Lipid fluorination enables phase separation from fluid phospholipid bilayers†

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To probe the effect of lipid fluorination on the formation of lipid domains in phospholipid bilayers, several new fluorinated and non-fluorinated synthetic lipids were synthesised, and the extent of phase separation of these lipids from phospholipid bilayers of different compositions was determined. At membrane concentrations as low as 1% mol/mol, both fluorinated and non-fluorinated lipids were observed to phase separate from a gel-phase (solid ordered) phospholipid matrix, but bilayers in a liquid disordered state caused no phase separation; if the gel-phase samples were heated above the transition temperature, then phase separation was lost. We found incorporation of perfluoroalkyl groups into the lipid enhanced phase separation, to such an extent that phase separation was observed from cholesterol containing bilayers in the liquid ordered phase.

Introduction

The inherent lateral asymmetry of biological membranes is evident in the complexity of cellular structures observed in Nature. However for over 30 years the behaviour of biological membranes was modelled with the Singer–Nicolson fluid mosaic membrane model, which predicted lateral and rotational freedom and random distribution of molecular components in the membrane.[1] It is now apparent that the cell membrane is not a homogenous mixture of proteins and phospholipids but is organised into a variety of domains or "lipid rafts" where different lipids constituting the membrane have phase-separated.[2] The idea that there could be isolatable structures containing lipids and proteins was supported by the observation that membrane microdomains, enriched in cholesterol and glycosphingolipids, seemed to survive detergent extraction.[3] Though initially controversial, as it was not clear if these were artefacts of the isolation technique, it is now apparent that these lipid rafts have distinct functional roles. These functions are relatively poorly understood, but are implicated in a range of biological processes such as signal transduction, endocytosis and cholesterol trafficking.[4,5]

Biomimetic systems that model the formation of lipid rafts have often been based upon mixtures of naturally occurring lipids that phase separate to form large lipid domains around 0.2–4.0 μm in diameter or larger.[6] Generally, the more structurally diverse the components of the lipid mixture, the more likely that mixture is to phase separate. Phosphocholines with significantly different acyl chain lengths, such as dilauroyl-L-α-phosphatidylcholine (DLPC) and dipalmitoyl-L-α-phosphatidylcholine (DPPC), are known to phase separate, as are mixtures of saturated phospholipids and sphingolipids.[7] Phospholipid phase separation can also be strongly influenced by cholesterol, a key component of most eukaryotic cell membranes; common eukaryotic biomembranes can contain up to 40% mol/mol cholesterol.[8]

The phase state and fluidity of the membrane can play a crucial role in determining the extent of lateral phase separation of the components. Three different phase states are commonly invoked to describe the properties of phospholipid bilayers in terms of the mobility of and interactions between membrane components.[9] The most structured phase is the gel-phase or solid ordered phase (s_o), where there are strong interactions between neighbouring phospholipids and little mobility in the membrane. When a solid ordered bilayer composed of pure phospholipid is heated, the bilayer undergoes a sharp phase transition at a characteristic temperature, the T_m, after which the bilayer "melts" and enters the liquid disordered phase (l_d). This phase is characterised by high mobility and little interaction between neighbouring components of the membrane. The addition of cholesterol, so crucial to biological membranes, to phospholipid bilayers creates a new fluid phase, the liquid ordered phase l_o, where despite high mobility there is a considerable degree of order and interaction between components within the membrane. This liquid ordered phase can co-exist with a surrounding liquid disordered phase, but at cholesterol concentrations of 50% mol/mol and above the entire membrane enters the liquid ordered phase.[8,10] Such liquid/liquid phase separation is of particular biological interest, as lipid rafts are believed to be liquid ordered cholesteric islands floating within a liquid disordered matrix.[4,11] As in Nature, these phase-separated domains can influence the action of enzymes significantly; for example, it has been shown that the action of phospholipase A_2 strongly depends on the phase state of the membrane.[12]

The phase separation of synthetic lipids from a phospholipid matrix can be efficient even at low concentrations of synthetic lipid, for example, 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine has been shown to phase separate from DPPC at membrane concentrations as low as 1% mol/mol, at temperatures below the T_m of DPPC.[13] The strong interactions between the DPPC lipids in the solid ordered phase may be the driving force for the exclusion of the synthetic additive. Other lipids, also with two alkyl chains embedded in the membrane, have also been shown to extensively phase separate from gel-state

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phospholipid bilayers. Sasaki *et al* used this effect to develop new ways of detecting metal ions; the degree of phase separation of chelating lipids in distearoyl-L-α-phosphatidylcholine (DSPC) vesicles decreased upon addition of metal ion.[14] Interestingly, utilising bilayers that are in a gel-like state at room temperature can prevent the bilayers from quickly reorganising in response to external stimuli, and indeed the response to an external binding event has been shown to take longer than 2 days at 25 °C.[15] Therefore phase separation from fluid bilayers that can respond rapidly to such external stimuli is potentially useful.[16] A potential solution could be to incorporate perfluoroalkyl groups into the synthetic lipids. Differential scanning calorimetry (DSC) studies have suggested fluorinated lipids extensively phase separate in bilayers composed of fluid phase dimyristoyl-L-α-phosphatidylcholine (DMPC), and visualization by electron microscopy under freeze fracture conditions showed large phase separated patches (150 nm in diameter), even at fluorinated lipid concentrations as low as 5% mol/mol.[17] However since direct visualisation with freeze fracture EM occurred at temperatures well below the phase transition temperature of DMPC, the degree of phase separation at room temperature is unclear.

As part of our studies into multivalent binding events at phospholipid bilayer membranes, we became interested in discovering structural features that would promote phase separation between biological and synthetic lipids in phospholipid membranes, particularly membranes in a fluid state.[18] Herein we describe the synthesis of and studies on a series of synthetic lipids that enabled us to determine some structural elements that promote extensive phase separation in phospholipid bilayers.

