing concentrations of PFOS resulted in drastic changes in the shape of the anisotropy versus temperature curves, the main phase transition temperature T_m , the width of the phase transition region and the fluidity in the liquid-crystalline and the gel phase of all the phosphatidylcholines. Comparable changes in these parameters were detected with both fluorescent probes.

Significant effects of PFOS were observed on the anisotropy values in the gel and liquid crystalline phase of all three phosphatidylcholines. Specifically, the anisotropy values decreased in the gel phase of DMPC (e.g., $< 15^\circ\text{C}$) as the concentration of PFOS increased (Figure 1). This effect was more drastic in the TMA-DPH measurements. Similar effects of PFOS on the gel phase were observed for DPPC [20,22] and DSPC (Figure S1). A slight

increase was noted for the anisotropy values in the liquid crystalline phase of DMPC (e.g., > 30°C) in the DPH measurements, whereas PFOS hardly showed an effect in the TMA-DPH measurements. Again, comparable effects of PFOS were noted on the liquid crystalline phase of DPPC and DSPC.

Addition of increasing concentration of PFOS resulted in a decrease in the temperature of the main phase transition T_m of all three phosphatidylcholines (Figure 2). The apparent partition coefficients of PFOS between the phosphatidylcholine bilayers and the bulk aqueous phase were estimated from the decrease of T_m using Eq. 1. As summarized in Table 1, the partition coefficients ranged from 4.4×10^4 to 8.8×10^4 and, thus, were independent of the chain length of the phosphatidylcholines.

The width of the phase transition increased significantly with increasing PFOS concentrations for all three phosphatidylcholines (Figure 2). This increase was primarily due to a decrease in the offset temperature (i.e., the ending of the transition from the liquid-crystalline to the gel phase of the phosphatidylcholines upon cooling) of the respective PFOS-phosphatidylcholine mixtures, whereas the onset temperature (i.e., the beginning of the transition from the liquid-crystalline to the gel phase of the phosphatidylcholines upon cooling) remained essentially constant up to a PFOS concentration of 372 $\mu\text{mol/L}$ ($31.8 \pm 0.32^\circ\text{C}$, $42.0 \pm 0.2^\circ\text{C}$ and $55.9 \pm 0.2^\circ\text{C}$ for DMPC, DPPC and DSPC, respectively; see Figures 2, S2 and S3). The only exception was the PFOS-DSPC system, where a slight decrease in the onset temperature was observed with the TMA-DPH probe at PFOS concentrations > 93 $\mu\text{mol/L}$. Despite these similarities, the changes in the width of the phase transition differed among the three phosphatidylcholines, with the effect of PFOS on the absolute transition width decreasing in the order DMPC > DPPC > DSPC (Figures 2, S2 and S3). Interestingly, the relative peak width (i.e., the peak width of the PFOS-phosphatidylcholine mixture adjusted for the peak width of the pure phosphatidylcholine) was comparable at lower PFOS concentrations (< 186 $\mu\text{mol/L}$), especially with the DPH probe (Figure 3). More complex and phosphatidylcholine-specific effects of PFOS on the relative transition width emerged especially at higher PFOS concentrations. For example, the relative peak width decreased in the DSPC > DPPC > DMPC at PFOS concentrations of 372 $\mu\text{mol/L}$.

