ELSEVIER

CPL
CHEMISTRY AND PHYSICS
OF LIPIDS

The structural and functional effects of Hg(II) and Cd(II) on lipid model systems and human erythrocytes: A review

CrossMark

Brandon J. Payliss, Mohamed Hassanin, Elmar J. Prenner*

Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada

ABSTRACT

The anthropogenic mobilization of mercury and cadmium into the biosphere has led to an increased and ineludible entry of these metals into biological systems. Here we discuss the impact of Hg(II) and Cd(II) on lipid model systems and human erythrocytes from a biophysical perspective. After a brief introduction to their implications on human health, studies that have investigated the effects of Hg(II) and Cd(II) on lipid model systems and human erythrocytes are discussed. In terms of lipids as toxicological target sites, predominantly variations in lipid head groups have been the source of investigation. However, as research in this field progresses, the effects of Hg(II) and Cd(II) on other structural features, such as acyl chain length and unsaturation, and other important lipid components and complex biomimetic lipid mixtures, will require further examinations.

This review provides an analysis of what has been learned collectively from the diverse methodologies and experimental conditions used thus far. Consequently, there is a need for more comprehensive and thorough investigations into the effects of Hg(II) and Cd(II) on lipid membranes under consistent experimental conditions such as pH, ionic strength, temperature, and choice of lipid model system.

© 2015 The Authors. Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Contents

1. Introduction

Industrialization and anthropogenic activities since the 1800s have gradually increased the mobilization of heavy metals, such as mercury and cadmium, from the earth's crust into the biosphere (Pacyna, 1996). As a result of the rising levels of toxic metals in the environment, certain organisms are exposed to higher daily doses than ever before. The increased net influx of toxic metals into living systems could disrupt certain biological functions. Indeed, mercury and cadmium have been associated with an increased prevalence of cardiovascular disease (Peters et al., 2010; Virtanen et al., 2007) and kidney damage (Järup, 2003; World Health Organization, 1992). The predominant route of entry for mercury and cadmium into the body is via the digestive tract (Gailer, 2007). Cadmium is thought to pass through the intestinal membrane by the divalent metal transporter 1, especially during iron deficiency (Park et al., 2002). This protein has also been implicated in the transport of other metals across the apical membrane of enterocytes that line the small intestine (Park et al., 2002; Gunshin et al., 1997). Competition between various metals or metal ions determines the extent and types of metals transported across the small intestine. As a result, deficiencies in iron or zinc could facilitate the passage of toxic metals across the intestinal lining into the bloodstream. This is an important aspect, since any deficiency in essential elements like iron and zinc may increase the absorption of toxic metals into the bloodstream (Park et al., 2002; Gunshin et al., 1997).

Once in the bloodstream, Hg(II) and Cd(II) can interact with various blood and cellular components (Fig. 1) such as human serum albumin (HSA) (Li et al., 2007), erythrocyte membrane proteins, glutathione, and intracellular proteins and metabolites (Trisak et al., 1990; Lou et al., 1991). Since it is well established that both Hg(II) and Cd(II) have a strong affinity for sulfhydryl groups (Hughes, 1957; Rabenstein et al., 1983; Rabenstein, 1989; Gwoździński, 1995; Bridges and Zalups, 2005), an appreciable amount of work has been directed directed toward better understanding the binding of Hg(II) and Cd(II) to endogenous proteins and metabolites. Comparatively less information, however, is available on how these toxic metals may adversely impact the cellular membrane.

Several studies show that environmental pollutants, such as cadmium and mercury, are nephrotoxic (Järup, 2003; Järup et al., 1998; Madden and Fowler, 2000; Zalups, 2000; Satarug et al., 2010) and cause adverse health effects in humans (Järup, 2003; Satarug et al., 2010; Tchounwou et al., 2003). For the general population, Hg(II) and Hg^0 exposure is primarily by dental amalgam fillings that contain mercury (World Health Organization, 1991; Barregard et al., 1995; Sallsten et al., 1996; Clarkson and Magos, 2006), but may also involve the utilization of skin-lightening cosmetic creams (McRill et al., 2000; Sin and Tsang, 2003; Chan, 2011). Dermal absorption of Hg(II) may occur across the epidermis (Chan, 2011; Park and Zheng, 2012) via the sebaceous gland, sweat glands, and hair follicles (Fig. 2). The organ which typically contains the highest concentration of Hg(II) is the kidney from absorption through the gastrointestinal tract (Park and Zheng, 2012). In addition, the oxidation of Hg^{2+} to Hg^0 by the enzyme catalase, a common mammalian intracellular protein, is also carried out by some bacteria found in the oral cavity and the gastrointestinal tract (Barkay et al., 2003). The resistance of bacteria to toxic metals has been reviewed (Summers, 2002), and non-occupational exposure to Hg(II) is most commonly via 'silver' dental amalgams, which can contain up to 50% of mercury. Exposure of bacteria to toxic metals
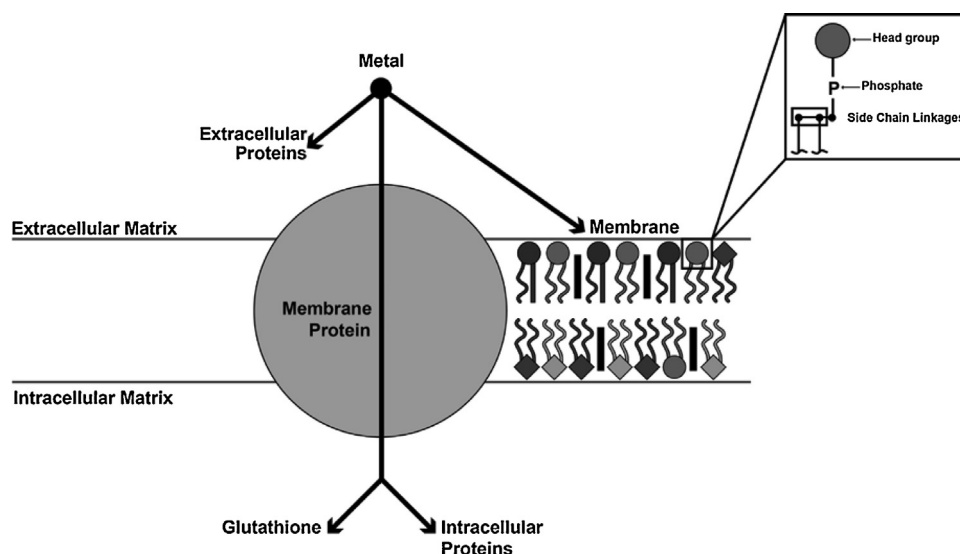

Fig. 1. Potential macromolecular targets of toxic metals in mammalian organisms.

has shown to increase antibiotic resistance (Summers et al., 1993; Lorscheider et al., 1995; Skurnik et al., 2010).

Human exposure to cadmium is predominantly attributed to the ingestion of food and the average daily uptake is estimated to be between 10 and 40 μg per day (Schwartz and Reis, 2000). Cigarette smokers are chronically exposed to cadmium and their blood cadmium levels are 4–5 times higher, on average, compared to non-smokers (Järup et al., 1998). Cadmium has been classified by the International Agency for Research on Cancer (IARC) as a group I human carcinogen (IARC, 1993), and has a biological half-life in the body estimated to be upwards of 15–30 years (Prozialeck et al., 2006). The concentrations of various toxic metals and metalloids in the blood can be measured accurately (Gailer, 2012), and circulating blood levels of Cd(II) have been estimated to be in the 10–100 nanomolar range (Järup et al., 1998; Hotz et al., 1999). It is plausible to hypothesize that a link may exist between toxic metals in the blood and the etiology of various diseases, which places importance on the insights that can acquired by studying metal–membrane interactions. Some of the diseases that may be associated with the exposure to either mercury and/or cadmium include allergies, autism, diabetes, and Parkinson's disease (Gailer, 2012). Cadmium has been reported to be cardiotoxic (Limaye and Shaikh, 1999), and has been identified as a potential risk factor for Alzheimer's disease (Notarachille et al., 2014).

1.1. Hg(II) and Cd(II) speciation in aqueous solution

Both Cd(II) and Hg(II) belong to the Group 12 elements, and although their general chemistries are quite similar, they behave quite differently in aqueous solutions. For example, $Cd(OH)_2$ is basic while $Hg(OH)_2$ is an extremely weak base (Cotton et al., 1999). There is considerable covalent character in mercury halides, with linear, planar, and tetrahedral geometries for $HgCl_2$, $HgCl_3^-$, and $HgCl_4^{2-}$. Cadmium halides tend to dissociate into ions, whereas mercury halides only dissociate weakly (Cotton et al., 1999). Both Hg(II) and Cd(II) only form compounds with a full d shell (d^{10}), so both are commonly regarded as non-transition elements. Typically, Hg(II) forms strong bonds to N, S, and P donors.

Elemental mercury (Hg^0) and Hg(II) are found naturally in the earth's crust, while Hg^0 is highly volatile and can evaporate readily as a consequence of its low vapor pressure (Bridges and Zalups, 2010). Mercury can exist in form of mercurous (Hg_2^{2+}) or mercuric (Hg^{2+}) mercury, often complexed with anionic species such as chloride, sulfide, and oxide ions (Bridges and Zalups, 2010). It is well established that both Hg(II) and Cd(II) can form various chemical species in aqueous solutions depending on the concentration, pH, temperature, and the concentration of counter-ions, such as chloride (Baes and Mesmer, 1976; Hahne and Kroontje, 1973). Only considering an $HgCl_2$ solution, the following major species may exist: Hg^{2+}, $HgOH^+$, $Hg(OH)_2$, $HgCl^+$, $HgCl_2$, $HgCl_3^-$, $HgCl_4^{2-}$, or HgOHCl, depending on the pH and chloride
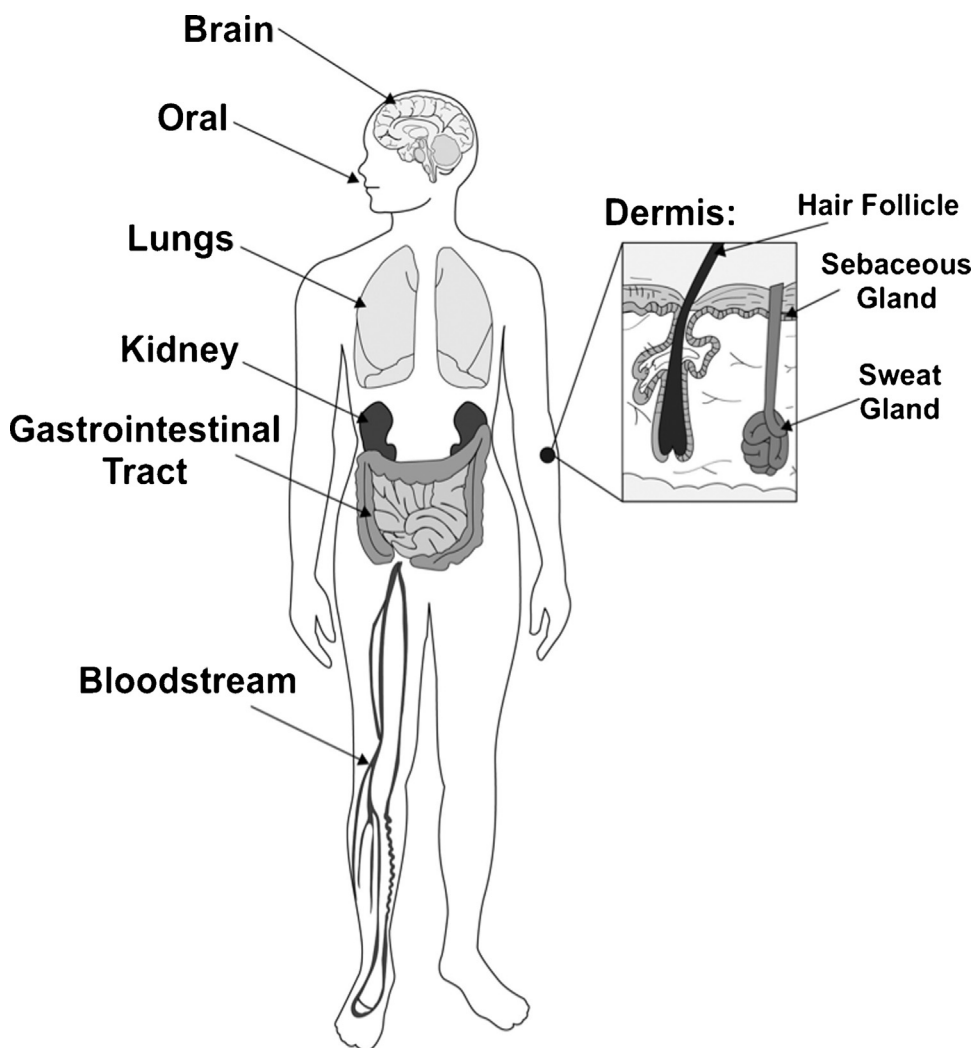

Fig. 2. Major organs and tissues implicated in the toxicology of metals in humans.

concentration (Hahne and Kroontje, 1973). These conditions also affect the extent to which $HgCl_2$ dissociates into ions, or exists as a neutral, triatomic molecule with linear geometry in solution (Wells, 2012). At physiological conditions in blood plasma (pH 7.4, $[Cl^-]$ = 105 mM), mercury is present in roughly equal quantities of $HgCl_2$, $HgCl_3^-$ and $HgCl_4^{2-}$ (Hahne and Kroontje, 1973). In the case of Cd(II), its chemical species may include: Cd^{2+}, $CdCl^+$, $CdCl_2$, $CdCl_3^-$, $CdCl_4^{2-}$, and hydrated complexes (Bazarkina et al., 2010) such as $Cd(H_2O)_6^{2+}$, $CdCl(H_2O)_5^+$, and $CdCl_3(H_2O)^-$. At the same pH (7.4) and chloride concentration (105 mM), the predominant species are $CdCl_2(H_2O)_2^0$ and $CdCl_3(H_2O)^-$ (Bazarkina et al., 2010). Notably, Cd(II) can form strong and possibly insoluble complexes with other anionic species found in the blood, such as carbonate and phosphate, which may exist in the form of $CdCO_3$ and $Cd_3(PO_4)_2$ (Santillan-Medrano and Jurinak, 1975), or as the hydrated complex, $Cd_5H_2(PO_4)_4$Ÿ$4H_2O$ (Ayati and Madsen, 2001).

