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Interactions of Cytochrome c with N-Acylated Phosphatidylethanolamine Lipids

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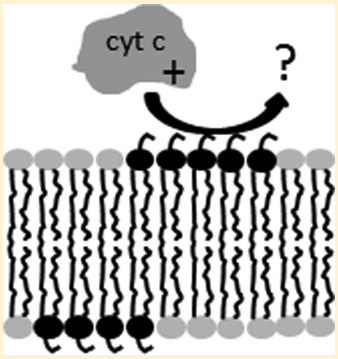
ABSTRACT: *N*-Acylphosphatidylethanolamines (NAPEs) are naturally occurring derivatives of phosphatidylethanolmine (PE) in which the PE amino group is attached to an acyl chain. Given their occurrence in natural systems, there is interest in knowing the effect of NAPEs on membrane dynamic structure and function. This study examines the ability of NAPEs to affect the association of the cytochrome c and Zn-heme cytochrome c with the surface of bilayer membranes. Fluorescence titration experiments show that cationic cytochrome c has the same high affinity for the surfaces of anionic vesicles that are rich in NAPEs or diplalmitoyphosphatidylglycerol (DPPG) but the protein/membrane interaction in each case is quite different. Cytochrome c adsorption to DPPG membranes is relatively irreversible due to the DPPG molecules adopting an extended conformation that promotes strong hydrophobic contact with the adsorbed protein. In contrast, cytochrome c association with *N*-acyl DPPE membranes is due primarily to reversible electrostatic interactions with the anionic headgroup, and not hydrophobic contact with the *N*-acyl chain. The presence of a small mole fraction of an *N*-propionyl derivative of DPPE (N-C3:0-DPPE) diminishes cytochrome c affinity for vesicles containing a large amount of DPPG apparently by relieving the membrane packing strain that drives the extended DPPG conformation.



INTRODUCTION

N-Acylphosphatidylethanolamines (NAPEs) are naturally occurring derivatives of phosphatidylethanolmine (PE) in which the PE amino group is attached to an acyl chain. NAPEs are found in plant, animal, microbes, and vertebrates, albeit in very small amounts relative to the total phospholipid content.[1,2] The amount of NAPEs, and the related *N*-acylethanolamines (NAEs), are known to increase when tissue is subject to a stress, such as wound formation in animals or dehydration/rehydration and hypoxia in plants.[3] Given their occurrence in natural systems, there is interest in knowing the biosynthetic pathways leading from NAPEs to NAEs, and also the effect of NAPEs on membrane dynamic structure and function.

Structurally, the conversion of a PE to a NAPE alters the charge of the phospholipid headgroup from zwitterionic to anionic and also introduces the bulky *N*-acyl chain. Infrared spectroscopy (IR), Raman, and electron spin resonance (ESR) studies have revealed the location and orientation of the *N*-acyl chain as a function of chain length in model membrane systems.[4−6] When the *N*-acyl chain has ten or more carbon atoms, it penetrates into the hydrophobic core of the bilayer and has the same orientation as the two glycero acyl chains. When the *N*-acyl chain is less than ten carbons, the chain is randomly oriented and located in the interfacial area, at the level of the glycero backbone. Differential scanning calorimetry (DSC), ^{31}P nuclear magnetic resonance (NMR), and IR studies have shown that the membrane gel-to-liquid transition temperature (T_m) is lowered when PE is replaced by short *N*-acyl chain NAPEs and raised when replaced by long chain NAPEs. In all cases, the *N*-acyl chain leads to a strong perturbation and restriction of the headgroup rotational mobility.[7−12]

This study addresses questions related to the ability of NAPEs to affect the association of the cytochrome c and Zn-heme cytochrome c with the surface of bilayer membranes. Cytochrome c is a highly conserved, highly water-soluble, heme-based protein with a molar mass of 12 400 Da.[13] Cytochrome c is the prototypical example of a peripheral protein and has been the focus of many studies examining it is interactions with membrane and model membrane structures.[14] Cytochrome c has +8 charge at physiological pH and been shown to bind to deprotonated acidic lipid domains loosely through electrostatic and more strongly through hydrophobic interactions.[15−18] The protein has a preferred orientation on the membrane surface and has been shown to undergo conformational changes upon adsorption.[18−25] The adsorption of cytochrome c also leads to the formation of microdomains that are rich in acidic lipids.[18,25−27] Some authors have reported that upon adsorption cytochrome c partially penetrates into the hydrophobic core of the bilayer while others report intramembrane penetration of the bilayer to such a depth that the