3.2. Differential scanning calorimetry

To confirm the findings from the fluorescence anisotropy studies, DSC was used to study the effect of PFOS on the pretransition and the major phase transition of all three phosphatidylcholines. Similar to the previously reported effect of PFOS in DPPC model membranes [20,22], the addition of small amounts of PFOS resulted in an increase in the width of the pretransition peak and a decrease of its onset (i.e., the beginning of the transition upon heating) and maximum temperature of DMPC and DSPC (Figures 4 and 5). At $X_{\text{DMPC}} < 0.99$, $X_{\text{DPPC}} < 0.97$ [20,22] and $X_{\text{DSPC}} < 0.94$ no pretransition peak could be observed. Changes in the width and T_m of the main phase transition resembled the effects observed in both the DPH and TMA-DPH fluorescence anisotropy experiments: The onset and T_m of the main phase transition peak in all three systems displayed a pronounced shift to lower temperatures in the mole fraction range under investigation. At the same time, the offset temperature (i.e., the ending of the transition from the gel to the liquid-crystalline phase of the phosphatidylcholines upon heating) showed no (DMPC) or only a small (DPPC and DSPC) decrease in this mole fraction range. This resulted in significant peak broadening, with the effect of PFOS on the relative half width of the phase transition being comparable at small mole fractions (Figure 6). Similar to the fluorescence anisotropy measurements, the absolute half width decreased in the rank order DMPC > DPPC > DSPC at phosphatidylcholine (Figures 4 and 5). For example, at a phosphatidylcholine mole fraction of approximately 0.9, the width of the DMPC mixture was

3-times broader compared to the DSPC mixture. At a mole fraction ≤ 0.8 of all three phosphatidylcholines, several phase transitions peaks were observed.

3.3. Effect of PFOS on membrane fluidity of cells

To investigate if PFOS also altered the fluidity of biological membranes, TMA-DPH fluorescence anisotropy measurements were employed to determine the effect of PFOS on cell membrane fluidity in the HL-60 cell line and lung macrophages isolated from female Sprague Dawley rats. The total phospholipids content of HL-60 cells was approximately $10 \mu\text{M}/1 \times 10^5$ cells (i.e., $988 \pm 70 \mu\text{M}/1 \times 10^7$ cells; $918\text{--}1060 \mu\text{M}/1 \times 10^7$ cells). The phospholipid content in alveolar macrophages was not determined because the cell numbers isolated were not sufficient for this determination. Therefore, comparable phospholipid contents for HL-60 cells and alveolar macrophages were assumed for the fluidity measurements described below.

In parallel, the effect of the structurally related potassium salt of PFOA was investigated in both HL-60 cells and alveolar macrophages. Range finding cytotoxicity studies with HL-60 cells showed no toxicity of PFOS (or PFOA) after 3h and 72h of exposure up to the highest concentration ($372 \mu\text{mol/L}$) tested (data not shown). However, a small decrease in cell viability was observed after the fluorescence experiments with both cell types. Cell viability (assessed with trypan blue exclusion assay) immediately before the fluorescence experiment was $92.9 \pm 2.3\%$ (HL-60) and $90.7 \pm 1.9\%$ (alveolar macrophages) and was only slightly lower (HL-60 cells: $90.1 \pm 2.1\%$; alveolar macrophages: $89.7 \pm 1.6\%$) after the fluorescence measurement. Comparable results were obtained with PFOA.

The addition of increasing PFOS concentrations resulted in a decrease in the TMA-DPH fluorescence anisotropy values in both HL-60 cells and alveolar macrophages (Figure 7); however, this effect was only statistically significant in HL-60 cell at PFOS concentrations above $37 \mu\text{mol/L}$. Similar to the DPPC-PFOS and DSPC-PFOS systems described above, the decrease in fluorescence anisotropy appeared to be linear up to a PFOS concentration up to $93 \mu\text{mol/L}$ in both HL-60 cells ($R^2 > 0.99$) and alveolar macrophages ($R^2 > 0.99$) and remained almost constant thereafter. Although PFOA decreased the TMA-DPH fluorescence anisotropy values, this effect did not reach statistical significance at any PFOA concentration investigated (Figure S4).