2. Membrane structure and model systems

One of the first points of contact upon exposure to a metal is the cellular membrane (Fig. 1) (García-Sevillano et al., 2014). Although in principle both lipids and proteins represent toxicological target sites in mammals, this review focuses on the former. Thus, it is necessary to introduce lipid structures and membrane properties.

2.1. Phospholipid structure and the erythrocyte membrane

The erythrocyte membrane contains approximately 39.5% protein and 35.1% lipids, by weight, where the remaining 19.5% and 5.8% correspond to water and carbohydrates, respectively

(Yawata, 2003). Biophysical studies on lipids such as cholesterol and sphingomyelin (SM) have demonstrated evidence of their involvement in microdomain formation and lateral organization in model membrane systems (Rinia et al., 2001; Giocondi et al., 2004; Prenner et al., 2007). Notably, the evidence supporting the existence of lipid microdomains or 'lipid rafts' has been discussed heavily in the literature (Edidin, 1997; Brown and London, 1998; Simons and Toomre, 2000; London, 2002; Simons and Ehehalt, 2002; Edidin, 2003; Simons and Gerl, 2010), and their existence still remains controversial (Munro, 2003; Jacobson et al., 2007).

The lipid composition of any mammalian membrane primarily consists of phospholipids, SM and cholesterol (Zachowski, 1993). Phospholipids can vary in head group structure (Fig. 3), ranging from major head group structures such as SM, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), to minor components such as phosphatidylinositol (PI) and phosphatidic acid (PA). Both PC and SM have the same phosphocholine head group, however, SM is built on a sphingosine backbone instead of a glycerol backbone, where the phosphocholine moiety is linked to the 1-hydroxyl group of ceramide. The hydrogen bonding capacities of PC and SM are distinct, since PC can only accept hydrogen bonds through the phosphate and ester groups. SM can act as a hydrogen bond acceptor, but also as a hydrogen bond donor by involvement of the 3-hydroxyl group. As a result, SM can form strong intermolecular interactions with neighbouring SM molecules through the mutual exchange of hydrogen bond donation and acceptance, leading to a more rigid membrane. By contrast, PC lipids form weaker intermolecular interactions with other neighbouring PCs, resulting in a more fluid membrane.
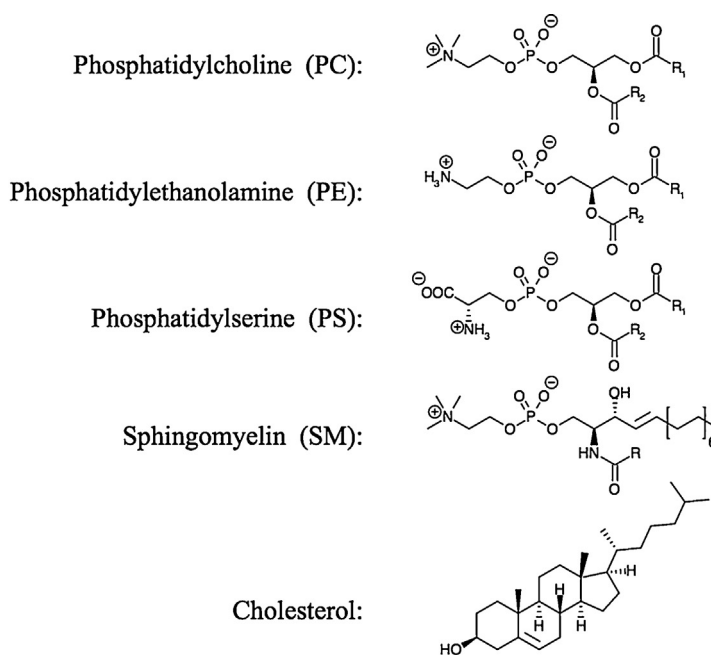
Percentage of Total Phospholipids (%)

	PC	PE	PS	SM
Outer Leaflet:	65 - 75	15-20	<4	>85
Inner Leaflet:	25-35	80-85	>96	<15

Fig. 3. Organic structures depicting the major lipids found in the erythrocyte membrane: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), and cholesterol. Chemical substituents 'R', 'R$_1$', and 'R$_2$' signify the positions where acyl chains can vary in length and degree of unsaturation. In addition, the percentage of total phospholipids in the outer and inner membrane leaflets is also noted (Zachowski, 1993). Structures are drawn as their predominant form at physiological pH (7.4).

It is important to note that lipids are asymmetrically distributed between the two leaflets of the erythrocyte membrane, and the quantity of cholesterol, SM and each glycerophospholipid, and can vary widely between cell types (Zachowski, 1993; Le et al., 2011). For example, in human erythrocytes, 65–75% of PC and 85% of SM is found in the outer leaflet of the membrane, while 80–85% of PE, >96% of PS, as well as >80% of PI and PA comprise the inner leaflet (Zachowski, 1993); cholesterol has an even distribution in both membrane leaflets. At physiological pH, PC, PE and SM are zwitterionic with a net charge of zero, while PS is net negatively charged, and cholesterol is uncharged. Phospholipids that contain zwitterionic or charged head groups are stabilized by the presence of water and counter-ions at the membrane surface. PE, owing to the presence of a primary amine in its head group, can also form intermolecular hydrogen bonding interactions with other neighbouring PE molecules, producing a more rigid membrane. In addition, some PE's can form non-lamellar inverted hexagonal structures (Zachowski, 1993).

The lipid acyl chains in phospholipids and sphingolipids vary in length and the degree of unsaturation. A decrease in acyl chain length and an increase in acyl chain unsaturation are both correlated with increased membrane fluidity. The most abundant lipid species in human erythrocytes, and the mammalian plasma membrane, is the partly unsaturated POPC, containing a saturated 16:0 (palmitic) acyl chain in the *sn*-1 and a monounsaturated 18:1 (oleic) acyl chain in the *sn*-2 position, accounting for approximately 25% of the total PC species (Leidl et al., 2008). For SM, the variable acyl chain is linked by an amide instead of an ester, where human erythrocyte SM most commonly has a fully saturated 16:0 acyl chain, followed by 24:1 and 24:0 (Leidl et al., 2008).

Ideally, a good biomimetic model of the erythrocyte membrane will examine not only the varying types of lipid head groups, different lengths of acyl chains, and variable degrees of unsaturation, but also would consider lipid mixtures based on their relative proportions within a particular leaflet of the membrane under investigation. Moreover, the addition of other macromolecules, such as proteins, will add further complexity. It is difficult to control and implement these considerations experimentally, which give rise to challenges in the analysis and interpretation of results.

3. Interactions of Hg(II) and Cd(II) with model membrane systems

The following section provides a summary of the physical effects of Hg(II) and Cd(II) on pure and mixed lipid systems, and has been divided into subsections based on the technique used or the physical characteristic assessed. Each subsection is followed by a 'Key Findings' section that summarizes some of the key points that were addressed. Most studies have investigated the effects of Hg(II) and Cd(II) on the membrane by monitoring changes or disruptions in biophysical characteristics, for example membrane fluidity, permeability, or melting temperature. Such membrane properties and their perturbations by metals can be monitored using various techniques and reporter molecules.

3.1. Fluorescence spectroscopy

Diphenylhexatriene (DPH) is a hydrophobic fluorescent probe that has been used in many studies to investigate the effects of Hg (II) and Cd(II) on the membrane fluidity of model membrane vesicles ((Delnomdedieu and Boudou, 1989; Delnomdedieu and Allis, 1993; Girault et al., 1998; Suwalsk et al., 2000,, 2004) as well as human (Suwalsk et al., 2000, 2004; Sorense et al., 1985; Amoruso et al., 1987) and rat (Delnomdedieu and Allis, 1993)and rat (Delnomdedieu and Allis, 1993) erythrocytes. Since an increase in DPH anisotropy is inversely proportional to membrane fluidity, it is proportional to the gel to liquid crystalline phase transition temperature (T_m). For simplicity, the effects of Hg(II) and Cd(II) on membrane fluidity are addressed here as either an increase, decrease or abolishment of the T_m. The results have been summarized by the choice of lipid model system: pure, mixed, and lipid extracts (Table 1). Notably, one inherent limitation in using DPH is that its position in the bilayer depends on the phase, for example, in the liquid crystalline phase, DPH can be oriented parallel to the bilayer surface and reside in the center of the bilayer (Lentz, 1989; Lentz, 1993). Indeed, this is an important limitation to be aware of, since the dynamic positioning of DPH in different part of the bilayer may produce fluorescence signals that do not reflect changes in membrane fluidity. To avoid this complication, it is preferable to use an amphipathic fluorescent probe such as

Table 1
Summary of melting temperature (T_m) results due to effects of Hg(II) and Cd(II) measured by DPH fluorescence anisotropy in pure and mixed lipids, or lipid extracts. All of the results reported below used MLVs as the model system (Delnomdedieu and Boudou, 1989; Delnomdedieu and Allis, 1993; Girault et al., 1998), unless otherwise indicated as LUVs (Suwalsky et al., 2000; Suwalsky et al., 2004). Experimental conditions are noted and descriptive symbols have been summarized below.

Head group	Lipid(s)	Hg(II)	Cd(II)	Ref.
PS	DMPS	×[a]	/	(Delnomdedieu and Boudou, 1989)
	DPPS	/	×[c,d,e]	(Girault et al., 1998)
	BBPS	×[a]	↑[c] Ø[d]	(Delnomdedieu and Boudou, 1989; Delnomdedieu and Allis, 1993; Girault et al., 1998)
PG	DPPG	↑[b]	↑[c] Ø[d]	(Delnomdedieu and Boudou, 1989; Girault et al., 1998)
PA	DMPA	×[a] Ø[f]	/	(Delnomdedieu and Boudou, 1989)
	DPPA	/	×[c] Ø[d] ↑[e]	(Girault et al., 1998)
PC	DMPC	↑[g,h]	Ø[c,h] ↓[g]	(Girault et al., 1998; Suwalsky et al., 2000; Suwalsky et al., 2004)
	DPPC	Ø[b]	/	(Delnomdedieu and Boudou, 1989)
Mixed	DMPE/egg-PC (1:1)	×[a]	Ø[c,d]	(Delnomdedieu and Boudou, 1989; Girault et al., 1998)
	DPPC/SA (1:1)	↓[a]	/	(Delnomdedieu and Boudou, 1989)

Symbols: / – no data; × – T_m abolished; ↑ – T_m increased; ↓ – T_m decreased; Ø – no change in T_m.
[a] pH 5.8, 74 mM acetate.
[b] pH 5.0, 74 mM acetate.
[c] pH 7.0, 50 mM MOPS.
[d] pH 7.0, 50 mM MOPS, 0.5 M KCl.
[e] pH 6.0, 50 mM MES.
[f] pH 7.8, 50 mM phosphate.
[g] Large unilamellar vesicles (LUVs), H$_2$O, pH not reported, 18 °C.
[h] Large unilamellar vesicles (LUVs), H$_2$O, pH not reported, 37 °C.