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protein can communicate with the inner membrane-water interface.[20,23,26,28−30]

To explain the hydrophobic contacts with cytochrome c, Kinnunen and co-workers have proposed that certain phospholipids, such as phosphatidylglycerol or cardiolipin, have ability to adopt an extended lipid conformation.[16,31−33] As shown in Figure 1, a phospholipid embedded in the outer layer

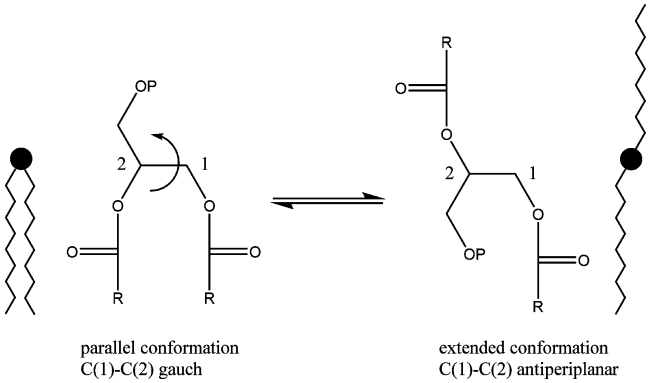

Figure 1. Conformation change experienced by normal diacyl phospholipids leading to the extended lipid conformation.

of a membrane undergoes a gauche to antiperiplanar conformational change allowing the nonpolar tails of a lipid to extend in opposite directions. The conformation change removes an acyl chain from the membrane outer monolayer and thus reduces phospholipid packing strain. It is favored by phospholipids that have a propensity to adopt an inverted hexagonal phase (H_{II}) and occurs when a membrane-bound peripheral protein presents a hydrophobic groove or channel to accept the exposed acyl chain. The extended lipid anchorage is potentially a universal mechanism for peripheral membrane protein−lipid interactions as well as vesicle fusion, but the phospholipid structural scope and the physiological relevance of this mode of interaction has not been broadly tested.[33−36] Here we describe the interaction of cytochrome c and Zn-heme cytochrome c with a series of vesicle membranes containing NAPEs with *N*-acyl chains of different length. We find that membrane association is quite strong but there is no evidence that the protein forms irreversible contacts with the *N*-acyl chains.

EXPERIMENTAL SECTION

Chemicals. DPPG, DOPG, and DOPC were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. The *β*-pyrene-C10-PG fluorescence label was purchased from Invitrogen (Carlsbad, CA). DPPE was purchased from Genzyme Pharmaceuticals (Cambridge MA). Propionyl chloride, 3-bromopropionyl chloride, octanoyl chloride, palmitoyl chloride were purchased from the Aldrich Chemical Co (Milwaukee, WI) and used as received. Beef heart cytochrome c (ferrichrome c) was purchased from Acros Organics USA (Morris Plains, NJ) and used as received. Zn-heme cytochrome *c* was prepared and purified according to the methods of Hirsch et al.[32]

N-C3:0-DPPE, N-C8:0-DPPE, N-C16:0-DPPE, and N-C3:0-Br-DPPE were prepared in a one-step reaction of DPPE with the corresponding acid chloride (propionyl chloride, octanoyl chloride, palmitoyl chloride, 3-bromopropionyl chloride) in anhydrous dimethylformamide and in the presence of *N*,*N*-diisopropylethylamine. The products of the N-acylation reactions were purified using column chromatography (silica gel, 90:10 chloroform/ethanol, v/v), and structure confirmed by mass spectrometry. The purity of each sample was checked by TLC immediately before use. The tetrabromo derivative of DOPG, 1,2-di(9,10-dibromo)stearoyl-*sn*-glycero-3-phosphoglycerol or D(diBr)SPG, was prepared by adding molecular bromine in hexane to a solution of DOPG in chloroform until the orange color of molecular bromine persisted. The resulting product was purified using column chromatography (silica gel, 70:30 chloroform/methanol, v/v) and the structures confirmed by ^{1}H NMR and mass spectrometry.