4. Discussion

4.1. Interaction of PFOS with phosphatidylcholine lipid bilayers

PFOS is an emerging persistent environmental contaminant that has been linked to neonatal mortality in rodents [10,11] and reduced birth weight in humans [12]. The adverse health effects in humans and animals by interacting with and disturbing of the normal properties of biological lipid assemblies. For example, the increased neonatal mortality observed in animal studies does not appear to be a receptor-mediated process [13], but may be due to the inhibition of pulmonary surfactant [22]. Since phosphatidylcholines are major constituents of pulmonary surfactant and cell membranes, the present study investigates the effect of PFOS on the phase behavior of three saturated phosphatidylcholines with different chain lengths and, therefore, different degrees of membrane cooperativity (i.e., DMPC, DPPC and DSPC) using steady-state fluorescence anisotropy measurements and DSC, with the objective to contribute to our fundamental understanding of PFOS-lipid interactions. DPH and TMA-DPH were selected for the fluorescence anisotropy experiments because both probes are well investigated and known to be located at different positions within the lipid membrane. DPH is preferentially located near the center of lipid bilayers, whereas TMA-DPH, a cationic derivative of DPH, is preferentially located near the polar head group region in the bilayers. For example, DPH and TMA-DPC are positioned approximately 7.8 and $10.9 \text{ \AA}$ away from the center of

dioleoylphosphatidylcholine membranes, respectively [45]. DSC was employed to complement the fluorescence anisotropy experiments because it allows an assessment of the phase behavior of PFOS-phosphatidylcholine mixtures without introducing a fluorescent probe.

Although fluorocarbon and hydrocarbon surfactants display non-ideal mixing and form fluorocarbon and hydrocarbon rich micelles in aqueous solution [46], PFOS [20,22,23], perfluorooctanoic acid [22,33] and other, structurally related perfluorinated surfactants [29, 47] readily partition into phosphatidylcholine lipid assemblies. In the case of surfactants with a short C₇ or C₈ perfluorinated chain (such as PFOS and perfluorooctanoic acid) addition of the surfactant decreases the T_m of the lipid bilayer. As shown in Figures 2 and 5, the T_m of DMPC, DPPC and DSPC model membranes decreased with increasing PFOS concentration in the DPH and TMA-DPH fluorescence anisotropy as well as the DSC measurements. The partition coefficients estimated from the decrease in T_m of the fluorescence experiments (Table 1) indicate that PFOS has a high tendency to partition into phosphatidylcholine bilayers, especially compared to short-chain hydrocarbon surfactants (e.g., potassium octanesulfonate: K = 3–5 × 10² [22]; sodium octanoate (pH=10): K = 1.5 × 10² [33]). Furthermore, the partitioning of PFOS into lipid assemblies appears to be independent of the molecular structure (i.e., chain length) of the phosphatidylcholine. This is a result of the pronounced hydrophobic effect of PFOS and other fluorinated surfactants [28,29], i.e., the incorporation of PFOS into the lipid bilayer is energetically highly favorable, especially compared to the incorporation of the corresponding hydrocarbon surfactants [33,48]. Analogously, the partitioning of fluorinated inhalation anesthetics into bilayers is independent of the chain-length of the phosphatidylcholine [49].

PFOS dramatically increased the width of the major phase transition of all three phosphatidylcholines in the fluorescence anisotropy and the DSC experiments, which is also in agreement with previous studies of the interaction of fluorinated surfactants with phospholipids [20,22,29,33]. The width of the phase transition decreased in the order DMPC > DPPC > DSPC at higher mole fractions of PFOS. Studies of the effect of other lipophilic compounds on the phase behavior of phosphatidylcholines, such as tamoxifen derivatives [50,51] and pyrethroid insecticides [52], show a similar rank order. These differences in the phase behavior of DMPC, DPPC and DSPC reflect an increase in van der Waals forces between the hydrophobic tails with increasing tail length, which results in an increased cooperativity of the lipid bilayer and, thus, an overall smaller transition width in the order DMPC > DPPC > DSPC. Interestingly, the relative effect of PFOS on the transition width in the fluorescence anisotropy and DSC experiments was comparable at lower PFOS concentrations, indicating a comparable relative effect of PFOS on the cooperativity of all three phosphatidylcholines (see below).