Laurdan or a DPH-phospholipid derivative, instead of a symmetrically hydrophobic probe like DPH.

It is evident from Table 1 that the reported effects of Hg(II) and Cd(II) on membrane fluidity and T_m are dependent on both the lipid composition and experimental conditions. Starting with the PS head group, Hg(II) abolished the T_m of both fully saturated 14:0 dimyristoylphosphatidylserine (DMPS) and the complex extract of bovine brain phosphatidylserine (BBPS) at pH 5.8 in 74 mM acetate buffer. Interestingly, Cd(II) abolished the T_m for saturated (16:0) dipalmitoylphosphatidylserine (DPPS) at both pH 6.0 and pH 7.0, and in the presence of 0.5 M KCl at pH 7.0. Contrastingly, the addition of Cd(II) (0.1 mM) caused an increase in the T_m of BBPS vesicles at pH 7.0 in 50 mM MOPS, but no detectable change in T_m for BBPS after the addition of 0.5 M KCl. In terms of acyl chain composition of BBPS, 18:0 and 22:6 are the most abundant acyl chains found in the sn-1 and sn-2 positions whereas 16:0 acyl chains are minor components in both positions, respectively (Yabuuchi and O'Brien, 1968). Importantly, although DPPS may only be at trace amounts in a BBPS extract, comparing these results illustrates the importance of considering both head group and acyl chain composition. Furthermore, the introduction of a high salt condition (0.5 M KCl) negated the effects of Cd(II) on the T_m of BBPS vesicles but maintained the effect of Cd(II) on DPPS vesicles. The difference observed here is likely due to the distinct physical properties between the bilayers formed by these two vesicle systems; DPPS vesicles have more rigid bilayers compared to BBPS, which can be deduced from the difference in T_m between BBPS (38 °C) and DPPS (54 °C) reported from DPH control experiments (Girault et al., 1998).

DPH anisotropy data describing the effects of Hg(II) and Cd(II) on PE are especially limited, possibly due to the practical limitations of preparing pure PE vesicles, as these lipids can form strong hydrogen bonds to glass and potentially exhibit non-lamellar phases. The only DPH anisotropy data reported is for mixed DMPE/egg-PC (1:1) vesicles tested with Hg(II) and Cd(II). (Delnomdedieu and Boudou, 1989; Girault et al., 1998) At pH 5.8 in 74 mM acetate buffer, Hg(II) abolished the T_m of DMPE/egg-PC vesicles, (Delnomdedieu and Boudou, 1989) while no effect was observed for Cd(II) at a pH of 7.0 in 50 mM MOPS (Girault et al., 1998). However, under the same conditions, both Hg(II) and Cd(II) caused the T_m of DPPG vesicles to increase. When Girault et al. (1998) tested the high salt condition (0.5 M KCl), the effect of Cd(II) on DPPG membrane fluidity was reduced, where the T_m slightly increased (ΔT_m = +1.5 °C) in the presence of Cd(II) relative to the low salt condition (ΔT_m = +13.5 °C).

Considering the PA head group, DPH anisotropy data has been reported for the effects of Hg(II) on saturated (14:0) dimyristoylphosphatidic acid (DMPA) of Hg(II) on DMPA and for Cd(II) on these differ (Delnomdedieu and Boudou, 1989) and for Cd(II) on saturated (16:0) dipalmitoylphosphatidic acid (DPPA) (Girault et al., 1998). Interestingly, a stronger effect was observed for both Hg(II) and Cd(II) on the T_m under slightly acidic conditions (pH ~6) compared to neutral or slightly alkaline conditions (pH ~8). Notably, the second dissociable proton (pK_{a2}) in the PA head group is dependent on the lipid environment, but lies in the physiological pH range (7 < pH < 8) (Kooijman et al., 2005; Kooijman and Burger, 2009). An explanation suggested by Girault et al. (1998) was that the weaker effect of Cd(II) on DPPA membrane fluidity at a higher pH could be a result of less binding of Cd(II) due to the neutralization of charge on the phosphate groups, and a reduction in membrane surface charge density (Girault et al., 1998).

For lipids containing the PC head group, there was no reported effect of Hg(II) on the T_m of saturated (16:0) dipalmitoyl-phosphatidylcholine (DPPC) MLVs (Delnomdedieu and Boudou, 1989), but an increase in T_m for saturated (14:0)

dimyristoylphosphatidylcholine (DMPC) LUVs (Suwalsky et al., 2000). As seen earlier for BBPS and DPPS, this comparison reiterates the role of acyl chain composition. Unfortunately, the differences in experimental conditions between both studies, such as the use of MLVs instead of LUVs, buffered as opposed to un-buffered conditions, pH, and temperature do not allow for any direct correlations to be made. However, while Hg(II) had no measurable effect on the T_m of DPPC under theses conditions, the addition of stearylamine (SA) at a 1:1 molar ratio resulted in a decrease of the T_m. SA consists of an 18:0 carbon acyl chain with a terminal primary amine, thus giving rise to positively charged vesicles under the conditions tested (pH 5.0 and 5.8). The authors reported a higher T_m (50.9 °C) for the DPPC/SA (1:1) system compared to pure DPPC (43.1 °C), and interestingly, it was shown that increasing concentrations of HgCl₂ progressively shifted the T_m of DPPC/SA (1:1) toward the T_m of pure DPPC. At a concentration of 2.5 mM HgCl₂ the DPPC/SA (1:1) system had the same T_m (43.1 °C) as pure DPPC. This could be explained by phase separation between Hg(II)-SA domains and pure DPPC. In addition to the known occurrence of Hg(II) complexation with amines (Cotton et al., 1999; Breitinger and Brodersen, 1970), these results support the likelihood of a specific interaction between Hg(II) and the primary amine of SA. Similarly, the abolished T_m observed in the DMPE/egg-PC (1:1) system may also be explained as Hg(II) binding to the primary amine in PE. With respect to Cd(II) and DMPC, only a T_m decrease was detected at 18 °C in the gel phase, indicating that Cd(II) disordered the bilayer when DMPC was below the T_m. No change of T_m for DMPC was reported in the liquid-crystalline phase (37 °C).

Bevan et al. (1983) examined perturbations in T_m induced by Hg (II) and Cd(II) by monitoring the fluorescence polarization of trans-parinaric acid methyl ester. This fluorescent molecule was selected since contains an esterified carboxyl group that effectively eliminates fluorescence polarization changes that could result from metal binding to the free carboxylate group, so changes in fluorescence polarization reflect only changes in membrane fluidity (Sklar et al., 1979). The model systems investigated in this study included pure DPPC and a DPPC/BBPS mixture (60:40) in 10 mM HEPES (pH 7.0) and 100 mM NaCl. In the presence of Cd(II) or Hg(II) the T_m increased, whereby Cd(II) had a larger effect (ΔT_m = +3.5 °C) compared to Hg(II) (ΔT_m = +2.2 °C) (Bevan et al., 1983). These results are consistent with the increase in T_m observed for the addition of Cd(II) to pure BBPS vesicles (Girault et al., 1998). Additionally, there was no detectable change in the T_m of pure DPPC vesicles after the addition of either Hg(II) or Cd(II), results which are consistent with those reported for Hg(II) and DPPC from a previous DPH study (Delnomdedieu and Boudou, 1989). Collectively, these results clearly demonstrate the preference of both Hg(II) and Cd(II) to bind BBPS and increase membrane rigidity. In addition, Bevan et al. (1983) reported the total percentage of carboxyfluorescein leakage that was observed for DPPC/BBPS vesicles after incubation with either Hg(II) and Cd(II) for 15 min. No leakage was reported for 0.1 mM Hg(II), but 14.7 % leakage for 0.1 mM Cd(II). Notably, the temperature used in the experiment (23 °C) was below the T_m of DPPC/BBPS, so these vesicles were in the gel phase. It is intriguing that both Hg(II) and Cd(II) caused a comparable increase in the T_m of DPPC/BBPS vesicles, but it was Cd(II) exclusively that induced carboxyfluorescein leakage. It is possible, based on this evidence, that the effects Hg(II) and Cd(II) on DPPC/BBPS could be mechanistically distinct events. Studies that have investigated the effects of Hg(II) and Cd(II) on vesicle permeability and analyte leakage are addressed in a separate Section 3.2 of this paper.

Le et al. (2009) used fluorescence spectroscopy to investigate the effects of Hg(II) and Cd(II) on LUVs comprised of pure POPC, POPC/POPS (85:15), POPC/POPE (85:15), or the erythrocyte biomimetic system of POPC/cholesterol/POPE/POPS (35:35:15:15)

in phosphate buffer (20 mM, pH 7.4) (Le et al., 2009). Binding affinities were quantified by fluorescence quenching of the fluorescent dye Phen Green™ SK (PGSK). This study found that membrane interactions with either Cd(II) or Hg(II) could be detected at sub micromolar concentrations ($\leq 1\,\mu M$). When ordering the effects of Hg(II) and Cd(II) on PGSK fluorescence from strongest to weakest, Hg(II) quenched the PGSK dye in the order of: POPC/POPS (85:15) > POPC/cholesterol/POPE/POPS (35:35:15:15) > POPC > POPC/POPE (85:15), while the opposite trend was observed for Cd(II). When considering the head group, these results demonstrate that Hg(II) preferred binding to PC/PS mixtures over PC/PE, while Cd(II) preferred PC/PE mixtures over PC/PS. Between either extreme for both Hg(II) and Cd(II), moderate fluorescence quenching of PGSK was observed for the pure POPC system, however, no quenching was reported for the more rigid POPC/cholesterol (85:15) vesicles when treated with Hg(II). Expectedly, the addition of 15 mol% cholesterol results in a more tightly packed bilayer through a condensing effect caused by increased van der Waals interactions between adjacent lipid acyl chains (Bhattacharya and Haldar, 2000). Although, the strength of these interactions is limited if *cis* unsaturated acyl chains are present (Ohvo-Rekilä et al., 2002), as is the case for the POPC/cholesterol (85:15) system. Recognizably, the fluorescence results discussed thus far have consistently reported no measurable effect of Hg(II) on the more rigid PC systems, such as the POPC/cholesterol (85:15) system, or in the DPH anisotropy results previously described for DPPC (Delnomdedieu and Boudou, 1989; Bevan et al., 1983). These results highlight the importance of considering physical characteristics like membrane fluidity and rigidity, but uniquely for lipids containing unsaturated acyl chains, another possibility could arise; the *cis* unsaturation in POPC could be a potential oxidative binding target for Hg(II) and Cd(II), resulting in membrane disruption via lipid peroxidation (Halliwell and Chirico, 1993; Stohs and Bagchi, 1995; Valko et al., 2005; Repetto et al., 1985). If lipid peroxidation is one of the underlying causes of membrane disruption, then a variety of methodologies ranging from chromatographic methods to fluorescence assays could be utilized to check for the presence of several different lipid peroxidation products (Halliwell and Chirico, 1993; Gutteridge and Halliwell, 1990; Girotti, 1985).

Another useful fluorescence technique involves measuring changes in the generalized polarization (GP) of the fluorescent dye laurdan, calculated from differences in the fluorescence intensity between environments of high or low polarity. The emission spectrum of this dye is highly sensitive to the presence of solvent dipoles, and GP may be used to probe the polarity of lipid head groups in a bilayer, whereby more rigid membranes exhibit higher GP values (Parasassi and Gratton, 1995). Theoretically, GP values can range between -1 for complete exposure of laurdan to the solvent, and 1 for no exposure to the solvent. However, experimentally determined GP values are typically found between -0.3 and 0.6 for pure and mixed lipids (Parasassi et al., 1998). Presently, laurdan has only been used for Hg(II) and Cd(II) studies with DMPC LUVs (Suwalsky et al., 2000; Suwalsky et al., 2004). A concentration-dependent increase in the GP of laurdan was measured over a Hg(II) concentration range (0–0.5 mM) at both 18 °C and 37 °C (70) which reflect temperatures below and well above the T_m of DMPC ($\sim$24 °C) (Koynova and Caffrey, 1998), so GP measurements were performed for both gel and liquid-crystalline phases of this lipid. Laurdan solvent exposure was reduced with increasing amounts of Hg(II) in both phases, with the greatest effect reported at 37 °C in the liquid crystalline phase. These results are consistent with the DPH results discussed earlier, where Hg(II) increased the T_m of DMPC LUVs (Suwalsky et al., 2000). On the other hand, over a concentration range of 0.01 mM to 1.8 mM, Cd(II) had the same observed effect as Hg(II) in the liquid crystalline

phase for DMPC, however Cd(II) disordered the head group region and increased the solvent exposure of laurdan in the gel phase. The Cd(II) induced disordering of the DMPC bilayer in the gel phase is consistent with the reduced T_m reported by DPH (Suwalsky et al., 2004). In contrast, no effect of Cd(II) on the liquid-crystalline phase DMPC at 37 °C was reported using DPH. The conflicting DPH and laurdan data could be a result of the experimental limitations addressed earlier surrounding the uncertainty in the positioning of DPH in the bilayer.