Results and discussion

Design

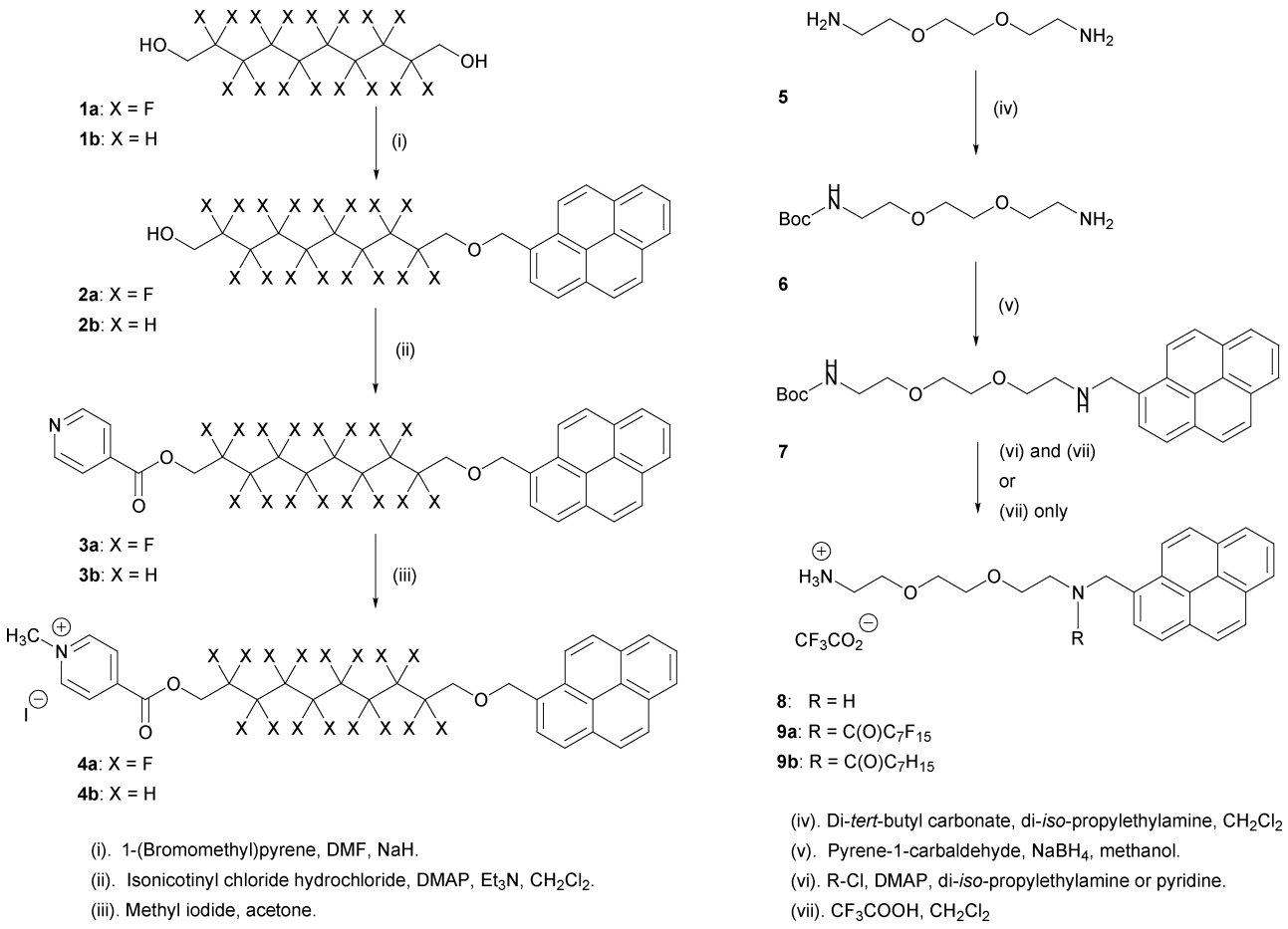
Fluorescence is a versatile technique to monitor the physical properties of biomembranes, and pyrene lipids in particular have been used to determine the phase separation of membrane components. This is mainly due to their distinctive photophysical properties, particularly their ability to form excimers, a process dependent upon the frequency of collisions between pyrene moieties. Excimers form when pyrene groups in the excited state collide with others in the ground state; the resultant excimer fluoresces at longer wavelengths than the monomer. The ratio of excimer to monomer emission intensities (the E/M ratio) is a measure of the fluidity or local concentration; the larger the ratio, either the more fluid the membrane or the higher the local concentration of the pyrene lipid.[19] Another important advantage of pyrene lipids is that the pyrene moiety is hydrophobic and does not strongly modify the hydrophobic character of the acyl chain that is embedded in the membrane. Measuring the E/M ratio has been used to study a variety of phenomena, including phase transitions, membrane fusion, lipid conformations and lateral structure within membranes. This latter effect is especially important; since the E/M ratio is directly proportional to the probe's local concentration, it makes it possible to interpret changes in this value and quantify the extent of temperature-induced phase separation or protein-induced domain formation in membranes.[20] For example, pyrene-labelled lipid probes have been used to demonstrate the co-existence of gel-like and fluid phases in vesicles composed of DPPC and 1-palmitoyl-2-oleoyl-L-α-phosphatidylcholine (POPC), with the pyrene-labelled lipid concentrated within the fluid phase.[21]

The observation of phase separation for lipids incorporating perfluoroalkyl groups and lipids with two alkyl chains as membrane anchors combined with the utility of pyrene as a probe for phase separation in phospholipid bilayers, led us to design pyrene containing lipids **3a** to **9b** (Scheme 1). These seven lipids were designed to examine the following questions: how does the structure of the membrane anchor and phase state of the surrounding phospholipid matrix combine to determine the degree of phase separation, and will the presence of perfluoroalkyl chains in membrane-bound lipids increase phase separation?

Synthesis

The bifunctional fluorinated alcohol **1a** was used as a convenient building block to construct the single chain perfluorolipids (Scheme 1). Using a Williamson ether synthesis, this diol was desymmetrised by reacting an excess of the diol with 1-(bromomethyl)pyrene, itself synthesized from reaction of pyren-1-ylmethanol with phosphorus tribromide.[22] Purification by column chromatography afforded the product, a pyrene perfluoroalcohol, which was then capped with a pyridyl headgroup. Reaction of the pyrene perfluoroalcohol with isonicotinyl chloride hydrochloride in the presence of triethylamine and DMAP as a catalyst proceeded smoothly to give the ester **3a** as a white solid in a yield of 79%. The non-fluorinated analogue **3b**, was synthesised in a similar manner starting from decane-1,10-diol, although purification of the desymmetrised decanediol proved to be more difficult. The pyridyl headgroups of both **3a** and **3b** were then further derivatised by reaction of the pyridyl group with methyl iodide, which gave the cationic pyridinium lipids **4a** and **4b** as yellow solids in yields of 66 and 52%, respectively.

The strategy adopted for the synthesis of the lipids with two membrane anchors, **9a** and **9b**, and the single chain precursor lipid **8**, was based around desymmetrisation of the commercially available di-amine *O*,*O*-bis(2-aminoethyl)ethyleneglycol **5**. Reaction with 0.67 equivalents of di-*tert*-butyl carbonate provided a mixture of protected products from which the desired monoprotected compound, *N*-Boc-3,6-dioxaoctane-1,8-diamine **6**, could be isolated by column chromatography. Reductive coupling of this amine to pyrene-1-carbaldehyde using $NaBH_4$ in methanol gave *tert*-butyl carbamate protected lipid **7**. The secondary amine in lipid **7** allowed the easy addition of a second membrane anchor, through acylation or alkylation with a variety of acid chlorides or alkylating agents. To carry out comparative investigations on the influence of perfluorination on lipid aggregation, both octanoyl chloride and perfluorooctanoyl chloride were used to acylate the secondary nitrogen, giving *tert*-butyl carbamate protected dual chain lipids. Quantitative deprotection of the non-acylated, octanoylated and perfluorooctanoylated lipids by trifluoroacetic acid gave the corresponding ammonium salts; lipids **8**, **9a** and **9b** were obtained as sticky oils with overall yields of 63, 62 and 62%, respectively. Lipids **8**, **9a** and **9b** are also convenient starting compounds for the synthesis of biomimetic membrane receptors *via* attachment of functional headgroups to the primary amine.



Scheme 1 Synthesis of compounds **1a** to **9b**.

Phase separation at 25 °C

The phase separation of these seven lipids, **3a**, **3b**, **4a**, **4b**, **8**, **9a** and **9b** was studied in unilamellar phospholipid vesicles of eight different compositions. These eight compositions were selected because they include the three main phase states and give bilayers with distinct physical properties. DMPC and DSPC are both saturated phospholipids, with gel to fluid phase transition temperatures (T_m) of 23 and 54 °C, respectively.[23] POPC has unsaturation in the C2 acyl chain, so POPC bilayers have a low T_m of −4 °C. Egg yolk phosphatidylcholine (EYPC) is a naturally occurring mixture of phospholipids, as found in biological membranes, and contains lipids with a mixture of saturated and unsaturated acyl chains of differing lengths, resulting in a broad T_m between −8 and −15 °C.[24] Thus at room temperature, 25 °C, DMPC, POPC and EYPC bilayers are above their transition temperatures and exist in a fluid phase, the liquid disordered phase, whilst DSPC bilayers are below their T_m value and are thus in the gel- or solid ordered phase. The remaining four compositions have cholesterol mixed with DMPC, DSPC, POPC and EYPC. The addition of cholesterol increases membrane rigidity for fluid phase membranes, yet decreases membrane stiffness and increases lipid lateral mobility in gel phase membranes, changes that typify entry into the liquid ordered phase (l_o).[25] The effect of cholesterol has been well studied and generic phase diagrams have been constructed for mixtures of cholesterol with many phospholipids including DMPC and DSPC.[26] The liquid ordered phase is in equilibrium with the liquid disordered phase up to about 30% mol/mol cholesterol, but only the liquid ordered phase persists at higher cholesterol levels. The addition of cholesterol also results in the thermal phase transition, T_m, of the bilayer disappearing.[8,10] Thus to obtain the liquid ordered phase, l_o, cholesterol was included at 50% mol/mol in DMPC, DSPC, POPC and EYPC bilayers.