Vesicle Preparation. Vesicle suspensions were prepared from stock solutions of the phospholipids in chloroform. Labeled vesicles contain the *β*-pyrene-C10-PG at a level of 1 mol %. The chloroform was removed in two steps, first using a stream of nitrogen followed by 20 min in a vacuum jar. The lipids were hydrated in 0.01 M pH 7.4 HEPES buffer for 30 min at 30 °C and then agitated for 30 s in an ultrasonic bath. Vesicles were formed by 21 passes through a track-etched polycarbonate membrane (0.1 *μ*m dia.) in a mini-extruder (Avanti Polar Lipids, Alabaster, AL) at 51 °C for vesicles containing dipalmitoyl lipids and room temperature for DOPG vesicles. The lipid suspensions were diluted as needed using 0.1 M pH 7.4 HEPES buffer.

Luminescence Measurements. All luminescence experiments were conducted in a manner similar to Kinnunen and co-workers using a FluorMax-3 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ) equipped with a Neslab RTE 7 refrigerated bath (Thermo Scientific, Newington, NH).[16,31,32,38,39] All experiments we conducted at 25 °C with a 1 nm excitation slit width and a 16 nm emission slit width. The association of lipid vesicles with cytochrome c was monitored via fluorescence quenching experiments between the lipophilic pyrene label and the heme of cytochrome c.[40,41] The *β*-pyrene-C10-PG label is excited (λ_{ex}) at 344 nm, and the emission is monitored (λ_{em}) at 398 nm. The experiments monitoring the association of luminescent Zn-heme cytochrome c with vesicles containing D(diBr)SPG or N-C3Br-DPPE are based on the well-established role of Br as a collisional quencher. The excitation wavelength of Zn-heme cytochrome c (λ_{ex}) is 420 nm, and the emission is monitored (λ_{em}) at 560 nm. All luminescence intensities were recorded after a period of stirring (∼1 min) and settling (∼1 min) following each addition of cytochrome or vesicles. This ensured that equilibration had occurred after each addition. All luminescence intensities are scaled to account for the dilution resulting from the increase in volume (≤10%) from the addition of aliquots of the competitive binding entity or the quencher. All data series depicted in the figures represent the average of at least two replicate experiments. Control experiments showed that the addition of cytochrome c to vesicle suspensions did not produce a change in turbidity (380−680 nm) over a period of 1 h, indicating that the cytochrome c did not induce vesicle aggregation. This observation is consistent with previous studies of cytochrome c adsorption on to anionic vesicles.[32]

RESULTS

Cytochrome c Adsorption. Adsorption of cytochrome c to the surface of pyrene labeled vesicles (20 *μ*M total phospholipid concentration) was monitored by the decrease in pyrene fluorescence intensity due to quenching by the heme

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moiety in the cytochrome c. The vesicles were composed of DOPC with 50 mol % DPPG, N-C3:0-DPPE, N-C8:0-DPPE, or N-C16:0-DPPE and 1% of the pyrene label (β-pyrene-C10-PG). The titration isotherms in Figure 2 indicate similar

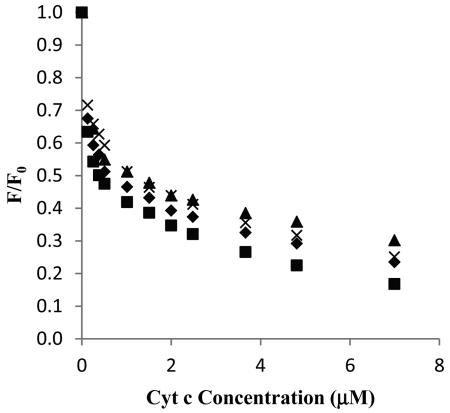

Figure 2. Relative fluorescence intensity upon addition of cytochrome c to pyrene labeled vesicles composed of DOPC with 50 mol % DPPG [◆], N-C3:0-DPPE [■], N-C8:0-DPPE [▲], and N-C16:0-DPPE [×]. The vesicle concentrations are 20 μM total phospholipid; λ_{ex} = 344 nm, λ_{em} = 398 nm.

cytochrome c affinities for all vesicle compositions. The association constants, K_a, were determined to be 2 × 10^6 M^{-1} for the vesicles containing DPPG or N-C3:0-DPPE and 1 × 10^6 M^{-1} for the vesicles containing N-C8:0-DPPE and N-C16:0-DPPE.