3.1.1. Fluorescence spectroscopy—key findings

- The effects of Cd(II) (0.1 mM) on the T_m of BBPS and DPPG were markedly reduced in the presence of high salt (0.5 M KCl), while the T_m was still abolished for DPPS under these conditions.
- Both Hg(II) and Cd(II) had a stronger effect on the T_m of DMPA and DPPA, respectively, under slightly acidic conditions (pH $\sim$6) compared to neutral or slightly alkaline (pH $\sim$8). This highlights the importance of head group charge on interactions formed with Hg(II) or Cd(II).
- The T_m reduction of DPPC/SA (1:1) vesicles toward the T_m of pure DPPC with increasing Hg(II) supports binding of Hg(II) to the primary amine in SA, and indicates a phase separation into Hg(II)-SA and DPPC.
- Both Hg(II) and Cd(II) binding to pure POPC LUVs was reported at concentrations as low as 1 μM, however, Hg(II) binding was not detected using the more rigid POPC/cholesterol (85:15) mixture.
- Increasing Hg(II) over the range of 0.1 mM to 0.5 mM reduced the solvent exposure of laurdan in both the gel (18 °C) and liquid-crystalline phases (37 °C) of DMPC LUVs suggesting that Hg(II) induced tighter lipid packing. The same effect was observed for Cd(II) (0.01 mM to 1.8 mM) in the liquid-crystalline phase; however, Cd(II) increased laurdan solvent exposure in the gel phase.
- Collectively, the results gathered here suggest that PS is a preferred binding target for Hg(II), in contrast to PE for Cd(II).

3.2. Leakage and permeability

One of the earliest papers that examined the interaction of Hg(II) with model phospholipid membranes was presented by Nakada et al. (1978). This study investigated the leakage of glucose from multilamellar vesicles (MLVs) after treatment with a range of HgCl$_2$ concentrations (0.1–10 μM) whereby glucose leakage was quantified using an enzyme assay as described by Kinsky et al. (1969a,b). MLVs comprised of egg-PC with varying molar ratios of dicetylphosphate (DCP) or SA, combined with cholesterol, were entrapped with glucose in the presence of 75 mM KCl and 75 mM NaCl. Structurally, DCP has a phosphate head group where two 18:0 carbon acyl chains are each bound by a phosphoester linkage. Notably, DCP and SA are both synthetic and do not occur naturally in mammalian cell membranes; these molecules were used as probes to investigate if the interaction of Hg(II) with the head group region of these lipid membranes was driven electrostatically. After incubating MLVs with HgCl$_2$ (10 μM) at 25 °C for 10 min, the percent leakage of glucose was 40% for the negatively charged egg-PC/cholesterol/DCP (1:0.3:0.1) and 26% for the positively charged egg-PC/cholesterol/SA (1:0.3:0.1) systems, respectively (Table 2). Under the same conditions, but in the presence of a higher mol ratio of cholesterol, the impact of HgCl$_2$ was less, whereby glucose leakage was approximately 32% for egg-PC/cholesterol/DCP (1:0.5:0.1) and 21% for egg-PC/cholesterol/SA (1:0.5:0.1). Interestingly, the authors stated that other divalent cations tested, such as Cd(II), Mn(II), Pb(II), and Zn(II) did not affect the permeability of both egg-PC/cholesterol/SA or DCP containing vesicles under the same conditions. Based on these results, it was

suggested by the authors that Hg(II) had a stronger effect on the more negatively charged DCP containing vesicles, compared to the more positively charged SA containing vesicles. However, some experimental complications could arise when adapting the use of the enzyme assay described by Kinsky et al. (1969a,b) to metal-membrane interaction studies. Although the authors state that the effect of Hg(II) on the enzyme activities of hexokinase and glucose-6-phosphate dehydrogenase was checked and determined negligible (Nakada et al., 1978), Tsuzuki and Yamada (1979) reported conflicting evidence, and demonstrated that $20\,\mu M$ of Hg(II) resulted in half-maximal inhibition (K_i) of glucose-6-phosphate dehydrogenase (Tsuzuki and Yamada, 1979). Furthermore, Tsuzuki and Yamada (1979) showed that Hg(II) can complex with nicotinamide adenine dinucleotide phosphate (NADP), another required reagent for this enzyme assay (Kinsky et al., 1969a,b). It is unclear if, and to what extent, these potential side reactions could have affected the results reported by Nakada et al. (1978). It is possible that an interaction with Cd(II) was not observed with this enzymatic assay, since Cd(II) forms aggregates with hexokinase (Olmo et al., 2002). Although these Cd(II)-hexokinase aggregates have been shown to have negligible effects on enzyme activity (Olmo et al., 2002), this enzyme is not suitable for testing Cd(II) since amount of free Cd(II) available for vesicle binding would be unknown. This serves as an example of the numerous experimental challenges and difficulties that can be encountered in metal-membrane interaction studies.

Gutknecht (1981) described the diffusion of Hg(II) through planar black lipid membranes (BLMs). Briefly, phospholipids are often dissolved in *n*-decane, or another organic solvent, and deposited onto a Teflon septum hole that separates two chambers. As the organic solvent evaporates, a unilamellar membrane is formed across the Teflon septum hole ($1\,mm^2$). Gutknecht (1981) tested the diffusion of Hg(II) through BLMs composed of egg-PC/cholesterol (1:1) by recording radioactivity measurements of ^{203}Hg. Once ^{203}Hg is introduced into the first chamber, it can diffuse through the BLM and enter the second chamber containing the radioactivity detector. The permeability coefficient (0.013 cm s^{-1}) of $HgCl_2$ ($0.04\,mM$ to $0.18\,mM$) was measured to be approximately 20-fold higher than the permeability coefficient of water (Walter and Gutknecht, 1986). Gutknecht (1981) speculated that $HgCl_3^-$, and $HgCl_4^{2-}$ were mercurial species that did not cross the membrane at a significant rate under the conditions tested (pH 7.0, pCl = 0 to 2, 3–5 mM HEPES). Using a similar methodology, Gutknecht (1983) tested the permeability of 0.1 mM $CdCl_2$ by tracing the flux of ^{109}Cd across BLMs comprised of diphytanoylphosphatidylcholine (DPhyPC). The free Hg^{2+} and Cd^{2+} cations were comparable in permeability coefficients, where both values were measured on the order of 10^{-11} cm s^{-1}. However, $CdCl_2$ permeability (4.1×10^{-8} cm s^{-1}) through the DPhyPC BLM was a factor of 10^6 less compared to $HgCl_2$ (0.013 cm s^{-1}) through egg-PC or egg-PC/cholesterol (1:1) BLMs (Gutknecht, 1981). These results could suggest a mechanistic distinction in toxicity between Hg(II) and Cd(II), and they should be cautioned from being considered analogous. These results suggest that $HgCl_2$ is a more membrane-permeable species compared to $CdCl_2$, likely due to the high degree of covalent character in Hg-Cl bonds compared to the more ionic character of Cd—Cl bonds. It is important to point out that DPhyPC has branched acyl chains, where four methyl groups are distributed on each of the *sn-1* and *sn-2* acyl chains, affording bilayers with different physical properties such as decreased water permeability and a higher molecular area compared to PC lipids from mammals (Tristram-Nagle et al., 2010). Overall, further study is required to understand the permeability behaviour of metal species across lipid bilayers or BLMs, preferably using lipids found in mammalian cell membranes.

Bienvenue and Boudou (1984) examined the effects of Hg(II) on membrane permeability using BLMs composed of egg-PC and egg-PC/cholesterol. Radioactivity measurements of the ^{203}Hg isotope were used to measure Hg(II) flux across the membrane. Under the conditions tested (acetate buffer pH 5.0; or phosphate buffer pH 9.5), a permeability coefficient for $HgCl_2$ was measured (1.5×10^{-4} cm s^{-1}), and this species showed a greater propensity to permeate the membrane at pH 5.0. The difference in permeability measurements as reported by Gutknecht (1981) for $HgCl_2$ (Table 2) was addressed by Bienvenue and Boudou (1984); attributing the variance to experimental conditions such as the stirring rate and the use of different organic solvents for lipid deposition. Of course, the significance of testing both pH values was that in acetate buffer (pH 5.0), the major mercurial species is $HgCl_2$, whereas in phosphate buffer (pH 9.5), the Hg(II) species is 100% $Hg(OH)_2$. It must be considered that the extremes of pH's tested could also affect the affinity of the metal for membrane binding targets, as a large difference of >4 pH units will undoubtedly affect head group charge and bilayer surface charge density. The BLMs composed of egg-PC/cholesterol (1:1) had an ~9-fold greater rate of flux measured for $HgCl_2$ ($0.5\,mM$) compared to $Hg(OH)_2$. The rate of $HgCl_2$ flux across a pure egg-PC BLM was the same as the egg-PC/cholesterol (1:1) system, indicating that the addition of 50 % cholesterol did not affect the rate of $HgCl_2$ flux. This result is unexpected due to the increased rigidity of cholesterol enriched

Table 2
Summary of results reported for analyte leakage (Nakada et al., 1978; Bevan et al., 1983) and flux experiments (Bienvenue and Boudou, 1984; Gutknecht, 1981; Gutknecht, 1983), whereby values are reported as either the percentage of analyte released (%) or as permeability coefficient (cm s^{-1}). Comparatively, the permeability coefficient of H_2O for PC unilamellar vesicles has been reported to be between 2.8×10^{-3} cm s^{-1} to 1.46×10^{-2} cm s^{-1}, depending on the acyl chain composition (Olbrich et al., 2000).

Lipid(s)	Metal	Value	Ref.
egg-PC/chol/DCP	$HgCl_2$	40 %[a]	(Nakada et al., 1978)
		32 %[b]	(Nakada et al., 1978)
egg-PC/chol/SA	$HgCl_2$	26 %[a]	(Nakada et al., 1978)
		21 %[b]	(Nakada et al., 1978)
egg-PC/chol	$HgCl_2$	3.2×10^{-2} cm s^{-1}[c]	(Gutknecht, 1981)
egg-PC/chol	$HgCl_2$ (pH 5.0)	1.5×10^{-4} cm s^{-1}[c]	(Bienvenue and Boudou, 1984)
	$Hg(OH)_2$ (pH 9.5)	0.6×10^{-4} cm s^{-1}[c]	(Bienvenue and Boudou, 1984)
diphytanoyl-PC	Hg^{2+}	$<3.8 \times 10^{-11}$ cm s^{-1}[d]	(Gutknecht, 1983)
	Cd^{2+}	$<1.1 \times 10^{-11}$ cm s^{-1}[d]	(Gutknecht, 1983)
	$CdCl_2$	4.1×10^{-8} cm s^{-1}[d]	(Gutknecht, 1983)

[a] MLVs—glucose leakage with enzyme assay. Lipid mol ratios of 1:0.3:0.1, respectively.
[b] MLVs—glucose leakage with enzyme assay. Lipid mol ratios of 1:0.5:0.1, respectively.
[c] BLM—^{203}Hg tracing experiments. Lipid mol ratios of 1:1, respectively.
[d] BLM—^{203}Hg tracing experiments. Pure lipid was used.

membranes, and directs the attention to other factors such as pCl and pH as important factors in HgCl$_2$ flux, aside from the membrane composition. Moreover, Bienvenue and Boudou (1984) observed that increasing the ionic strength in either chamber of the BLM apparatus reduced the rate of HgCl$_2$ flux. This can be explained by the shielding of membrane surface charge caused by an increase in the presence of counter-ions at the membrane surface, but also that high ionic strength environments could reduce the population of HgCl$_2$ and increase the populations of Hg(II) species that do not cross the membrane as easily, such as HgCl$_3^-$ and HgCl$_4^{2-}$, thereby reducing the rate HgCl$_2$ flux.

3.2.1. Leakage and permeability—key findings

- Higher molratios of cholesterol in mixed MLVs containing DCP or SA, in combination with egg-PC, reduced Hg(II) induced leakage (0.1–10 μM) of glucose from MLVs. In addition, glucose leakage was higher for DCP containing MLVs compared to SA.
- The permeability coefficient of HgCl$_2$ (0.04–0.5 mM) across BLMs composed of egg-PC, egg-PC/cholesterol, and DPhyPC was measured on the order of 10^{-2} to 10^{-4} cm s^{-1}. On theother hand, the permeability coefficient for CdCl$_2$ (10^{-8} cm s^{-1}) acrossDPhyPC was measured to be orders of magnitude less.
- No change inthe measured permeability coefficient of HgCl$_2$ was reportedbetween the pure egg-PC and the 1:1 egg-PC/cholesterol systems; increasing the mol % of cholesterol to 50% in egg-PC did not affect the measured permeability coefficient of HgCl$_2$.