Unilamellar vesicles (0.8 μm diameter, 20 mM lipid in 20 mM MOPS buffer, pH 7.4) were made up incorporating lipids **3a** to **9b** in the membrane at a membrane concentration of 1% mol/mol.[27] At this relatively low concentration the synthetic lipids were fully incorporated into the membranes, and this low concentration is also a challenging test of the degree to which the synthetic lipids will phase separate.[28] At higher concentrations phase separation is easier to observe, but the E/M ratio is less sensitive to changes in the structure or charge of the synthetic lipids and therefore less useful for sensing applications. The unilamellar phospholipid vesicles were formed using standard techniques; suspensions of multilamellar vesicles (MLVs) were extruded through polycarbonate membranes with 800 nm pores. The synthetic lipids were mixed at 1% mol/mol with the phospholipids prior to the hydration of the phospholipid thin films. After a 1000-fold dilution (20 μM lipid), the fluorescence spectra of the vesicle suspensions were recorded and the pyrene excimer emission (at 480 nm) to monomer emission (at 378 nm) ratio (E/M) was calculated for each.

Table 1 Excimer/monomer ratios determined at pH 7.4 and 25 °C for lipids **3a**, **3b**, **4a**, **4b**, **9a** and **9b** at 1% mol/mol in phospholipid vesicles of different compositions

	Lipid excimer/monomer ratio[a]					
Bilayer composition	**3a**	**3b**	**4a**	**4b**	**9a**	**9b**
DSPC	1.0	0.3	0.9	—	0.4	0.4
DSPC–cholesterol	0.5	—	0.7	—	—	—
DMPC–cholesterol	0.5	—	0.4	—	—	—
POPC–cholesterol	0.5	—	0.3	—	—	—

[a] A dash indicates that no excimer was detected.

The fluorescence spectra of the fluorinated/non-fluorinated pair of lipids **3a** and **3b** in DMPC, POPC and EYPC vesicles, showed that neither lipid formed measurable amounts of excimer in these fluid phospholipid bilayers at 25 °C. A low level of excimer was detected for lipid **3b** in DSPC (E/M = 0.25), but the fluorinated lipid **3a** formed considerable amounts of excimer in DSPC (E/M = 1.0) even at 1% mol/mol, confirming literature suggestions that fluorination does promote the phase separation of synthetic lipids (Table 1).[17] The pyridyl groups of lipids **3a** and **3b** have a pK_a of only ~3.5 in solution,[29] so at pH 7.4 this group should be unprotonated in the membrane (*ca.* 0.01% protonated). However, at more acidic pH values, a change in the charge state of the lipid *via* protonation may result in changes in the distribution of this lipid in the membrane, and suggest some possible sensing applications. Indeed, other methods of changing headgroup charge, such as metal ion coordination, are known to strongly affect the phase separation of pyrene lipids and been used to create sensors for the detection of heavy metal ions.[14] Thus the E/M ratios were determined for **3a** and **3b** in DSPC vesicles at pH 6.0 (MES buffer). The fluorinated analogue **3a** showed no significant change in excimer or monomer fluoresence at pH 6.0; the E/M ratio remains at 1. However despite the relatively small decrease in pH, excimer fluorescence disappeared for **3b** in DSPC at pH 6.0. At pH 6.0 the number of protonated lipids would be still be very low (a protonation level of *ca.* 0.5% for **3b**), so it seems more likely the change in the amount of phase separation is due to changes in the physical characteristics of the membrane at the more acidic pH. As an alternative to changing the pH, the incorporation of a permanent charge in the lipids, as found in the *N*-methylated lipids **4a** and **4b**, would be expected to decrease the amount of excimer through charge–charge repulsion. As anticipated, when incorporated into DSPC vesicles **4b** did not exhibit any excimer in the fluorescence spectrum. However, in keeping with the greater tendency of the fluorinated analogues to phase separate, fluorinated *N*-methylated lipid **4a** still showed considerable amounts of excimer in DSPC bilayers, although the E/M ratio had decreased to 0.9.[30] No excimer was observed for either **4a** or **4b** in liquid disordered EYPC, DMPC or POPC bilayers.

When the non-fluorinated lipids **3b** and **4b** were included into vesicles comprised of DMPC, DSPC, POPC and EYPC mixed 50% mol/mol with cholesterol, no excimer was observed, even in vesicles composed of DSPC and cholesterol. However fluorinated analogues **3a** and **4a** showed significant excimer emission in DSPC–cholesterol vesicles, but with reduced E/M ratios of 0.5 and 0.7, respectively (Table 1). Furthermore, strong excimer emission was now observed for both **3a** and **4a** in POPC–cholesterol and DMPC–cholesterol vesicles (E/M ratios between 0.3 and 0.5). Curiously, no excimer was observed for either **3a** or **4a** in EYPC–cholesterol bilayers.

The observation of excimer for the fluorinated lipids **3a** and **4a** in phospholipid–cholesterol bilayers suggests that it is not membrane fluidity that prevents domain formation, but instead the degree of membrane order may be the determining factor. The phospholipid bilayers that promoted phase separation of the fluorinated lipids **3a** or **4a** were those in an ordered liquid crystalline state, such as solid ordered s_o (DSPC) or liquid ordered l_o (DSPC, DMPC or POPC mixed with cholesterol). Unlike their fluorinated analogues, the non-fluorinated lipids **3b** and **4b** were generally miscible with ordered bilayers, with only **3b** weakly phase separating from solid ordered DSPC. Bilayers in the liquid disordered state l_d (EYPC, POPC and DMPC) were good solvents for the synthetic lipids and no phase separation was observed in these bilayers for any of our synthetic lipids. The observation that fluorinated lipids are immiscible with an ordered matrix is consistent with the physical properties of perfluoroalkanes. Perfluoroalkanes exhibit exceptionally weak interactions both with themselves and with other compounds, as evidenced by their low surface tensions, low enthalpies of solubilisation in alkanes and immisciblity with most organic solvents.[31] Phospholipids in an ordered bilayer have maximised their interactions with each other and with other lipids embedded within the bilayer.[8,9,10] This process would maximise unfavourable interactions between the perfluoroalkyl chain in lipids **3a** or **4a** and the alkyl chains of the phospholipids, interactions minimized by the phase separation of these two fluorinated lipids.

Synthetic lipids with more than one membrane anchor have been shown to phase separate in DSPC bilayers.[14,15] If this dual membrane anchor effect is additive to the fluorination effect we have already found, we may be able to develop lipids where the ability to phase separate is maximised. As expected, lipid **8**, with a single membrane anchor, gave no excimer when incorporated at 1% mol/mol into vesicles comprised of any of the phospholipid mixtures tested. However **9b**, with two membrane anchors, displayed significant excimer emission in DSPC vesicles at 1% mol/mol (E/M ratio of 0.4), but no excimer was observed in any of the other phospholipid compositions. Interesting, perfluorination of the sidechain, as in the analogue **9a** did not give any significant increase in the excimer emission from DSPC vesicles, exhibiting an identical E/M ratio of 0.4. Furthermore, unlike **3a** and **4a**, no excimer was observed for **9a** in any of the other phospholipid compositions, even those including cholesterol. It appears that fluorination in this case does not give any change in the extent of phase separation in DSPC bilayers and does not cause phase separation from liquid ordered bilayers, suggesting that phase separation of **9a** and **9b** is driven by crystallisation of the DSPC matrix.

Effect of temperature on phase separation.

Several of our synthetic lipids phase separate in gel-phase bilayers, but at room temperature DSPC vesicles may be unable to respond rapidly to external changes in the environment, particularly binding and adsorption events at the membrane, a field in which we are particularly interested.[32] Given that membrane fluidity

should increase with temperature, we investigated the dependence of excimer emission intensity on temperature for lipids **3a**, **4a**, **9a** and **9b** in DSPC vesicles. By heating to 60 °C, we should also be able to confirm that phase separation of these lipids is dependent upon the phospholipid matrix being in an ordered phase. Furthermore, since adding cholesterol to bilayers removes this phase transition, studying the change in excimer intensity observed for **3a** and **4a** in DMPC–cholesterol bilayers also offers an excellent control experiment.