Cytochrome c Desorption. Figure 3 shows the relative fluorescence for a series of titration experiments using pyrene labeled vesicles (20 μM total phospholipid concentration) that were initially treated with enough cytochrome c (2−4 μM) to lower the fluorescence intensity to 35−40% and then titrated with unlabeled DOPG vesicles. The fluorescence intensities (F) are relative to the initial fluorescence intensity (F_0) of the

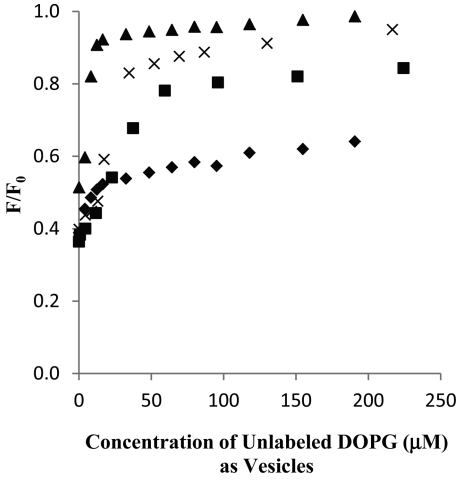

Figure 3. Relative fluorescence intensity for pyrene labeled vesicles composed of DOPC with 50 mol % DPPG [◆], N-C3:0-DPPE [■], N-C8:0-DPPE [▲], and N-C16:0-DPPE [×]. Initially, the samples were treated with enough cytochrome c to lower the relative pyrene fluorescence to ~38% (F_0 is prior to the addition of cytochrome c) and then titrated with unlabeled DOPG vesicles. The pyrene labeled vesicle concentrations was 20 μM total phospholipid; λ_{ex} = 344 nm, λ_{em} = 398 nm.

pyrene labeled vesicle suspension prior to the addition of the cytochrome c. The added DOPG vesicles desorb the cytochrome c that is bound electrostatically to the pyrene labeled vesicles and produce an increase in fluorescence intensity. The plots show that the concentration of DOPG vesicles needed to desorb cytochrome c from the pyrene labeled vesicles depended strongly on vesicle membrane composition and followed the order of N-C8:0-DPPE < N-C16:0-DPPE < N-C3:0-DPPE < DPPG. In other words, cytochrome c was most weakly bound to vesicles containing 50 mol % N-C8:0-DPPE and most strongly bound to vesicles containing 50 mol % DPPG.

In Figure 4 are the results for a similar set of cytochrome c desorption experiments but now the pyrene labeled lipid

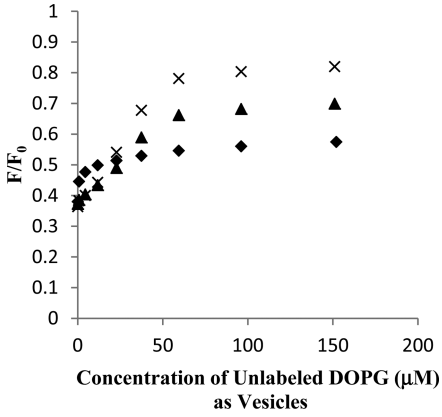

Figure 4. Relative fluorescence intensity for pyrene labeled vesicles composed of DOPC with 50 mol % DPPG [◆], 5 mol % N-C3:0-DPPE and 45 mol % DPPG [▲], or 50 mol % N-C3:0-DPPE [■]. Initially, the samples were treated with enough cytochrome c to lower the relative pyrene fluorescence to ~38% (F_0 is prior to the addition of cytochrome c) and then titrated with unlabeled DOPG vesicles. The pyrene labeled vesicle concentrations were 20 μM total phospholipid,; λ_{ex} = 344 nm, λ_{em} = 398 nm.