3.3. NMR and Raman spectroscopy

The binding of Hg(II) to MLVs comprised of egg-PC, egg-PE, or bovine spinal chord PS (BSPS) was reported using ^{199}Hg nuclear magnetic resonance (NMR) spectroscopy (Delnomdedieu et al., 1992). The ^{199}Hg isotope has a spin 1/2 nuclei and has a natural abundance of 16.9% (Lide, 2004). Changes in chemical shift after metal addition indicated binding of Hg(II) to the phospholipids. ^{199}Hg chemical shifts varied by up to several hundred ppm depending on the concentration of Hg(II) or by changes in pH and pCl. The authors tested a range of HgCl$_2$ concentrations (0–100 mM, pH 5.5, pCl 2.8), where roughly 90% of the Hg(II) species exists in the form of HgCl$_2$ (Delnomdedieu et al., 1992). It was reported that Hg(II) bound to PE and PS in either a weak and 'labile' state, or a stronger and more 'rigid' state. The labile form refers to

the Hg(II)-lipid complex which has was comparable to the properties of a liquid, and the rigid form refers to a situation in which Hg(II) was more strongly bound. The binding of Hg(II) to PE at a 1:1 stoichiometric ratio was labile, whereas a ratio of 1:3 (Hg/PE) resulted in the rigid state. Labile Hg(II) binding to PS occurred at a stoichiometric ratio (Hg/PS) of 1:2 whereas a ratio of 1:3 resulted in the rigid binding state. No perturbations in ^{199}Hg chemical shift was detected when Hg(II) binding was tested on MLVs comprised of egg-PC. Comparing Hg(II) binding to PE and PS in the labile state, with stoichiometric ratios (Hg/lipid) of 1:1 and 1:2 respectively, it is interesting to note that a stronger binding event of Hg(II) to PE was reported, although binding to PS occurred at a higher stoichiometric ratio (Lide, 2004). The authors also built upon their previous work (Delnomdedieu and Boudou, 1989) by suggesting that the carboxylate group in PS could reduce the accessibility of Hg(II) to bind the amine group. Furthermore, Delnomdedieu et al. (1992) demonstrated the reversibility of Hg(II) binding to PS and PE at high chloride concentrations (pCl ≤0.5), by measuring the disappearance of Hg(PE) and Hg(PS)$_2$ complexes. The release of chloride ions was detected in the bulk solvent upon increasing concentrations of lipid, which could imply that the displacement of chloride ions from Hg(II) could be of mechanistic importance in Hg(II)-phospholipid binding.

Fourier transform ^{1}H-NMR experiments were performed by Shinada et al. (1991) on dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) dispersions, and some of the chemical shift data has been summarized (Table 3) for further discussion. Most notably, HgCl$_2$ binding induced the largest ^{1}H-NMR change in chemical shift between control and super saturated HgCl$_2$ experiments for the amine protons in DOPE. Conversely, the protons in the CH$_2$OP linkage on the glycerol backbone side resulted in the largest change in chemical shift ($\Delta\delta$) between the control and super saturated HgCl$_2$ experiments for DOPC. It is also noteworthy that there was little difference measured between the observed chemical shifts in the control and the 1:1 mol ratio experiments for HgCl$_2$ (2.69 mM) and DOPC. However, an upfield shift ($\Delta\delta = -0.08$ ppm) was observed for the amine protons in DOPE for the 1:1 experiment relative to the control. Interestingly for DOPC, the protons in the CH$_2$OP group oriented toward the head group or the glycerol backbone shifted downfield ($\Delta\delta = +0.11$ and $+0.15$ ppm, respectively) in the super saturated HgCl$_2$ experiment. Evidently, these results further support Hg(II) binding to the amine head group in PE, but also demonstrate the

Table 3
^{1}H-NMR chemical shift (δ, ppm) data reported for HgCl$_2$ binding to DOPE and DOPC (2.69 mM) dispersions (Shinada et al., 1991). Experiments were performed at 27 °C using a JEOL GSX270 (270 MHz, 6.35 T) instrument.

Structure

| Lipid | Condition | Proton chemical shift (ppm) in respective positions (A, B, C, or D) | | | |
		A	B	C	D
DOPE (R = —NH$_3$)	Control[b] (CDCl$_3$)	8.35	3.19	4.36	3.95
	Mol ratio[a] (1:1)	8.27	3.20	4.36	3.95
	Super saturated HgCl$_2$	8.14	3.28	4.38	4.00
DOPC (R = N(CH$_3$)$_3$)	Control (D$_2$O)	3.23	3.65	4.36	3.92
	Mol ratio[a] (1:1)	3.26	3.67	4.37	3.93
	Super Saturated HgCl$_2$	3.23	3.79	4.47	4.07

[a] Mol ratios are reported as HgCl2/lipid.
[b] The DOPE control in pure D2O was reported as very broad, so CDCl3 was used.
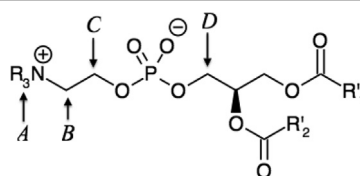
penetrative capacity of Hg(II), with binding detected at the phosphate region in DOPC.

The interaction of Hg(II) with egg-PC, BBPS, or egg-PE was examined using ^{31}P-NMR (Girault et al., 1995; Girault et al., 1996). It is important to note that both studies prepared these lipids with the addition of 10% (w/v) Triton X-100, causing the lipids to be in a micellar phase (Girault et al., 1995; Girault et al., 1996). Of course, ^{31}P is 100 % naturally abundant, making it convenient for monitoring binding interactions to or within the vicinity of the phosphate moiety. The conditions used in this experiment were acetate buffer (74 mM, pH 5.8) with a pCl of 2.8, thus greater than 95% of the Hg(II) species exists as HgCl$_2$, with less than 5 % from HgClOH and HgCl$_3^-$ (Hahne and Kroontje, 1973; Girault et al., 1995). Results from using different ratios of HgCl$_2$ to phospholipid showed a progressive, non-linear decrease in isotropic peak area with increasing HgCl$_2$ concentration, whereby the peak area decreased in the order of egg-PE > BBPS > egg-PC. A decrease in isotropic peak area was related to the amount of phospholipid that was tightly bound to mercury, resulting in the formation of Hg(II)-lipid aggregates. When HgCl$_2$ was added to the detergent mixed micelles of egg-PE/egg-PC (1:1) and BBPS/egg-PC (1:1) at HgCl$_2$/lipid ratios of 0.8:1, 1.6:1, and 2.4:1, a progressive peak area reduction was observed. The peak area signals obtained for the mixed systems were similar to the pure PE and PS systems. The authors proposed that under the conditions tested that Hg(II) did not bind the phosphate group, since a binding event to or in the vicinity of the phosphate group would have resulted in an observed ^{31}P change in chemical shift on the order of several ppm (James, 1975). It was proposed that Hg(II) likely bound to a group distant from the phosphate, presumed to be the amine in the head groups of PE and PS. The authors also suggested that the decreased motion of PC molecules in the mixed PE/PC and PS/PC micelles was likely a direct result of Hg(II) binding to PE or PS aggregates. Girault et al. (1996) also reported the ^{31}P-NMR chemical shift anisotropies ($\Delta\sigma$) for egg-PE, BBPS, egg-PC, and egg-PE/egg-PC (1:1) MLVs in the presence or absence of Hg(II) (Table 4) (Girault et al., 1996). The authors proposed that the increase in $\Delta\sigma$ values observed upon the addition of Hg(II) are indicative of a reduction in local motions of the phosphate group, or by a change in the head group angle with respect to the bilayer normal (Seelig, 1978; Rajan et al., 1981; Dufourc et al., 1992). It was speculated that Hg(II) complexation with the lipids could disrupt intermolecular interactions; such as hydrogen bonding and electrostatic pairing, between neighbouring lipid head groups, or that surface binding of Hg(II) to the phospholipids, or covalent binding to the primary amine in PS and PE, could drive head group reorientation. The authors also showed that a high concentration of NaCl (6.2 M) reversed the Hg(II) induced hexagonal phase formation of egg-PE, and under the same conditions, no effect of Hg(II) was observed on BBPS and egg-PC (Table 4). It should be clearly recognized that a dramatic change in chemical speciation of Hg(II) would occur in 6.2 M NaCl, with nearly 100 % of Hg(II) species in the form of HgCl$_4^{2-}$ (Hahne and Kroontje, 1973; Delnomdedieu et al., 1992).

Table 4
^{31}P-NMR chemical shift anisotropies ($\Delta\sigma$, in ppm) reported by Girault et al. (1996) for MLVs treated with or without Hg(II), in the absence or presence of NaCl (Girault et al., 1996). The accuracy of $\Delta\sigma$ values is within 3 ppm. Experiments were performed at 23 °C in 74 mM acetate (pH 5.8), and ^{31}P-NMR was recorded at 162 MHz using a Bruker AM 400 spectrometer.

	BBPS	egg-PE	egg-PC	egg-PE/egg-PC (1:1)
Control	−49	−40	−44	−40/−44
+ Hg(II)[a]	−34	−41	−34	−34
+ Hg(II)[a] + 6.2 M NaCl	−49	−37	−44	−41/−43

[a] Hg(II) was added at a Hg(II)/lipid ratio of 2.4:1.

In addition to ^{31}P-NMR, ^{113}Cd-NMR was also used to examine the binding affinity of Cd(II) and its effects on the membrane fluidity of MLVs using a variety of lipids (Girault et al., 1998). The Cd(NO$_3$)$_2$ salt in the absence of chloride was used, so approximately 90 % of Cd(II) species were in the form of Cd^{2+} with the remaining 10% as CdNO$_3^-$ (Girault et al., 1998). The temperature at which egg-PE is in the hexagonal phase was measured at 35 °C, however, the addition of Cd^{2+} to MLVs composed of egg-PE induced hexagonal phase formation at 24 °C. It is worth noting that increasing ionic strength, the effects of dehydration (Webb and Steponkus, 1993; Claessens et al., 2004), or a reduction in head group area relative to acyl chain area (Cullis and De Kruijff, 1978), are all important factors in hexagonal phase formation. The DMPG and DMPS samples immediately formed solid precipitates upon the addition of Cd^{2+} at a Cd^{2+}/lipid ratio of 0.5, an effect that was also observed to a lesser extent for DMPA over a few hours. The authors showed that 0.8 M NaCl was sufficient to remove 70 % of Cd(II) bound to DMPG, and that 1.8 M NaCl released 100 % of Cd(II) bound to DMPG and DMPA, but only 50 % of Cd(II) was removed from DMPS. Thus, Girault et al. (1998) ranked the binding affinity of Cd(II) in the order of PS >> PA > PG. It was also reported that Cd(II) interactions with DMPG were reduced with increasing membrane fluidity, in contrast to DMPA, where increasing membrane fluidity enhanced the magnitude of Cd(II) binding. The authors suggested that the latter effect could be the result of Cd(II) binding to a deeper region of the bilayer, possibly near the phosphate region or near the glycerol backbone.

Raman spectroscopy has been used to characterize the effects of Cd(II) (1 M) on DMPC and DPPC dispersions (Lis et al., 1975). Lis et al. (1975) reported the ratio of Raman intensities (I_{1064}/I_{1089}) at wavenumbers of 1064 cm^{-1} (I_{1064}) and 1089 cm^{-1} (I_{1089}) to discern that the magnitude at which metal ions decreased the proportion of gauche character in the lipid acyl chains was in the following order: Cd^{2+} ~ Ca^{2+} > Mg^{2+} > Ba^{2+}. Furthermore, Lis et al. (1975) also demonstrated that cadmium chloride salts, such as CdCl$_2$ and CdCl$^+$, increased the I_{1064}/I_{1089} ratio, while Cd(acetate)$_2$ and CdSO$_4$ salts were approximately the same. This is likely the result of two effects from changing the anion: changes in chemical speciation, and attractive forces of the anion toward the positively charged amine in the PC head group.