It has been found for pyrene probes in DPPC membranes below the T_m of 41 °C that E/M gradually increased with temperature until the T_m, at which point there was a sharp drop in E/M. The increase in E/M resumed as the temperature increased beyond the T_m due to the increased frequency of collisions between excited state pyrene moieties in the now fluid matrix.[21,33] This sharp decrease in E/M at the T_m was ascribed to dispersion of pyrene-rich domains as the bilayer becomes more fluid. The increase in E/M below the T_m was attributed to either domain formation or orientational effects caused by the highly ordered structure of the gel phase.[33,34]

At 25 °C, **3a**, **4a**, **9a** and **9b** are phase separated in gel-phase DSPC, but not in fluid phase DMPC (T_m = 23 °C).[23,35] Thus we used DMPC as a control, as it revealed the increase in pyrene excimer fluorescence caused by increasing collisional frequency with temperature, which can be compared with studies in DSPC where phase separation is expected to be the dominant contributor to excimer emission. None of the lipids showed significant excimer emission at 1% mol/mol in DMPC at room temperature, and the change in the emission intensity at 480 nm as the temperature was raised from 25 to 60 °C was <5% for lipids **3a**, **4a** and **9b**, whilst **9a** showed a linear 28% increase. This suggests that at a membrane concentration of 1% mol/mol the increase in the rate of collision between pyrene moieties is only a small contributor to the overall change in pyrene fluorescence.

In contrast to previous literature studies, the intensity of pyrene excimer emission for lipids **3a**, **4a**, **9a** and **9b** in DSPC strongly decreased as the temperature was raised from 25 to 60 °C (Figs. 1 and 2). For lipids **3a**, **9a** and **9b** this decrease appeared exponential in form, with a rapid decrease in excimer emission just prior to the transition temperature at 54 °C.[36,37] Beyond 54 °C there was no excimer emission and only background light scatter was observed. However **4a** behaved differently as the temperature increased; the decrease in the amount of excimer initially showed a weaker dependence on temperature, yet as the temperature approached the phase transition temperature of DSPC there was an abrupt decrease in the amount of excimer, showing that as with lipids **3a**, **9a** and **9b**, lipid **4a** is very much more soluble in fluid phase DSPC. In all cases, after the transition temperature the observed response was similar to that in DMPC and there was no excimer.

The dependence of excimer formation on the phase state of the bilayer is further exemplified by the behaviour of lipids **3a** and **4a** in vesicles composed of a 1 : 1 mixture of DMPC and cholesterol (Fig. 1). Lipids **3a** and **4a** phase separate in DMPC–cholesterol vesicles at room temperature, both displaying E/M ratios of around 0.4. For both of these perfluorinated lipids, as the temperature was increased to 60 °C there was only a gradual decrease in the intensity of the excimer fluorescence, consistent with the observation that phospholipid bilayers with 50% mol/mol cholesterol do not undergo any phase transitions below 60 °C.[9]

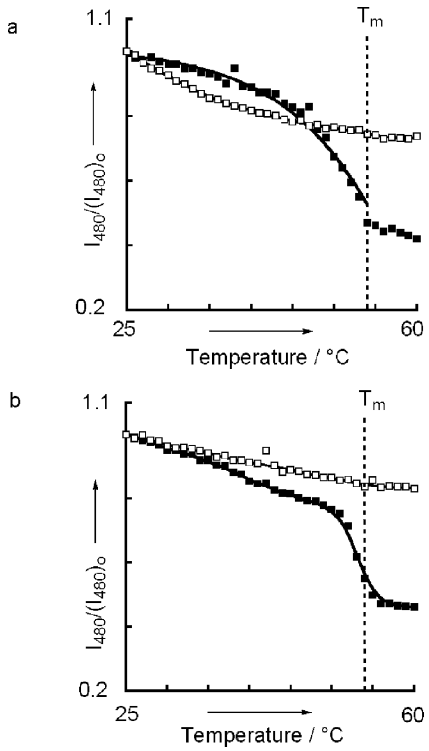


Fig. 1 Relative change in excimer emission at 480 nm with temperature for lipids **3a** and **4a** in vesicles: (a) **3a** in DSPC (■) and **3a** in 1 : 1 DMPC–cholesterol (□); (b) **4a** in DSPC (■) and **4a** in 1 : 1 DMPC–cholesterol (□).

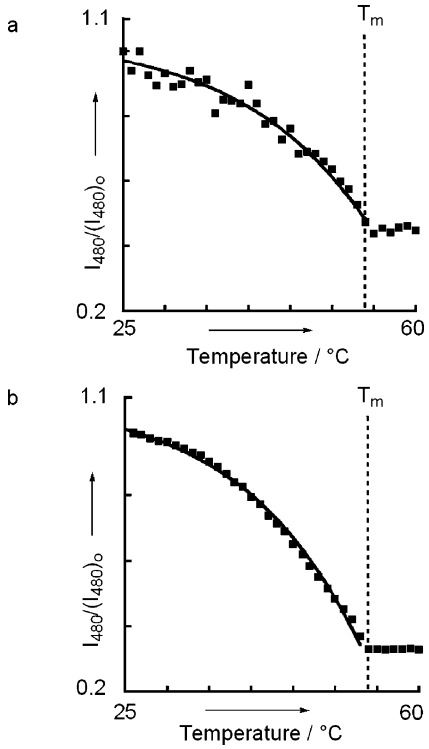


Fig. 2 Relative change in excimer emission at 480 nm with temperature for lipids **9a** and **9b** in DSPC vesicles: (a) **9a** in DSPC (■); (b) **9b** in DSPC (■).

Therefore, between 25 and 60 °C lipids **3a** and **4a** both remain phase separated in DMPC–cholesterol vesicles. The small decrease in the amount of excimer suggests the lipids are more soluble in the liquid ordered matrix at higher temperatures, consistent with increasing disorder at higher temperatures. This insensitivity to temperature may enable us to develop vesicle-based biosensors that can operate at a range of temperatures, which may be particularly important when the emission of a signal is dependent upon the rate of reorganisation of sensor lipids in the membrane.

Conclusions

We have synthesised several pyrene containing fluorinated and non-fluorinated lipids and discovered a structural motif that promotes phase separation from membranes in a fluid phase. Lipids with a single pyrene–perfluoroalkyl membrane anchor phase separated from liquid ordered phospholipid bilayers containing cholesterol, even at membrane concentrations of 1% mol/mol. Lipids with this pyrene–perfluoroalkyl motif also formed domains at 1% mol/mol in gel-phase DSPC bilayers. Lipids with two membrane anchors were found to phase separate from gel-phase DSPC bilayers only, and in this case fluorination of one of the membrane anchors made little difference to the extent of phase separation. In all cases phase separation from DSPC was dependent upon the bilayer being in the solid ordered phase; heating past the transition temperature caused the lipids to completely disperse. Indeed, none of our synthetic lipids phase separated from bilayers in the liquid disordered phase.

Now that we have found a structural motif that enables phase separation of synthetic lipids from fluid phospholipid bilayers, we aim to develop vesicle-based fluorescent sensors that will rapidly respond to external binding events. Such systems will be applied to the development of new types of sensors for biomolecules and should also allow investigation of the effect of phase separation on multivalent interactions with membrane-bound receptors.

Experimental

NMR spectra were recorded on Bruker AC 300 or AMX 400 spectrometers, and fluorescence spectra on a Hitachi F-4500 Fluorescence Spectrophotometer with a Julabo F25-HE water bath. Vesicle suspensions were diluted 1 in 1000 prior to measurement of the fluorescence spectra. ES^+ and EI^+ mass spectra were obtained on Micromass Prospec and Micromass Platform spectrometers.