vesicles are composed of DOPC with 50 mol % DPPG, 50 mol % N-C3:0-DPPE, or a combination of 5 mol % N-C3:0-DPPE and 45 mol % DPPG. The data demonstrate that cytochrome c is strongly and irreversibly bound to the surface of vesicles containing 50 mol % DPPG but weakly and reversibly bound to the surface of vesicles containing 50 mol % N-C3:0-DPPE. Furthermore, the presence of a small mole fraction (5 mol %) of N-C3:0-DPPE substantially weakens the association of cytochrome c to vesicles that still contain a large amount (40 mol %) of DPPG.

Zn-Heme Cytochrome c Experiments. In Figure 5 are the results from two titration experiments with Zn-heme cytochrome c, a luminescent variant of cytochrome c that has a similar primary structure.[42,43] In each experiment the luminescence signal from a 1 μM solution of Zn-heme cytochrome c is monitored as increasing aliquots of vesicles composed of either D(diBr)SPG (the tetrabromo derivative of DOPG) or N-C3:0-Br-DPPE (bromo derivative of N-C3:0-DPPE) are sequentially added. The Zn-heme cytochrome c luminescence signal is partially quenched only by the D(diBr)SPG vesicles. In other words, the 3-bromopropionoyl chain of the N-C3:0-Br-DPPE is unable to quench the fluorescence of the Zn-heme cytochrome c adsorbed to the vesicle surface.

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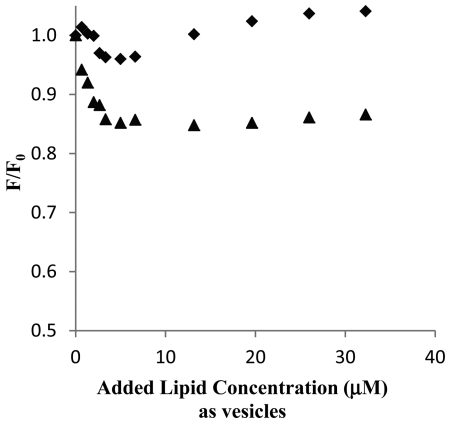

Figure 5. Relative luminescence intensity for 1 μM Zn heme cytochrome c solutions upon the addition of vesicles composed of N-C3:0-Br-DPPE [◆] or D(diBr)SPG [▲]; λ_{ex} = 395 nm, λ_{em} = 590 nm.

DISCUSSION

The fluorescence titration experiments in Figure 2 show that cytochrome c has a similar affinity for vesicles composed of DOPC mixed with 50 mol % DPPG or any one of the three N-acylated DPPE lipids (N-C3:0-DPPE, N-C8:0-DPPE, or N-C16:0-DPPE). The membrane surfaces of all four vesicle compositions are highly anionic and favor adsorption by the positively charged cytochrome c. There is literature precedence that these vesicles are laterally phase separated into microdomains that are either rich in zwitterionic DOPC or the anionic N-acylated DPPEs due to a combination of headgroup differences, acyl chain differences, and the adsorption of cytochrome c.[37−40]