3.3.1. NMR and Raman spectroscopy—key findings
- ^{199}Hg-NMR revealed that Hg(II) (0 to 100 mM) bound to egg-PE and BSPS first with a weak, labile binding event, followed by a second stronger and more rigid binding event. No binding to MLVs comprised of egg-PC was reported.
  - Hg(II) bound PE at a stoichiometric ratio (Hg/PE) of 1:1 and 1:2 for the labile and rigid conditions, where Hg(II) bound for PS at a stoichiometric ratio (Hg/PS) of 1:2 and 1:3, respectively.
- Hg(II) binding to PE and PS was reversible at high chloride concentrations (pCl ≤0.5), an effect also observed at a high concentration of NaCl (6.2 M).
- Chloride ions were released into the media upon increasing lipid concentrations
- ^{1}H NMR experiments on Hg(II) binding to DOPE (2.69 mM) dispersions showed an upfield chemical shift (~0.1 ppm) in the amine protons of PE at a 1:1 stoichiometric ratio (Hg/PE). For DOPC, Hg(II) binding was only detected in super saturated HgCl$_2$ conditions, where a downfield chemical shift was detected in both C–H protons immediately neighbouring the phosphate group.
  - These results suggest Hg(II) binding to PE could occur via the amine head group, and Hg(II) binding to the phosphate moiety in PC.
- Using ^{31}P-NMR, it was reported that the binding strength of Hg(II) (0–36 mM) to lipids mixed with Triton X-100 followed an order from strongest to weakest: egg-PE > BBPS > egg-PC.

- Binding to the phosphate was not detected, as a binding event would have resulted in a ^{31}P change in chemical shift of several ppm
- Changes in chemical shift anisotropy of ^{31}P upon Hg(II) binding was considered to be a result of either disrupting hydrogen bonding and electrostatic pairing between lipid head groups, or by head group reorientation via covalent bonding to a group distant from the phosphate.
- ^{113}Cd-NMR and ^{31}P-NMR experiments using $Cd(NO_3)_2$ showed that:
  - The order of Cd(II) binding affinity was ranked: PS >> PA > PG.
- No change in ^{113}Cd peak area was detected for Cd(II) binding to DMPC
- Temperature-induced hexagonal phase formation in egg-PE was recorded at 35 °C, however, hexagonal phase formation was evident at 24 °C in the presence of Cd(II) at a Cd/lipid ratio of 0.5.
- Raman spectroscopy was used to demonstrate the magnitude to which metal ions decreased the proportion of gauche character, where Ca(II) and Cd(II) had the strongest observed effect.

3.4. Langmuir

The Langmuir technique, which has been reviewed in detail elsewhere (Giner-Casares et al., 2014), can be used to measure the physical properties of a lipid monolayer. Briefly, lipids are deposited onto the surface of an aqueous phase contained in a Langmuir trough. A movable barrier allows for expansion and compression of the lipid monolayer formed at the air–water interface, whereby lipid head groups are positioned below the air/water interface in the aqueous phase, and the hydrophobic lipid acyl chains are in the gas phase. This technique can be used to measure the compressibility of lipids, where lipids with a low compressibility cannot be as densely packed in a lateral arrangement as those having a high compressibility. Broniatowski et al. (2010) used this technique to examine the effects of Hg(II) on DPPC, DPPG, lyso-PC, and SM monolayers. The authors reported that neither DPPG or DPPC complexed strongly with Hg(II) and they did not observe Hg(II) accumulation in the monolayer. The surface-pressure mean molecular area $(\pi - A)$ isotherm for DPPG was nearly identical in the presence or absence of either $HgCl_2$ or $Hg(NO_3)_2$ at 0.5 mM. Comparatively, Hg^{2+} only weakly affected the structural organization of the DPPC monolayer. On the other hand, $HgCl_2$ shifted the $\pi - A$ isotherm considerably for DPPC relative to the control in pure water. From the $\pi - A$ isotherm data, the authors also calculated the compression moduli, which provides information on the physical state of the monolayer (Gaines, 1966) and can be used as a qualitative measure for the degree of order in the monolayer. These data showed that $HgCl_2$ disordered the DPPC, SM, and lyso-PC monolayers, and had little effect on DPPG. The authors hypothesized that since $HgCl_2$ typically is limited in its dissociation into ions in aqueous solution, and given that $HgCl_2$ is a salt of a strong acid and a weak base, that negatively charged complexes such as $[Hg(H_2O)Cl_2OH]^-$ could be present in solution. These complexes could then interact favorably with the positively charged choline head group in DPPC, SM, and lyso-PC, and might explain why Hg(II) only weakly affected DPPG monolayers.

The interactions of $HgCl_2$ and $CdCl_2$ with PC, PE, and PS monolayers were examined by Le et al. (2013) by monitoring changes in surface pressure-area isotherms. In these experiments, 0.1 mM $HgCl_2$, $CdCl_2$, or a 1:1 mixture of $HgCl_2/CdCl_2$ was added to the sub-phase containing 100 mM NaCl and 1 mM phosphate (pH 7.4) before the deposition of lipid films. The lipid systems used in this study (in molar ratios) were: pure POPC, POPC/POPE (85:15), POPC/POPS (85:15), and the biomimetic erythrocyte system composed of POPC/cholesterol/POPE/POPS (35:35:15:15). The pure

POPC isotherm was not affected by the addition of Hg(II) or Cd(II), however, isotherms of systems containing POPE or POPS shifted to larger molecular areas. Hg(II) and Cd(II) preferentially bound PS > PE versus PE > PS, respectively. Moreover, Hg(II) and Cd(II) increased rigidity and monolayer compressibility in all systems containing PE or PS. It has to be emphasized that a 1:1 mixture (100 μM) of Hg(II)/Cd(II) had the strongest effect on all systems relative to those tested with Hg(II) or Cd(II) alone, and even shifted the pure POPC isotherm, in addition to all other isotherms, to larger average molecular areas. This is a very important observation, as it indicates that the exposure to one toxic metal should not be seen in isolation. Indeed, humans are regularly exposed to mixtures of various metals and the impact of metal mixtures has not been sufficiently examined in the biophysical investigations of metal–membrane interactions.

3.4.1. Langmuir—key findings
- After examining the stability of lyso-PC, SM, and DPPC monolayers, $HgCl_2$ (0.5 mM) measurably disordered all three monolayer systems, while $Hg(NO_3)_2$ (0.5 mM) negligibly affected DPPC, lyso-PC, and only weakly affected SM. A monolayer of DPPG was also tested, to which neither $HgCl_2$ or $Hg(NO_3)_2$ had a measured effect.
- Both $HgCl_2$ (0.1 mM) and $CdCl_2$ (0.1 mM) increased rigidity and compressibility in monolayers containing PC/PE or PC/PS. The effects observed for Hg(II) and Cd(II) was ranked PS > PE and PE > PS, respectively. No effect was measured for either Hg(II) or Cd(II) with pure POPC.
- A 1:1 mixture (0.1 mM) of $HgCl_2/CdCl_2$ had a pronounced effect on pure POPC, and all other monolayer systems tested, compared to $HgCl_2$ or $CdCl_2$ alone. This evidence underlines the impact of toxic of metal mixtures.

3.5. X-ray diffraction

An early X-ray diffraction study (Lis and Parsegian, 1981) characterized the degree at which of Cd(II), Ca(II), and other divalent metal ions bound to a variety of different PC lipids. Both Cd(II) and Ca(II), along with Mn(II), bound DPPC to a greater extent than other metals tested, such as Co(II), Mg(II), or Ba(II). The authors showed that the binding of the tested divalent metals was dependent on metal concentration, variations in lipid acyl chain composition, the presence or absence of NaCl, but also by the amount of bilayer separation between opposing bilayers contained in MLVs (Lis and Parsegian, 1981). To account for the differences in bilayer separation, Lis and Parsegian (1981) suggested that as opposing bilayers approach one another, they could electrostatically induce a reorientation of the PC head group relative to the plane of the bilayer, since the PC head group has a large dipole moment. If head group reorientation affects metal binding affinity, as previously suggested, then a highly dynamic environment of approaching and distancing bilayers of MLVs in solution could have dramatic effects on the measured metal binding affinity. Thus, for a more accurate determination of binding constants in metal-membrane interactions studies, the use of model vesicle systems with single bilayers, such as LUVs, would be a more suitable choice compared to MLVs.

In continuation of the Broniatowski et al. (2010) study, this group investigated the effects of $HgCl_2$ and $Hg(NO_3)_2$ on DPPC, SM, and lyso-PC using X-ray reflectivity (XR) and grazing incidence X-ray diffraction (GIXD). From the GIXD and XR data collected, it was discerned that neither $HgCl_2$ and $Hg(NO_3)_2$ affected the structural properties of the DPPC monolayer. In the case of the GIXD data collected for SM, both Hg(II) salts, especially $HgCl_2$, introduced a high degree of disorder into the SM

monolayers. From the XR data, the authors did not calculate any additional electrons in the head group region of SM or lyso-PC after being treated with 0.1 mM $Hg(NO_3)_2$, indicating no permanent complexation with Hg^{2+}. In contrast, 56 additional electrons were calculated in the head group region of SM, and with a total of 114 electrons in $HgCl_2$, a 2:1 complex of SM/ $HgCl_2$ was proposed. However, the authors note that the number of $HgCl_2$ molecules is likely lower since some of these electrons likely belong to water molecules. In the case of lyso-PC, 111 additional electrons were calculated in the head group region of lyso-PC, and 1:1 binding complex of lyso-PC/$HgCl_2$ was proposed. It has to be emphasized that $HgCl_2$ was the only compound that produced a measurable interaction with SM and lyso-PC (Broniatowski et al., 2010), in contrast to $Hg(NO_3)_2$.

X-ray diffraction experiments have also been reported for the effects of $HgCl_2$ and $CdCl_2$ on MLVs composed of DMPC and DMPE (Suwalsky et al., 2000; Suwalsky et al., 2004). Interestingly, when a range of $HgCl_2$ concentrations (0.01 mM to 0.1 mM) was tested on MLVs of DMPC, alternating disordered and ordered phases were detected, seen by a reduction or an increase in small angle reflections, respectively; at 0.1 M $HgCl_2$ no reflections were detected for DMPC indicating bilayer destruction. In the case of DMPE, an increase in the concentration of Hg(II) was directly proportional with disordering of the lipid membrane, indicated by a reduction in the intensity of small angle reflections. Conversely, the bilayer of DMPC was destroyed in the presence of 1 mM $CdCl_2$ seen by the disappearance of small and wide angle reflections. Contrastingly, high-angle reflections were still detected for DMPE in the presence of 0.4 M $CdCl_2$. For the effects of Cd(II) on DMPC and DMPE, it was evident from the reduction in small angle reflection intensities that Cd^{2+} concentrations as low as 10 μM were sufficient to perturb the head group region for both DMPE and DMPC LUV's. After treatment with 1 mM $CdCl_2$ the small angle reflections of DMPC disappeared, indicating that the bilayer was completely destroyed. On the other hand, DMPE was more resistant to Cd(II)-induced bilayer destruction; increasing concentrations of Cd(II) gradually reduced the small angle reflection intensities of the DMPE bilayer, and were still detected even in the presence of 0.4 M Cd(II). With a smaller polar group in PE compared to PC, PE has a higher effective charge than PC. In addition, PE head groups can pack together tightly due to hydrogen bonding which is not the case for PC head groups. Thus, DMPE forms more stable multilayer arrangements compared to DMPC. Moreover, Suwalsky et al. (2004) explained that the increase of bilayer width between the dry film (54.5 Å) and fully hydrated (64 Å) DMPC, when in the gel phase, could have promoted the incorporation of Hg(II) or Cd(II) into the inter-bilayer spaces of DMPC MLVs. Furthermore, Suwalsky et al. (2004) stated that Cd(II), followed by Pb(II), induced the stronger structural perturbations of DMPE and DMPC compared to any other metals studied, such as Hg(II), Cu (II), and Zn(II).

3.5.1. X-ray diffraction—key findings
- The effect of Hg(II) and Cd(II) on binding to MLVs composed of PC lipids was dependent on acyl chain composition, ionic strength, metal and lipid concentrations, and the extent of bilayer separation between bilayers in MLVs.
- $HgCl_2$ (0.5 mM) had markedly stronger effects on DPPC, SM, and lyso-PC compared to Hg^{2+}. Binding of $HgCl_2$ was detected in the head group regions of SM and lyso-PC, at a 2:1 and a 1:1 lipid/ $HgCl_2$ ratio, respectively.
- Both small and wide angle reflections disappeared for MLVs composed of DMPC in the presence of 0.1 M $HgCl_2$, indicating bilayer destruction. In contrast, small and wide angle reflections were still present for MLVs composed of DMPE at 0.1 M $HgCl_2$.
- Small and wide angle reflections for MLVs composed of DMPC disappeared in the presence of 1 mM $CdCl_2$, whereas DMPE was more resistant to bilayer destruction, as small and wide angle reflections for DMPE were still detected at 0.4 M $CdCl_2$.