Column chromatography was carried out on 60 mesh silica gel. Water was distilled HPLC grade. MES, MOPS, Egg yolk phosphatidylcholine EYPC (Type XVI from fresh egg yolk), DSPC and DMPC were used as purchased from Sigma, POPC was used as purchased from Avanti Polar Lipids. *N*-Boc-3,6-dioxaoctane-1,8-diamine (**6**) and 1-(bromomethyl)pyrene were prepared according to literature procedures.[22,38]

2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9-Hexadecafluoro-10-(pyren-1-ylmethoxy)decan-1-ol (2a)

To sodium hydride (10 mg, 0.417 mmol, 0.8 eq.) dissolved in dry DMF (3 mL) at a temperature of 10 °C was added 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9-hexadecafluorodecan-1,10-diol (705 mg, 1.526 mmol, 3 eq.), dissolved in dry DMF (1.5 mL), and mixture was stirred for 10 min. The mixture was chilled to 0 °C and 1-(bromomethyl)pyrene (50 mg, 0.508 mmol, 1 eq.), dissolved in DMF (1.5 mL) was added. The resultant solution was warmed to room temperature and left to react for 24 h. The DMF was then removed by evaporation *in vacuo*. The crude organic product was purified by addition of ethyl acetate (18 mL) and water (3 mL) followed by separation of organic layer. The aqueous layer was then washed a further three times with ethyl acetate (9 mL). The organic layers were combined and dried over magnesium sulfate and the ethyl acetate removed by evaporation *in vacuo*. Flash chromatography (SiO_2, eluent ethyl acetate–cyclohexane 2 : 7) of the crude product gave the pure product as a white solid (50 mg, 18%). R_f 0.35 (ethyl acetate–cyclohexane 1 : 2); ν_{max}(Nujol)/cm^{-1} 3355 (OH), 1600 (ArH), 1596 (ArH), 1382 (CF), 1297 (CF), 1207 (COC), 1147 (CF), 840 (ArH), 707 (ArH); δ_H (400 MHz, acetone-d_6, 25 °C) 4.18 (2 H, t, ^3J (F,H) = 14.0 Hz, CF_2CH_2OAr), 4.37 (2 H, tt, ^3J (H,H) = 7.0 Hz, ^3J (F,H) = 14.0 Hz, CF_2CH_2OH), 5.23 (1 H, t, ^3J (H,H) = 7.0 Hz, OH), 5.52 (2 H, s, CH_2Ar), 8.1–8.5 (9 H, m, Ar); δ_C (75 MHz, acetone-d_6, 25 °C): 61.2 (^3J (F,C) = 22 Hz, CF_2CH_2OH), 68.3 (^3J (F,C) = 22 Hz, CF_2CH_2OAr), 74.2 ($ArCH_2O$), 102–115 (8 × CF_2), 125.4 (CH), 126.6 (CH), 126.7 (CH), 127.5 (CH), 128.3 (CH), 129.5 (CH), 129.7 (CH), 129.9 (CH), 131.5 (C), 132.3 (C), 133.1 (C), 133.5 (C), 133.9 (C). *m/z* (EI) 676 (16%, M^+) 215 (100%, $PyreneCH_2^+$). *m/z* (EI-HRMS) 476.0887 (M^+. $C_{27}H_{16}O_2F_{16}^+$ requires 476.0889).

2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9-Hexadecafluoro-10-(pyren-1-ylmethoxy)decyl isonicotinate (3a)

To a solution of 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9-hexadecafluoro-10-(pyren-1-ylmethoxy)decan-1-ol (50 mg, 0.07 mmol, 1 eq.) in dry dichloromethane (1 mL) was added isonicotinyl chloride hydrochloride (26.3 mg, 0.15 mmol, 2.14 eq.), triethylamine (20.1 μL) and DMAP (3 mg). The mixture was left stirring overnight. Evaporation of the dichloromethane *in vacuo* gave a white residue which was purified by flash column chromatography (SiO_2, ethyl acetate–cyclohexane 1 : 1) to give the pure product as a white solid (44 mg, 79%) R_f 0.3 (ethyl acetate–cyclohexane 1 : 2); ν_{max}(Nujol)/cm^{-1}: 1739 (C=O), 1603 (CH_{Ar}), 1561 (CH_{Ar}), 1374 (CF), 1215 (CO); δ_H (300 MHz, $CDCl_3$, 25 °C) 4.04 (2 H, t, ^3J (F,H) = 14.0 Hz, CF_2CH_2OAr), 4.86 (2 H, t, ^3J (F,H) = 14.0 Hz, $CF_2CH_2OC(O)$), 5.43 (2 H, s, CH_2Ar), 7.88 (2 H, d, ^3J (H,H) = 7.5 Hz, pyridyl C*H*), 8–8.39 (9 H, m, Ar), 8.85 (2 H, ^3J (H,H) = 7.5 Hz, pyridyl C*H*); δ_C (75 MHz, $CDCl_3$, 25 °C): 61.3 (CF_2CH_2Opy), 66.5 (CF_2CH_2OAr), 73.9 ($ArCH_2O$), 109–117 (m, 8 × CF_2), 124.0 (pyridyl *C*H), 124.1 (C), 125.6 (CH), 125.7 (CH), 126.6 (C), 127.2 (2 × CH), 128.4 (CH), 129.0 (CH), 129.3 (CH), 130.2 (C), 131.2 (C), 132.0 (C), 132.3 (C), 133.0 (C), 136.6 (CH), 143.8 (C), 152.2 (pyridyl *C*H), 165.0 (C); *m/z* (ES-HRMS) 782.1187 ($M + H^+$. $C_{33}H_{19}F_{16}NO_3^+$ requires 782.1188).

2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9-Hexadecafluoro-10-(pyren-1-ylmethoxy)decyl-*N*-methylisonicotinium iodide (4a)

To a concentrated solution of 2,2,3,3,4,4,5,5,6,6,7,7,8,8,9,9-hexadecafluoro-10-(pyren-1-ylmethoxy)decyl isonicotinate (63 mg, 0.09 mmol, 1 eq.) dissolved in acetone (2 mL) was added methyl iodide (130 μL, 0.9 mmol, 10 eq.) and the mixture

left to stir. After 3 d, the solid that formed was filtered and washed with acetone to give the pure product as an off-white solid (43 mg, 66%), R_f 0.61; ν_{max}(solid KBr disk)/cm^{-1} 3449, 3038, 2924, 2854, 1763 (C=O), 1639, 1460, 1383, 1281, 1204; δ_H (300 MHz, acetone-d_6, 25 °C) 4.36 (2 H t, ^3J (F,H) = 22 Hz, CF_2CH_2OAr), 4.81 (3 H, s, NCH_3), 5.24 (2 H t, ^3J (F,H) = 22 Hz, $CF_2CH_2OC(O)$), 5.52 (2 H, s, CH_2Ar), 8.08–8.75 (11 H, m, Ar + pyridyl C*H*), 9.51 (2 H, d, ^3J (H,H) = 7.5 Hz, pyridyl C*H*); δ_C (100 MHz, acetone-d_6, 25 °C) 50.3 (CH_3), 62.7 (^3J (F,C) = 26 Hz, CF_2CH_2OH), 67.9 (^3J (F,C) = 26 Hz, CF_2CH_2OAr), 73.7 ($ArCH_2O$), 109–117 (m, 8 × CF_2), 123.9 (C), 124.7 (CH), 125.7 (C), 125.9 (CH), 126.8 (CH), 127.5 (2 × CH), 128.7 (CH), 128.8 (CH), 129.0 (CH), 129.1 (CH), 130.7 (C), 131.5 (C), 132.1 (C) 132.6 (C), 133.0 (C), 149.0 (C), 152.4 (pyridyl *C*H), 162.2 (C); *m*/*z* (ES-HRMS) 796.1344 (M + H$^+$. $C_{34}H_{21}F_{16}NO_3^+$ requires 796.1339).