In Figure 3 are the results of titration experiments in which DOPG vesicles are used to dissociate cytochrome c from the surface of pyrene labeled vesicles containing 50 mol % DPPG or *N*-acyl DPPE. Previous work has shown that cytochrome c could not be removed from labeled vesicles containing phosphatidylglycerol (PG) lipids or cardiolipin, a result that was attributed to the ability of PG or cardiolipin to adopt an extended lipid conformation and form a strong hydrophobic contact with the cytochrome c.[32,38,39] Consistent with this picture, we find that only a small fraction of the cytochrome c could be removed from labeled vesicles that were rich in DPPG. In contrast, it was much easier to remove the cytochrome c from vesicles that were rich in *N*-acyl DPPE. The highly reversible association of cytochrome c with the surfaces of vesicles that are rich in *N*-acyl DPPE suggests that the protein/membrane interaction is primarily electrostatic, with the *N*-acyl chain providing no measurable hydrophobic contribution. The apparent inability of *N*-acyl DPPEs to adopt an extended conformation is consistent with their known self-assembly properties. As proposed by Kinnunen and co-workers,[16,31−34,38,39] the extended lipid conformation is a packing strain relief mechanism for frustrated phospholipids that prefer an inverted hexagonal phase (H_{II}) but are forced to reside in a constraining lamellar phase. Previous studies have shown that N-acylated DPPE lipids with saturated *N*-acyl chains ranging from 2 to 18 carbons form lamellar phases when dispersed in aqueous buffers and thus there is little inherent driving to adopt conformations that favor nonlamellar phases.[7,9] Furthermore, the data in Figure 4 suggest that *N*-acyl DPPEs may actually prevent DPPG from adopting an extended conformation. The data show that the presence of a small amount of N-C3:0-DPPE is enough to greatly attenuate cytochrome c affinity for vesicles containing a large amount of DPPG. This composition change does not alter the membrane surface charge and thus must be due to a change in the membrane packing. NAPEs are known to perturb the interfacial environment depending on the chain length and location. Thus, it appears that the presence of N-C3:0-DPPE increases packing disorder within the DPPG domains and reduces the driving force for DPPG to adopt an extended conformation that favors binding with cytochrome c.

A final question is whether the N-acylated lipids promotes unfolding of cytochrome c that is adsorbed to the vesicle surface.[22,23] Titration experiments added Zn-heme cytochrome c to lipid vesicle suspensions prepared with either N-C3:0-Br-DPPE or D(diBr)SPG. The Zn-heme cytochrome c is a phosphorescent variant of cytochrome c that has been used a structural analogue for cytochrome c, and its luminescence is known to be quenched by agents containing heavy atoms like Br.[42] As shown in Figure 5 the luminescence from the Zn-heme cytochrome c is quenched by the association with D(diBr)SPG vesicles but not by association with the N-C3Br-DPPE vesicles. The results for the experiments with D(diBr)SPG are similar to those reported previously, although the extent of quenching with our vesicle system is not as great.[16] The quenching of Zn-heme cytochrome occurs when the Br-substituted acyl chain inserts into the core of the protein and contacts the Zn-heme center.[16] Our results with vesicles composed of N-C3:0-Br-DPPE show no quenching of the Zn-heme luminescence, suggesting no close interaction between the *N*-acyl chain and any unfolded protein adsorbed at the membrane interfacial region.

SUMMARY

Cationic cytochrome c has the same high affinity for the surfaces of anionic vesicles that are either rich in N-acylated DPPE or rich in DPPG, but the protein/membrane interaction is quite different. Cytochrome c adsorption to DPPG membranes is relatively irreversible due to the DPPG adopting an extended conformation that promotes strong hydrophobic contact of a glycero acyl chain with the adsorbed protein. In contrast, the association of cationic cytochrome c with *N*-acyl DPPE membranes is quite reversible and due primarily to electrostatic interactions with the anionic headgroup, and not hydrophobic contact with the *N*-acyl chain. The presence of a small amount of N-C3:0-DPPE diminishes cytochrome c affinity for vesicles containing a large amount of DPPG by relieving the membrane packing strain that drives the extended DPPG conformation. The results suggest that NAPEs, a class of natural products, can potentially effect the association of peripheral proteins to membrane surfaces by changing the surface charge and also by altering the membrane packing strain.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIAITONS

NAPES, N-acylated phospholipids; cyt c, cytochrome c; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol); DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPG, 1-palmitoyl-2-oleyll-*sn*-glycerol-3-phosphoglycerol; D(diBr)SPG, 1,2-di(9,10-dibromo)stearoyl-*sn*-glycero-3-phosphoglycerol; N-C3:0-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-(*N*-propionyl)-phosphoethanolamine; N-C8:0-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-(*N*-octanoyl)phosphoethanolamine; N-C16:0-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-(*N*-palmitoyl)phosphoethanolamine; N-C3:0-Br-DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-(*N*-3-bromopropionyl)phosphoethanolamine; *β*-pyrene-C10-PG, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol; HEPES, hydroxyethyl piperazineethanesulfonic acid

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