In addition to its effect in the maximum and width of the major phase transition of phosphatidylcholines, PFOS decreased the fluorescence anisotropy values in the gel phase, but increased them in the liquid crystalline phase of all three phosphatidylcholines. The effect of PFOS on the gel phase was very pronounced in the TMA-DPH measurements and suggests an increase in the fluidity of the lipid assemblies present in the gel phase. This observation is in agreement with the effect of PFOS on DPPC monolayers at the air-water interface [23]. For example, PFOS has a pronounced effect on the transition of DPPC monolayers from a liquid-expanded to a liquid-condensed phase at concentrations as low as 10 μmol/L. PFOS completely abolishes this phase transition at a concentration of 10 μmol/L, resulting in a monolayer in a liquid-expanded state. PFOS also reduces the maximum compression modulus of DPPC monolayers from 191 mN/m to 150 mN/m, which indicates the formation of liquid-like DPPC monolayers [23]. Together, these studies suggest that PFOS has a fluidizing effect on some phosphatidylcholine assemblies. In the case of phosphatidylcholine bilayers, this fluidizing

effect is more predominant in the head group region, as indicated by the more pronounced effect in the TMA-DPH experiment. In contrast, PFOS has an ordering effect on the liquid crystalline phase, as indicated by an increase in the anisotropy values. The later observation is in agreement with molecular dynamics simulations, which show an improved packing of the lipid molecules after penetration of PFOS into DPPC bilayers at 52 °C [23]. Thus, the effect of PFOS on phosphatidylcholine appears to depend, at least to some extent, on the state of the lipid assemblies of interest (e.g., gel versus liquid crystalline phase in bilayers).

Overall, the effect of PFOS on the phase behavior of DMPC, DPPC and DSPC model membranes is characteristic for mixtures of phosphatidylcholines with many amphiphilic molecules, such as C₅ and higher alkanols, alkanolic acids and some nonionic detergents [24], and indicates a loss of cooperativity in the phase transition due to the incorporation of PFOS. As we suggested previously [20,22], PFOS is most likely oriented parallel to the acyl chains in phosphatidylcholine bilayers, with the SO₃⁻ group anchored at the water-lipid interface. This assumption is indirectly supported by an NMR relaxation study showing such an orientation for the structurally related perfluorooctanoate anion in a phosphatidylcholine bilayer [20,22]. The orientation of PFOS within the bilayer disturbs the highly ordered packing of the acyl chain of the phospholipid in the cooperative region (approximately C₁ through C₈). This results in a loss of van der Waals interactions between acyl chains within a cooperative unit and the introduction of free volumes into the phosphatidylcholine bilayer. Furthermore, the introduction of negatively charged SO₃⁻ groups in the head group region also contributes to a loss of cooperativity [33,53]. As a result, the cooperative units in PFOS-containing bilayers are smaller and undergo a phase transition not only at lower temperatures but also over a broader temperature range.

4.2. Interactions of PFOS with mammalian cell membranes

One of the intriguing observations of our studies with model membranes is the fact that the membrane partition behavior of PFOS is independent of the chemical structure (i.e., chain length) of the phospholipid and, consequently, the cooperativity of the model membrane. Since the hydrophobic force is the driving force for the partitioning of PFOS out of the aqueous bulk phase into lipid assemblies, PFOS is expected to have a significant effect on biological bilayers in the same concentration range that affects model membranes. To test this hypothesis, we investigated the effect of PFOS on cell membrane fluidity in HL-60 cells and alveolar macrophages. Both cell types were selected because the phospholipid composition of their cell membranes is well investigated [42,54]. TMA-DPH was used as a fluorescence probe for these studies because it has been widely used to assess the fluidity of biological membranes [44]. TMA-DPH offers the advantage that it remains in the superficial layers of the cell membrane and is hardly internalized into the cells during the measurement period.

As expected, PFOS exhibited a dose-dependent increase in cell membrane fluidity in both the human HL-60 leukemia cells and the normal rat lung macrophages, which reached statistical significance at PFOS concentrations of 37 µmol/L (20 mg/L) in HL-60 cells. Although a similar change in membrane fluidity was seen in alveolar macrophages, this effect did not reach statistical significance due to the higher standard deviations with these freshly isolated primary cells. Hu et al. reported that PFOS increased membrane fluidity in fish leukocytes in a dose-dependent fashion, with the lowest effective concentration causing a significant change in membrane fluidity observed at 5–15 mg/L [24]. Interestingly, PFOA did not have a statistically significant effect on the membrane fluidity of HL-60 cells and alveolar macrophages. The later observation suggests that PFOS may have a more pronounced effect on biological lipid assemblies, which is in agreement with its more pronounced effect on model mono- and bilayers [22,23].