10-(Pyrene-1-ylmethoxy)decan-1-ol (2b)

1,10-Decandiol (264 mg, 1.515 mmol, 3 eq.) dissolved in dry DMF (1.5 mL) was added to sodium hydride (14 mg, 0.711 mmol, 1.4 eq.) dissolved in dry DMF (3 mL) at a temperature of 10 °C, and the resultant mixture stirred for 10 min. The mixture was chilled to 0 °C and 1-(bromomethyl)pyrene (50 mg, 0.505 mmol), dissolved in DMF (1.5 mL), was added. The resultant solution was warmed to room temperature and left to react for 24 h. The DMF was removed by evaporation *in vacuo* to give an off-white residue. The crude organic product was purified by addition of ethyl acetate (18 mL) and water (3 mL) followed by separation of organic layer. The aqueous layer was then washed further with ethyl acetate (3 × 9 mL). The organic layers were combined, dried over magnesium sulfate, filtered and the ethyl acetate removed from the filtrate by evaporation *in vacuo*. The pure product was obtained as a white solid after purification by flash column chromatography (SiO_2, eluent ethyl acetate–cyclohexane 2 : 5), (30 mg, 15%). R_f 0.39 (ethyl acetate–cyclohexane 1 : 1); ν_{max}(Nujol)/cm^{-1} 3223 (OH), 1720 (C=O), 1596 (CH_{Ar}), 1374 (CO), 1300 (C–O); δ_H (300 MHz, $CDCl_3$, 25 °C) 1.24 (12 H, br m, alkyl CH_2), 1.44 (2 H, br m, alkyl CH_2), 1.52 (2 H, t, ^3J (H,H) = 6.5 Hz, alkyl CH_2), 2.06 (2 H, s, $OH + 1/2H_2O$), 3.63 (4 H, t, ^3J (H,H) = 6.5 Hz, 2 × CH_2O), 5.23 (2 H, s, CH_2Ar), 8–8.23 (9 H, m, Ar); δ_C (75 MHz, $CDCl_3$, 25 °C) 26.1 (CH_2), 26.6 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 30.2 (CH_2), 33.2 (CH_2), 63.4 (CH_2OH), 71.0 (OCH_2), 71.9 ($ArCH_2O$), 123.9 (CH), 124.9 (CH), 125.1 (CH), 125.3 (CH), 126.3 (CH), 127.3 (CH), 127.7 (CH), 128.0 (CH), 129.7 (C), 131.2 (C), 131.6 (C), 132.2 (C); *m*/*z* (ES-HRMS) 411.2292 (M + H$^+$. $C_{27}H_{32}O_2Na^+$ requires 411.2295).

10-(Pyren-1-ylmethoxy)decyl isonicotinate (3b)

To a solution of 10-(pyrene-1-ylmethoxy)decan-1-ol (30 mg, 0.077 mmol, 1 eq.) in dry dichloromethane (1 mL) was added isonicotinyl chloride hydrochloride (15.8 mg, 0.088 mmol, 1.14 eq.), triethylamine (20.1 μL) and DMAP (3 mg). The reaction mixture was left stirring overnight at room temperature. Evaporation of the dichloromethane *in vacuo* gave an off-white solid, which was purified by flash column chromatography (SiO_2, eluent ethyl acetate–cyclohexane 2 : 5). This gave the pure product as a white solid (12 mg, 31%). R_f 0.59 (eluent ethyl acetate–cyclohexane 2 : 5); ν_{max}(Nujol)/cm^{-1} 1732 (C=O), 1596 (CH_{Ar}), 1553 (CH_{Ar}), 1376 (CO), 1273 (CO); δ_H (300 MHz, $CDCl_3$, 25 °C) 1.26 (12 H, m, alkyl CH_2); 1.44 (2 H, br m, alkyl CH_2), 1.70 (2 H, t, ^3J (H,H) = 7.5 Hz, alkyl CH_2), 3.60 (2 H, t, ^3J (H,H) = 7.4 Hz, CH_2O), 4.33 (2 H, t, ^3J (H,H) = 7.5 Hz, $CH_2OC(O)$), 5.23 (2 H, s, CH_2Ar), 7.84 (2 H, d, ^3J (H,H) = 7.6 Hz, pyridyl C*H*); 8.02–8.38 (9 H, m, Ar), 8.78 (2 H, d, ^3J (H,H) = 7.6 Hz, pyridyl C*H*); δ_C (75 MHz, $CDCl_3$, 25 °C): 26.3 (CH_2), 26.6 (CH_2), 28.9 (CH_2), 29.6 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 30.2 (CH_2), 66.5 (CH_2), 70.9 (CH_2), 71.9 (CH_2), 123.5 (CH), 123.9 (CH), 124.9 (CH), 125.1 (CH), 125.3 (CH), 125.5 (CH), 126.3 (CH), 127.3 (CH), 127.7 (CH), 127.8 (CH), 128.0 (CH), 129.7 (C), 130.1 (CH), 131.2 (C), 131.6 (C), 131.6 (C), 132.2 (C), 150.3 (CH), 165.3 (C); *m*/*z* (ES-HRMS) 494.2695 (M + H$^+$. $C_{33}H_{36}NO_3^+$ requires 494.2690).

10-(Pyren-1-ylmethoxy)decyl-*N*-methylisonicotinium iodide (4b)

Methyl iodide (130 μL, 1.2 mmol, 50 eq.) was added to a concentrated solution of 10-(pyren-1-ylmethoxy)decyl isonicotinate (12 mg, 0.024 mmol, 1 eq.) in acetone (250 μL) and the mixture left to stir for 3 d. The solvent was then evaporated *in vacuo* to give the product as a white solid that required no further purification (6.4 mg, 52%). ν_{max}(KBr disk)/cm^{-1} 3440, 2924, 1726 (C=O), 1633 (CH_{Ar}), 1384 (CO), 1127 (CO); δ_H (300 MHz, $CDCl_3$, 25 °C) 1.1–1.9 (16 H, m, CH_2 alkyl), 3.58 (2 H, t, ^3J (H,H) = 7.5 Hz, CH_2O), 4.30–4.80 (5 H; t, $CH_2OC(O)$ + s, NCH_3), 5.20 (2 H, s, OCH_2Ar), 7.92–8.48 (9 H, m, Ar), 9.0–9.43 (4 H, m, pyridyl C*H*); δ_C (75 MHz, $CDCl_3$, 25 °C) 24.7 (CH_2), 25.1 (CH_2), 27.2 (CH_2), 27.9 (CH_2), 28.1 (CH_2), 28.2 (CH_2), 28.7 (CH_2), 49.0 (CH_3), 66.8 (CH_2), 69.8 (CH_2), 70.5 (CH_2), 71.9 (CH_2), 122.6 (CH), 123.5 (CH), 124.2 (CH), 124.3 (CH), 125.0 (CH), 126.0 (CH), 126.2 (CH), 126.4 (CH), 126.6 (CH), 128.2 (C), 129.7 (C), 130.1 (C), 130.9 (C), 143.3 (C), 145.6 (pyridyl *C*H), 160.0 (C=O); *m*/*z* (ES-HRMS) 508.2852 (M + H$^+$. $C_{34}H_{38}NO_3^+$ requires 508.2846).