3.6. Interactions of Hg(II) and Cd(II) with erythrocytes

One of the earliest studies examined the effects of Hg(II) and Cd (II) on the efflux of potassium ions (K^+) from human erythrocytes that were incubated with either Hg(II) or Cd(II) for 60 min at 37 °C (Vincent and Blackburn, 1958). Both Hg(II) and Cd(II) promoted release of K^+ from healthy human erythrocytes. Expressed as a ratio of micro-equivalents of K^+ (μEq K^+) released per 0.1 μmol/mL of metal added, Vincent and Blackburn (1958) report that this ratio was 200 to 800 times higher for Hg(II) compared to Cd(II). It is worth pointing out that, as described earlier, both Hg(II) and Cd(II) can bind sulfhydryl groups in membrane proteins. However, Vincent and Blackburn (1958) reported that the release of K^+ did not change in the presence of non-metallic thiol inhibitors, suggesting that K^+ release was independent of any interactions that could be formed between Hg(II) and any free sulfhydryl groups on erythrocyte membrane proteins. This demonstrates that Hg(II) induced leakage of K^+ from erythrocytes is a result of alterations in lipid structure and organization in the bilayer, or through disrupting the osmotic balance of erythrocytes due to the rapid uptake of $HgCl_2$ and inhibition of aquaporins (Savage and Stroud, 2007). To elaborate further on the uptake of $HgCl_2$, Weed et al. (1962) measured the influx of $HgCl_2$ into human erythrocytes by monitoring ^{203}Hg uptake and desorption. This study demonstrated that metal binding to erythrocytes inhibited glucose uptake, and was proposed to induce the loss of intracellular K^+. The uptake of $HgCl_2$ was achieved quickly as equilibrium was reached in the course of 3–5 min. Furthermore, these results are consistent with a ^{1}H-NMR study performed later by Rabenstein and Isab (1982), where it was shown that $HgCl_2$ added to intact human erythrocytes could cross the membrane and reach equilibrium concentrations in 4 min.

Using scanning electron microscopy, Suwalsky et al. (2000) showed that human erythrocytes incubated with 1 mM Hg(II) for one hour at 37 °C exhibited changes in their morphology, leading to the formation of echinocytes and stomatocytes (Fig. 4). Sheetz and Singer (1974) proposed the bilayer couple hypothesis (Sheetz and Singer, 1974), which suggested that a shape-changing agent, in this case, a metal ion, may preferentially perturb one membrane leaflet over the other. The binding of a metal to the outermost leaflet could increase its surface area, inducing the formation of irregular bulges on the membrane surface and result in echinocyte formation. In contrast, for cell membrane invaginations or stomatocyte formation, metals interact more strongly with the inner membrane leaflet. Based on the bilayer couple hypothesis, the observations made by Suwalsky et al. (2000) are consistent with Hg(II) interacting with both leaflets of the erythrocyte membrane, although echinocyte formation was most prominent. Suwalsky et al. (2004) also used scanning electron microscopy to demonstrate that 1 mM Cd(II) induced echinocyte formation in human erythrocytes.

Fluorescence spectroscopy studies have also been used to study the effects of Hg(II) and Cd(II) on erythrocyte membrane fluidity through the incorporation of DPH or Laurdan. Suwalsky et al. (2000) used both dyes to assess the impact of metals on the fluidity of the less polar acyl chain region, monitored by DPH, versus the more polar interface monitored by Laurdan. Hg(II) was added in a range of concentrations (0.1 mM and 0.5 mM), where

Discoid

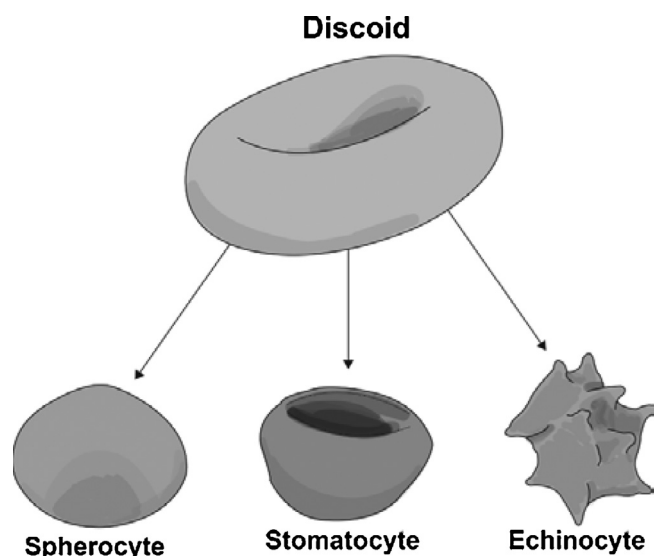**Spherocyte**   **Stomatocyte**   **Echinocyte**

Fig. 4. Erythrocyte cellular morphologies implicated with exposure to toxic metals. Healthy erythrocytes have a discoid morphology, however, upon exposure to toxic metals, erythrocytes may adopt spherocyte, stomatocyte, or echinocyte forms.

concentrations above 0.1 mM Hg(II) increased the ordering of both the acyl chains as well as the phosphate region in human erythrocytes at 37 °C. These results are consistent with the Delnomdedieu and Allis (1993) study, which also reported an increase in DPH fluorescence polarization, or an increase in membrane rigidity, for rat erythrocytes treated with Hg(II).

The effects of Cd(II) on the DPH anisotropy of human erythrocytes was investigated by Sorensen et al. (1985), testing metal concentrations between 50 and 500 μM on human erythrocytes at 24 °C. This study showed that Cd(II) at all concentrations caused a decrease in the motional freedom of the DPH fluorescent probe, where the effects of Cd(II) were reported to be 3–10 times stronger than those of Ca(II). The authors also experimentally demonstrated that the effects of Cd(II) on the erythrocyte membrane were reduced in the presence of Ca(II) (Sorensen et al., 1985), highlighting a degree of competitive binding between Ca(II) and Cd(II) for phospholipid binding targets. Similarly, Amoruso et al. (1987) found that the membrane fluidity of human erythrocyte ghosts decreased, shown by an increase in DPH fluorescence polarization, when subject to increasing concentrations of Cd(II) (10 μM to 0.1 M) at 37 °C.

Fluorescence polarization of unsealed erythrocyte membranes performed at 37 °C revealed that increasing amounts of Cd(II) (0 to 1.8 mM) decreased both the general polarization (GP) of laurdan and DPH anisotropy, which together imply a disordering effect at both the head group and acyl chain regions. This was proposed to be a consequence of Cd(II) binding to the negatively charged phosphates located in the head group region, as indicated by previous studies (Girault et al., 1998; Sundaralingam and Jensen, 1965). It is likely that Suwalsky et al. (2004) reported a decrease in DPH anisotropy since this study used of unsealed human erythrocytes, where the membrane leaflets of the erythrocyte membrane have been inverted (Agre, 1989). In contrast, Amoruso et al. (1987) used resealed erythrocyte ghosts where the membranes leaflets are maintained in their native orientation. These results highlight the importance of considering the asymmetric distribution of lipids between the inner and outer membrane leaflets in the human erythrocyte, as the effects of Cd(II) are clearly different depending on which membrane leaflet is exposed to the aqueous environment.

3.6.1. Interactions of Hg(II) and Cd(II) with erythrocytes—key findings

- The effects of Hg(II) on K⁺ release from human erythrocytes was 200–800 times greater compared to Cd(II). This effect was dependent on the amount of metal per unit of red blood cells.
- The presence of non-metallic thiol inhibitors did not change the Hg(II) induced efflux of intracellular K⁺, indicating that binding of Hg(II) to erythrocyte membrane proteins was independent of Hg(II) induced K⁺ release.
- Independent ^{1}H-NMR and ^{203}Hg tracing studies showed that erythrocyte uptake of $HgCl_2$ was rapid, achieving equilibrium concentrations in less than 5 min.
- Scanning electron microscopy of human erythrocytes incubated in Hg(II) or Cd(II) adopted predominantly echinocyte morphology, however stomatocytes were also present.
- Concentrations above 0.1 mM Hg(II) increased order in both the acyl chain and head group regions of human erythrocytes.
- Cd(II) (10 μM to 0.1 M) reportedly caused an increase in membrane rigidity for erythrocytes having their membrane outer leaflets in their native orientation. However, for unsealed erythrocytes where the inner and outer leaflets have been inverted, Cd(II) increased membrane fluidity and disordered the head group region.

4. Recommendations

4.1. Metal speciation and experimental conditions

After reviewing the effects of Hg(II) and Cd(II) on lipid model systems and erythrocytes, it is evident that metal speciation in aqueous solution plays a critical role. In order to provide sufficient information for a reader it is important that experimental conditions are clearly defined. The most critical factors are pH, chloride concentration, ionic strength, and metal concentrations. Furthermore, it is important to test the pH of the sample solution after the addition of a metal as some metals will change the pH. Such changes affect speciation, lipid protonation states, membrane charge localization, and surface charge density. The choice of buffer in metal-membrane interaction studies also deserves careful consideration to avoid non-specific binding and precipitation of metal salts. Since most metals strongly complex with buffers from the Tris and bis(2-hydroxyethyl) amine families, these buffers should be avoided (Ferreira et al., 2015). Recently, metal-buffer interactions has been reviewed and should be consulted when searching for a suitable buffer (Ferreira et al., 2015). With respect to fluorescence techniques, it is important to assess dye localization in the membrane, but also to examine if any undesirable interactions, such as fluorescence quenching, arise between the metal and dye. Ideally, such interactions should be minimal and need to be corrected during data analysis. We also caution the use of enzymatic assays in metal-membrane interaction studies without comprehensive control experiments, especially for metals such as Hg(II) and Cd(II) that demonstrate a strong affinity for cysteine residues. Finally, due to the dependence of metal-membrane interactions on metal/lipid and lipid/lipid ratios, it is also critically important that metal and lipid concentrations are clearly stated, as some studies have not included this important information.

4.2. Lipid structure and methodology

Lipid structure, including head group, backbone, and acyl chain composition, is inherently coupled to bilayer structure and function, influencing various biophysical properties including charge localization, surface charge density, membrane fluidity or

rigidity, bilayer thickness, and permeability. Such properties influence metal-membrane interactions, so it is of critical importance that lipid structure is well known and clearly indicated. This is especially important for the use of lipid extracts, which contain lipids with mixed acyl chains that vary in distribution between lipid extract sources. We also recommended the use of LUV model systems over MLVs or lipid dispersions when possible, since metal binding is affected by the distance between bilayers in an MLV (Lis and Parsegian, 1981), a problem that is avoided with the use of LUVs. Furthermore, LUV model systems are prepared by methods such as extrusion (Hope et al., 1985; MacDonald et al., 1991), and the size distribution of vesicles can be checked using methods such as dynamic light scattering (Hallett et al., 1991). Vesicle size and curvature affect the accessibility of the lipid head group and backbone regions, which serve as potential metal-binding targets and impact the extent of leakage of vesicle contents (Hope et al., 1985): thus working with LUVs of a narrow size distribution would improve reproducibility between studies. It is also clear that the concentrations of metal and lipid used vary appreciably between methodologies; for instance, NMR provides detailed structural information but is less sensitive and requires higher concentrations of metal and lipid compared to fluorescence techniques. Thus, it is important to select a method appropriate for the range of targeted metal concentrations, which will also vary depending on the circumstances. Cases of acute metal exposure will involve higher concentrations compared to more long-term chronic metal exposure, while both will affect human health and well-being

5. Concluding remarks

In the context of the chronic low-level exposure of humans to toxic metals, the biophysical effects of Hg(II) and Cd(II) on lipid model systems and human erythrocytes, and their impact on biomembrane structure and function, may have important toxicological implications. Moreover, it has been demonstrated here that methodologies, sample preparations, and experimental conditions regarding metal–membrane interaction studies have varied considerably. This could explain some of the conflicting results reported in the literature. Thus, a more comprehensive investigation of Hg(II) and Cd(II) interactions is needed, and the use of more standardized model systems and consistent experimental conditions such as physiological salt, pH and temperature should be considered in the planning of future studies to allow for a better comparison of results. Furthermore, the effects of Hg(II) and Cd(II) in the presence of other biologically abundant metals such as Ca(II), Mg(II), Zn(II), and Fe(III), could provide mechanistic and toxicological insights. As previously mentioned, one study reported that a Hg(II) and Cd(II) mixture had the most dramatic effect on the lipids tested when compared to the action of either Hg(II) or Cd(II) alone (Le et al., 2013).

It is important to point out that many of the studies investigating Hg(II) and Cd(II) interactions have only tested a small fraction of the phospholipids present in the mammalian membrane, and have been assessed thus far primarily based on head group composition. The effects of Hg(II) and Cd(II) on other important lipid components such as phosphoinositides, ceramides, plasmalogens, and cerebrosides, have yet to be reported in the literature. A more comprehensive analysis on the effects of Hg(II) and Cd(II) on membrane and lipid structure is required. Such studies could investigate the dependence of Hg(II) and Cd(II) interactions on physical properties such as T_m, surface charge, head group spacing, bilayer thickness, acyl chain composition, length, and unsaturation. The effects of lipid/lipid ratio, and the use of mixed acyl chains and more complex biomimetic lipid mixtures

should also be addressed. Vesicle disruption, leakage, and fusion studies could also provide further insight.