5. Conclusions

Although the lipid composition of cell membranes is different compared to the composition of pulmonary surfactant, the observed changes in membrane fluidity demonstrate the ability of PFOS to readily partition into various lipid assemblies, including model and cell membranes as well as monolayers of pulmonary surfactants. After incorporation, PFOS alters the fluidity and potentially the functions of the lipid assembly in a manner that depends on the cooperativity and state (e.g., gel versus liquid phase) of the respective lipid assembly. The later finding is of particular importance for understanding the proposed inhibitory effect of PFOS on pulmonary surfactant, which undergoes rapid changes from a liquid expanded to a liquid condensed state during each respiratory cycle. Therefore, further studies of the effect of PFOS on the dynamic function of pulmonary surfactant both *in vitro* and *in vivo* are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Temperature dependent changes of the DPH (A) and TMA-DPH (B) fluorescent anisotropy $\langle r \rangle$ of mixtures of DMPC with selected concentrations of PFOS. As indicated by the arrows, samples of the respective phosphatidylcholine ($10 \mu\text{mol/L}$) were cooled at a rate of $0.2^\circ/\text{minute}$. Each anisotropy-versus-temperature curve is the average of three independent measurements.

Figure 2. Partial phase diagrams of (A) DMPC-PFOS, (B) DPPC-PFOS [22] and (C) DSPC-PFOS mixtures obtained from the DPH. As indicated by the arrows, all samples were cooled at a rate of 0.2°/minute. The data points represent averages of at least three experiments $\pm$ one standard deviation. ■ onset of main phase transition; Δ maximum of the main phase transition; ● offset of the main phase transition.

Figure 3. Concentration dependence of the relative width of the phase transition of DMPC, DPPC and DSPC mixtures in the presence of 0 to 372 μM PFOS using DPH (A) and TMA-DPH (B) as fluorescent probe. All data points are averages of at least three experiments $\pm$ one standard deviation. Samples were cooled at a rate of $0.2^\circ/\text{minute}$.

Figure 4.

Typical calorimetric scans for mixtures of (A) DMPC, (B) DPPC [22] and (C) DSPC with PFOS in excess water. The mole fraction of the respective phosphatidylcholine is indicated besides each scan. The heating rate was 5°/minute from 4°C to 80°C (only the part of the curve with a phase transition is shown).

Figure 5. Partial phase diagram of mixtures of (A) DMPC-PFOS, (B) DPPC-PFOS [22] and (C) DSPC-PFOS mixtures in excess water upon heating. All data points are averages of at least three experiments $\pm$ one standard deviation. ◇ onset of pretransition; ● onset of main transition; ▽ maximum of main transition; ▲ and maxima within main transition; ■ offset of main transition.

Figure 6.

In the DSC experiments, the relative half width of the main phase transition is concentration dependent for mixtures of PFOS with DMPC, DPPC and DSPC. All data points are averages of at least three experiments $\pm$ one standard deviation.

Figure 7.

PFOS alters the membrane fluidity of HL-60 cells and alveolar macrophages at 37°C in TMA-DPH fluorescence anisotropy measurements. All data are averages of at least three experiments $\pm$ one standard deviation. * significantly different from control by multiple comparison test ($p < 0.05$).

Table 1

Apparent partition coefficients (K) of PFOS between phosphatidylcholine model membranes and the bulk aqueous phase.

Phosphatidylcholine	DPH ($\times 10^4$)	TMA-DPH ($\times 10^4$)
DMPC	6.3	4.4
DPPC	5.7 [20,22]	8.8 [22]
DSPC	5.7	5.9