N-Boc-*N*′-(pyren-1-ylmethyl)-3,6-dioxaoctane-1,8-diamine[39] (7)

To a solution of 1-pyrenemethanol (400 mg, 1.7 mmol, 1 eq.) and *N*-Boc-3,6-dioxaoctane-1,8-diamine (550 mg, 2.2 mmol, 1.3 eq.) in dichloroethane (10 mL) containing 4 Å molecular sieves (2.4 g) was added activated manganese oxide (1.5 g, 17.2 mmol, 10 eq.). The reaction mixture was stirred at room temperature for at least 4 h and reaction progress monitored by TLC. Sodium borohydride (100 mg, 2.6 mmol, 1.5 eq.) was added, followed by methanol (2 mL) at 0 °C. The reaction mixture was stirred for 30 min at room temperature and filtered on a short Celite® pad. The solvents were evaporated *in vacuo* and the resulting residue was purified by flash chromatography on silica gel, using ethanol–ethyl acetate–triethylamine 40 : 59.5 : 0.5 as the eluent, to give the product as a colorless oil (710 mg, 89%). ν_{max}(neat)/cm^{-1} 3500–3100, 3000–2800, 1709, 1515, 1455, 1365 1250, 1172; δ_H (300 MHz, $CDCl_3$, 25 °C) 1.42 (s, 9 H), 2.01 (br s, 1 H), 2.99 (t, ^3J (H,H) = 5.2 Hz, 2 H), 3.27 (m, 2 H), 3.48 (t, ^3J (H,H) = 5.0 Hz, 2H), 3.58 (s, 4 H), 3.67 (t, ^3J (H,H) = 5.2 Hz, 2 H), 4.53 (s, 2 H), 5.10 (br s, 1 H), 7.97–8.0 (m, 4 H), 8.11–8.19 (m, 4 H), 8.38 (d, ^3J (H,H) = 9.2 Hz, 1 H); δ_C (75 MHz, $CDCl_3$, 25 °C) 28.8 (CH_3), 40.7 (CH_2), 49.5 (CH_2), 52.0 (CH_2), 70.6 (CH_2), 71.0 (CH_2), 79.5 (C), 123.1 (CH), 125.3 (CH), 125.7 (CH), 125.9 (CH), 126.0 (CH), 126.5 (CH), 127.8 (CH), 127.8 (C), 128.0 (CH), 128.2 (CH), 128.6 (CH), 129.5

(C), 131.1 (C), 131.2 (C), 131.7 (C), 156.4 (C); *m/z* (ES-HRMS) 463.2585 (M + H$^+$. $C_{28}H_{35}N_2O_4^+$ requires 463.2597).

N-(Pyren-1-ylmethylamino)-3,6-dioxaoctane-1,8-diamine (8)

To a solution of *N*-Boc-*N*′-(pyren-1-ylmethyl)-3,6-dioxaoctane-1,8-diamine (60 mg, 0.130 mmol, 1 eq.) in dichloromethane (1 mL) at room temperature was added trifluoroacetic acid (300 μL, 4 mmol, 31 eq.). The reaction was stirred for 3 h then the solvent evaporated *in vacuo* at room temperature to provide the crude product as an oil. (78 mg, 99%). ν_{max}(neat)/cm^{-1} 3446, 3049, 1780, 1683, 1457, 1354, 1204, 850, 798, 724, 707; δ_H (300 MHz, $CDCl_3$, 25 °C) 3.07 (br m, 2 H), 3.26 (br m, 2 H), 3.53 (t, ^3J (H,H) = 5.2 Hz, 2 H), 3.55 (s, 4 H), 3.65 (t, ^3J (H,H) = 4.9 Hz, 2 H), 4.99 (br t, ^3J (H,H =2 Hz, 2 H), 7.39 (br s, 2 H), 8.07 (m, 3H), 8.16 (d, ^3J (H,H) = 9.1 Hz, 1 H), 8.19 (d, ^3J (H,H) = 8.0 Hz, 1 H), 8.22 (s, 2 H), 8.26 (d, ^3J (H,H) = 7.6 Hz, 2 H); *m/z* (ES-HRMS) 363.2061 (M + H$^+$. $C_{23}H_{27}N_2O_2^+$ requires 363.2072).

N′-2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-Pentadecafluorooctanoyl-*N*-Boc-*N*′-(pyren-1-ylmethyl)-3,6-dioxaoctane-1,8-diamide

To a solution of *N*-Boc-*N*′-(pyren-1-ylmethyl)-3,6-dioxaoctane-1,8-diamine (80 mg, 0.173 mmol, 1 eq.) in dichloroethane (1 mL) at room temperature was added dry pyridine (42 μL, 0.519 mmol, 3 eq.), DMAP (2 mg, 0.017 mmol, 0.1 eq.) and perfluorooctanoyl chloride (75 mg, 0.173 mmol, 1 eq.). The reaction was stirred for 3 h and the solvent evaporated *in vacuo*. The residue was purified by flash chromatography on silica gel using ethyl acetate–cyclohexane 2 : 5 as the eluent to provide the product as a colorless oil (144 mg, 97%). In the NMR spectra a 1 : 0.7 mixture of rotamers about the perfluorooctanamide bond was observed. ν_{max}(neat)/cm^{-1} 3382, 2977, 1720 (C=O), 1683 (C=O), 1507, 1366, 1018; δ_H (300 MHz, $CDCl_3$, 25 °C) 1.37 (s, 9 H), 3.33 (br s, 2.1 H), 3.50–3.75 (m, 10.4 H), 4.92 (br s, 0.8 H), 5.29 (s, 0.8 H), 5.63 (s, 2 H), 7.80 (d, J = 7.9 Hz, 0.4 H), 7.95 (d, J = 7.8 Hz, 0.6 H), 8.00–8.24 (m, 8 H); δ_C (75 MHz, $CDCl_3$, 25 °C) 28.7 (CH_3), 40.8 (CH_2), 45.9 (CH_2), 47.7 (CH_2), 49.1 (CH_2), 50.2 (CH_2), 50.3 (CH_2), 50.3 (CH_2), 53.8 (CH_2), 69.0 (CH_2), 70.6 (CH_2), 70.7 (CH_2), 70.8 (CH_2), 71.0 (CH_2), 71.1 (CH_2), 79.6 (C), 121.8 (CH), 123.0 (CH), 124.4 (CH), 125.1 (CH), 125.4 (CH), 125.8 (CH), 125.9 (CH), 126.1 (CH), 126.5 (CH), 126.6 (CH), 127.6 (CH), 127.7 (CH), 128.0 (CH), 128.1 (CH), 128.2 (CH), 128.7 (CH), 128.8 (CH), 129.9 (C), 131.1 (C), 131.6 (C), 131.8 (C), 156.3 (C), 158.7 (C), 159.0 (C); *m/z* (ES-HRMS) 881.2028 (M + Na$^+$. $C_{36}H_{33}F_{15}N_2O_5Na^+$ requires 881.2048).

N′-2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-Pentadecafluorooctanoyl-8-(pyren-1-ylmethylamino)-3,6-dioxaoctanamide (9a)

To a solution of *N*′-2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-pentadecafluorooctanoyl *N*-Boc-*N*′-(pyren-1-ylmethyl)-3,6-dioxaoctane-1,8-diamide (130 mg, 0.15 mmol, 1 eq.) in dichloromethane (2 mL) at room temperature was added trifluoroacetic acid (375 μL, 5 mmol, 33 eq.). The reaction was stirred for 3 h and the solvent evaporated *in vacuo* at room temperature to provide the crude product as a viscous oil (133 mg, 99%). In the NMR spectra a 1 : 0.7 mixture of rotamers about the perfluorooctanamide bond was observed. ν_{max}(neat)/cm^{-1} 3600–2400, 1779, 1682, 1515, 1456, 1203; δ_H (300 MHz, $CDCl_3$, 25 °C) 3.25 (br s, 1.88 H), 3.50–3.75 (m, 9.94 H), 5.47 (s, 0.80 H), 5.52 (s, 1.1 H), 7.29 (br s, 2.6 H), 7.76 (d, ^3J (H,H) = 7.9 Hz, 0.58 H), 7.85 (d, ^3J (H,H) = 7.8 Hz, 0.42 H), 8.00–8.25 (m, 8H); δ_C (75 MHz, $CDCl_3$, 25 °C) 40.1 (CH_2), 40.4 (CH_2), 45.5 (CH_2), 45.5 (CH_2),47.0 (CH_2), 48.7 (CH_2), 49.1 (CH_2), 66.0 (CH_2), 66.2 (CH_2), 67.6 (CH_2), 68.2 (CH_2), 70.0 (CH_2), 70.2 (CH_2), 70.2 (CH_2), 70.4 (CH_2), 120.8 (CH), 122.0 (CH), 123.8 (CH), 124.7 (CH), 125.1 (CH), 125.7 (CH), 126.0 (CH), 126.3 (CH), 126.4 (CH), 127.2 (CH), 127.2 (CH), 128.0 (CH), 128.4 (CH), 128.8 (CH), 129.3 (C), 130.9 (C), 131.7 (C), 131.9 (C), 132.1 (C), 160–165 (br, C); *m/z* (ES-HRMS) 759.1669 (M + H$^+$. $C_{31}H_{26}F_{15}N_2O_3^+$ requires 759.1704].

N′-Octanoyl-*N*-Boc-*N*′-(pyren-1-ylmethyl)-3,6-dioxaoctane-1,8-diamide

To a solution of *N*-Boc-*N*′-(pyren-1-ylmethyl)-3,6-dioxaoctane-1,8-diamine (200 mg, 0.432 mmol, 1 eq.) in dichloroethane (2 mL) at 0 °C was added di-*iso*-propylethylamine (151 μL, 0.865 mmol, 2 eq.), DMAP (5.3 mg, 0.043 mmol, 0.1 eq.) and octanoyl chloride (96 μL, 0.562 mmol, 1.3 eq.). The reaction mixture was warmed to room temperature and left stirring for 3 h. The solvent was evaporated *in vacuo* and the residue purified by flash chromatography on silica gel using ethyl acetate–cyclohexane 2 : 1 as the eluent. This provided the product as a colorless oil (242 mg, 96%). In the NMR spectra a 1 : 0.83 mixture of rotamers about the octanamide bond was observed. ν_{max}(neat)/cm^{-1} 3342, 3042, 2928, 1716 (C=O), 1646, 1506, 1456, 1417, 1365, 1250, 1173; δ_H (300 MHz, $CDCl_3$, 25 °C) 0.81 (br t, ^3J (H,H) = 6.6 Hz, 1.38 H), 0.89 (br t, ^3J (H,H) = 6.0 Hz, 1.66 H), 1.39 (s, ~9 H), 1.18–1.40 (br m, ~10 H), 1.56–1.80 (br m, 2.12 H), 2.39 (t, ^3J (H,H) = 7.4 Hz, 0.90 H), 2.50 (t, ^3J (H,H) = 7.5 Hz, 1.11 H), 3.28 (br m, 1.99 H), 3.37–3.55 (m, 8.56 H), 3.74 (s, 1.84 H), 4.98 (br m, 0.94 H), 5.28 (s, 0.71 H), 5.43 (s, 0.85 H), 5.45 (s, 1.04 H), 7.77 (d, ^3J (H,H) = 7.9 Hz, 0.46 H), 7.90 (d, ^3J (H,H) = 7.8 Hz, 0.57 H), 7.97–8.23 (m, 7.68 H), 8.31 (d, J = 9.3, 0.59 H); δ_C (75 MHz, $CDCl_3$, 25 °C) 14.4 (CH), 14.5 (CH_2), 22.9 (CH_2), 23.1 (CH_2), 25.3 (CH_2), 25.7 (CH_2), 26.02 (CH_2), 28.8 (CH), 29.3 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 29.9 (CH_2), 32.0 (CH_2), 32.2 (CH_2), 33.6 (CH_2), 33.8 (CH_2), 34.4 (CH_2), 40.7 (CH_2), 46.3 (CH_2), 46.7 (CH_2), 46.8 (CH_2), 50.9 (CH_2), 69.7 (CH_2), 70.2 (CH_2), 70.6 (CH_2), 70.7 (CH_2), 71.0 (CH_2), 79.6 (C), 121.9 (CH), 123.4 (CH), 123.7 (CH), 125.0 (CH), 125.1 (C), 125.4 (CH), 125.6 (CH), 125.7 (CH), 125.9 (CH), 126.4 (CH), 126.5 (CH), 127.7 (CH), 127.8 (CH), 128.0 (CH), 128.3 (CH), 128.5 (CH); 131.4 (C), 131.6 (C), 156.4 (C), 174.1 (C), 174.8 (C); *m/z* (ES-HRMS) 611.3453 (M + Na$^+$. $C_{36}H_{48}N_2O_5Na^+$ requires 611.3461).

N′-Octanoyl 8-(pyren-1-ylmethylamino)-3,6-dioxaoctanamide (9b)

To a solution of *N*′-octanoyl-*N*-Boc-*N*′-(pyren-1-ylmethyl)-3,6-dioxaoctane-1,8-diamide (60 mg, 0.1 mmol, 1 eq.) in dichloromethane (1 mL) at room temperature was added trifluoroacetic acid (250 μL, 3.3 mmol, 33 eq.). The reaction was stirred for 3 h and the solvent evaporated *in vacuo* at room temperature to provide the pure product as a viscous oil (64 mg, 99%). In the NMR spectra a 1 : 0.7 mixture of rotamers about the octanamide bond was observed. ν_{max}(neat)/cm^{-1} 3600–2600, 1779, 1622, 1456, 1207. δ_H (300 MHz, $CDCl_3$, 25 °C) 0.79 (t, ^3J (H,H) = 6.9 Hz, 1.97 H); 0.85 (t, ^3J (H,H) = 7.1 Hz, 1.2 H), 1.10–1.35 (m, 8.4 H), 1.64 (br m, 2.38 H), 2.46 (t, ^3J (H,H) = 7.3 Hz, 1.4 H), 2.63

(t, ^3J (H,H) = 7.8, 0.7 H), 3.36 (m, 2.1 H), 3.55–3.85 (m, 10.1 H), 5.38 (s, 1.8 H), 5.45 (s, 0.8 H), 7.50 (br s, 2.6 H), 7.74 (d, ^3J (H,H) = 7.9, 0.6 H), 7.84 (d, ^3J (H,H) = 7.8 Hz, 0.4 H), 8.00–8.27 (m, 8.34 H); δ_C (75 MHz, $CDCl_3$, 25 °C) 14.2 (CH_3), 14.3 (CH_2), 22.8 (CH_2), 22.9 (CH_2), 26.1 (CH_2), 26.4 (CH_2), 29.0 (CH_2), 29.2 (CH_2), 29.5 (CH_2), 29.7 (CH_2), 31.8 (CH_2), 31.9 (CH_2), 33.7 (CH_2), 33.7 (CH_2), 40.6 (CH_2), 40.8 (CH_2), 46.8 (CH_2), 47.1 (CH_2), 47.2 (CH_2), 50.7 (CH_2), 66.3 (CH_2), 66.5 (CH_2), 68.0 (CH_2), 68.5 (CH_2), 70.3 (CH_2), 70.5 (CH_2), 70.6 (CH_2), 70.9 (CH_2), 121.2 (CH), 122.6 (CH), 123.0 (CH), 124.9 (CH), 125.4 (CH), 126.0 (CH), 126.1 (CH), 126.4 (CH), 126.7 (CH), 126.8 (CH), 127.7 (CH), 128.3 (CH), 128.9 (CH), 129.1 (CH) 129.1 (C), 130.9 (C), 131.6 (C), 131.7 (C), 131.8 (C), 160–165 (br, C); *m/z* (ES-HRMS) 489.3134 ($M + H^+$. $C_{31}H_{41}N_2O_3^+$ requires 489.3117).

Phospholipid vesicle synthesis

The required amount of lipids **3a**–**9b** to give a 1% mol/mol membrane concentration were added to the appropriate phosphatidylcholine–cholesterol mixture and the mixture dissolved in spectroscopic grade ethanol-free chloroform, followed by removal of the solvent to give a thin film of phospholipid on the interior of a round-bottomed flask. MOPS buffer, pH 7.4 (20 mM) was added to give a final total lipid concentration of 20 mM, then phospholipid suspensions prepared by vortex mixing the thin film of phospholipid with the buffer. Unilamellar vesicles doped with lipids **3a**–**9b** (1% mol/mol) were synthesised by extrusion of this phospholipid suspension through polycarbonate membranes with 800 nm pores at temperatures above the respective phase transition temperatures (25 °C for EYPC, POPC, DMPC and phospholipid–cholesterol mixtures, 60 °C for DSPC).

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