Although this review has assessed the progress made in this field to date, it also highlights the need for further studies to investigate the biophysical effects that Hg(II) and Cd(II) pose on cell membranes. It is comprehensible that further research in this area is important in the field of medicine; future studies may provide greater insight into the influences of Hg(II) and Cd(II) on the membrane structure and function, to better understand how toxic metals influence disease etiology.

References

Agre, P., 1989. Red Blood Cell Membranes: Structure: Function. Clinical Implications..

Amoruso, M.A., Witz, G., Goldstein, B.D., 1987. Alteration of erythrocyte membrane fluidity by heavy metal cations. Toxicol. Ind. Health 3, 135–144.

Ayati, M., Madsen, H., 2001. Solubility product of the cadmium phosphate $Cd_5H_2(PO_4)_4 4H_2O$ at 37°C. J. Chem. Eng. Data 46, 113–116.

Baes, C., Mesmer, R., 1976. Hydrolysis of Cations. Wiley, pp. 287–312.

Barkay, T., Miller, S.M., Summers, A.O., 2003. Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol. Rev. 27, 355–384.

Barregard, L., Sallsten, G., Jarvholm, B., 1995. People with high mercury uptake from their own dental amalgam fillings. Occup. Environ. Med. 52, 124–128.

Bazarkina, E.F., Pokrovski, G.S., Zotov, A.V., Hazemann, J.-L., 2010. Structure and stability of cadmium chloride complexes in hydrothermal fluids. Chem. Geol. 276, 1–17.

Bevan, D., Worrell, W., Barfield, K., 1983. The Interaction of Ca^{2+}, Mg^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+} with phospholipid bilayer vesicles. Colloids Surf. 6, 365–376.

Bhattacharya, S., Haldar, S., 2000. Interactions between cholesterol and lipids in bilayer membranes. Role of lipid headgroup and hydrocarbon chain-backbone linkage. Biochim. Biophys. Acta 1467, 39–53.

Bienvenue, E., Boudou, A., 1984. Transport of mercury compounds across bimolecular lipid membranes: effect of lipid composition, pH and chloride concentration. Chem. Biol. Interact. 48, 91–101.

Breitinger, D., Brodersen, K., 1970. Development of and problems in the chemistry of mercury–nitrogen compounds. Angew. Chem. Int. Ed. 9, 357–367.

Bridges, C.C., Zalups, R.K., 2005. Molecular and ionic mimicry and the transport of toxic metals. Toxicol. Appl. Pharmacol. 204, 274–308.

Bridges, C.C., Zalups, R.K., 2010. Transport of inorganic mercury and methylmercury in target tissues and organs. J. Toxicol. Environ. Health B Crit. Rev. 13, 385–410.

Broniatowski, M., Flasin, M., Dynarowicz-ła, P., 2010. Grazing incidence diffraction and X-ray reflectivity studies of the interactions of inorganic mercury salts with membrane lipids in Langmuir monolayers at the air/water interface. J. Phys. Chem. B 114, 9474–9484.

Brown, D., London, E., 1998. Functions of lipid rafts in biological membranes. Rev. Cell Dev. Biol. 14, 111–136.

Chan, T.Y.K., 2011. Inorganic mercury poisoning associated with skin-lightening cosmetic products. Clin. Toxicol. (Phila) 49, 886–891.

Claessens, M.M.A.E., van Oort, B.F., Leermakers, F.A.M., Hoekstra, F.A., Cohen Stuart, M.A., 2004. Charged lipid vesicles: effects of salts on bending rigidity, stability, and size. Biophys. J. 87, 3882–3893.

Clarkson, T.W., Magos, L., 2006. The toxicology of mercury and its chemical compounds. Crit. Rev. Toxicol. 36, 609–662.

Cotton, F.A., Wilkinson, G., Murillo, C.A., Bochmann, M., 1999. Advanced Inorganic Chemistry, 6th ed. John Wiley & Sons, Inc.

Cullis, P., De Kruijff, B., 1978. he polymorphic phase behaviour of phosphatidylethanolamines of natural and synthetic origin. A ^{31}P NMR study. Biochim. Biophys. Acta 513, 31–42.

Delnomdedieu, M., Allis, J.W., 1993. Interaction of inorganic mercury salts with model and red cell membranes: Importance of lipid binding sites. Chem. Biol. Interact. 88, 71–87.

Delnomdedieu, M., Boudou, A., 1989. Interaction of mercury chloride with the primary amine group of model membranes containing phosphatidylserine and phosphatidylethanolamine. Biochim. Biophys. Acta 986, 191–199.

Delnomdedieu, M., Boudou, A., Georgescauld, D., Dufourc, E.J., 1992. Specific interactions of mercury chloride with membranes and other ligands as revealed by mercury-NMR. Chem. Biol. Interact. 81, 243–269.

Dufourc, E.J., Mayer, C., Stohrer, J., Althoff, G., Kothe, G., 1992. Dynamics of phosphate head groups in biomembranes. Comprehensive analysis using phosphorus-31 nuclear magnetic resonance lineshape and relaxation time measurements. Biophys. J. 61, 42–57.

Edidin, M., 1997. Lipid microdomains in cell surface membranes. Curr. Opin. Struct. Biol. 7, 528–532.

Edidin, M., 2003. The state of lipid rafts: from model membranes to cells. Annu. Rev. Biophys. Biomol. Struct. 32, 257–283.

Ferreira, C.M.H., Pinto, I.S.S., Soares, E.V., Soares, H.M.V.M., 2015. (Un)suitability of the use of pH buffers in biological, biochemical and environmental studies and their interaction with metal ions—a review. RSC Adv. 5, 30989–31003.

Gailer, J., 2007. Arsenic–selenium and mercury–selenium bonds in biology. Coord. Chem. Rev. 251, 234–254.

Gailer, J., 2012. Probing the bioinorganic chemistry of toxic metals in the mammalian bloodstream to advance human health. J. Inorg. Biochem. 108, 128–132.

Gaines, G., 1966. Insoluble Monolayers at Liquid–gas Interfaces. Interscience Publishing, 144.

García-Sevillano, M.A., García-Barrera, T., Navarro, F., Gailer, J., Gómez-Ariza, J.L., 2014. Use of elemental and molecular-mass spectrometry to assess the toxicological effects of inorganic mercury in the mouse Mus musculus. Anal. Bioanal. Chem. 406, 5853–5865.

Giner-Casares, J.J., Brezesinski, G., Möhwald, H., 2014. Langmuir monolayers as unique physical models. Curr. Opin. Colloid Interface Sci. 19, 176–182.

Giocondi, M.-C., Boichot, S., Plénat, T., Le Grimellec, C.C., 2004. Structural diversity of sphingomyelin microdomains. Ultramicroscopy 100, 135–143.

Girault, L., Lemaire, P., Boudou, A., Dufourc, E., 1995. Inorganic mercury interactions with lipid components of biological membranes: P-NMR study of Hg (II) binding to headgroups of micellar phospholipids. Mercur. Glob. Pollut. 80, 95–98.

Girault, L., Lemaire, P., Boudou, A., Debouzy, J.C., Dufourc, E.J., 1996. Interactions of inorganic mercury with phospholipid micelles and model membranes. A ^{31}P-NMR study. Eur. Biophys. J. 24, 413–421.

Girault, L., Boudou, A., Dufourc, E.J., 1998. Cd-, P-NMR and fluorescence polarization studies of cadmium(II) interactions with phospholipids in model membranes. Biochim. Biophys. Acta 1414, 140–154.

Girotti, A.W., 1985. Mechanisms of lipid peroxidation. J. Free Radic. Biol. Med. 1, 87–95.

Gunshin, H., Mackenzie, B., Berger, U., 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388, 6264–6268.

Gutknecht, J., 1981. Inorganic mercury (Hg^{2+}) transport through lipid bilayer membranes. J. Membr. Biol. 61, 61–66.

Gutknecht, J., 1983. Cadmium and thallous ion permeabilities through lipid bilayer membranes. Biochim. Biophys. Acta 735, 185–188.

Gutteridge, J.M., Halliwell, B., 1990. The measurement and mechanism of lipid peroxidation in biological systems. Trends Biochem. Sci. 15, 129–135.

Gwoździński, K., 1995. Structural changes in erythrocyte components induced by copper and mercury. Radiat. Phys. Chem. 45, 877–882.

Hahne, H.C.H., Kroontje, W., 1973. Significance of pH and chloride concentration on behavior of heavy metal pollutants: mercury(II), cadmium(II), zinc(II), and lead (II). J. Environ. Qual. 2, 444.

Hallett, F.R., Watton, J., Krygsman, P., 1991. Vesicle sizing—number distributions by dynamic light scattering. Biophys. J. 59, 357–362.

Halliwell, B., Chirico, S., 1993. Lipid peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr. 57, 715S–724.

Hope, M.J., Bally, M.B., Webb, G., Cullis, P.R., 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane. Biochim. Biophys. Acta 812, 55–65.

Hotz, P., Buchet, J., Bernard, A., Lison, D., Lauwerys, R., 1999. Renal effects of low-level environmental cadmium exposure: 5-year follow-up of a subcohort from the Cadmibel study. Lancet 354, 1508–1513.

Hughes, W.L., 1957. A physicochemical rationale for the biological activity of mercury and its compounds. Ann. N. Y. Acad. Sci. 65, 454–460.

IARC, 1993. Beryllium, Cadmium, Mercury, and Exposures in the Glass Manufacturing Industry, vol. 58. Beryllium Cadmium Mercury and Exposures in the Glass Manufacturing Industry International Agency for Research on Cancer.

Järup, L., Berglund, M., Elinder, C., 1998. Health effects of cadmium exposure—a review of the literature and a risk estimate. Scand. J. Work. Environ. Health 24, 1–52.

Järup, L., 2003. Hazards of heavy metal contamination. Br. Med. Bull. 68, 167–182.

Jacobson, K., Mouritsen, O.G., Anderson, R.G.W., 2007. Lipid rafts: at a crossroad between cell biology and physics. Nat. Cell Biol. 9, 7–14.

James, T.L., 1975. Nuclear Magnetic Resonance in Biochemistry. Elsevier.

Kinsky, S., Haxby, J., Zopf, D., Alving, C., Kinsky, C., 1969a. Complement-dependent damage to liposomes prepared from pure lipids and Forssman hapten. Biochemistry 8, 4149–4158.

Kinsky, S., Haxby, J., Zopf, D., Alving, C., Kinsky, C., 1969b. Corrections-complement-dependent damage to liposomes prepared from pure lipids and forssman hapten. Biochemistry 9.

Kooijman, E.E., Burger, K.N.J., 2009. Biophysics and function of phosphatidic acid: a molecular perspective. Biochim. Biophys. Acta 1791, 881–888.

Kooijman, E.E., Carter, K.M., van Laar, E.G., Chupin, V., Burger, K.N.J., de Kruijff, B., 2005. What makes the bioactive lipids phosphatidic acid and lysophosphatidic acid so special? Biochemistry 44, 17007–17015.

Koynova, R., Caffrey, M., 1998. Phases and phase transitions of the phosphatidylcholines. Biochim. Biophys. Acta 1376, 91–145.

Le, M.T., Gailer, J., Prenner, E.J., 2009. Hg^{2+} and Cd^{2+} interact differently with biomimetic erythrocyte membranes. Biometals 22, 261–274.

Le, M.T., Hassanin, M., Mahadeo, M., Gailer, J., Prenner, E.J., 2013. Hg- and Cd-induced modulation of lipid packing and monolayer fluidity in biomimetic erythrocyte model systems. Chem. Phys. Lipids 170–171, 46–54.

Leidl, K., Liebisch, G., Richter, D., Schmitz, G., 2008. Mass spectrometric analysis of lipid species of human circulating blood cells. Biochim. Biophys. Acta 1781, 655–664.

Lentz, B.R., 1989. Membrane fluidity as detected by diphenylhexatriene probes. Chem. Phys. Lipids 50, 171–190.

Lentz, B.R., 1993. Use of fluorescent probes to monitor molecular order and motions within liposome bilayers. Chem. Phys. Lipids 64, 99–116.

Li, Y., Yan, X., Chen, C., 2007. Human serum albumin—mercurial species interactions. J. Proteome Res. 6, 2277–2286.

Lide, D.R., 2004. CRC Handbook of Chemistry and Physics, 85th ed. CRC Press Section 11.

Limaye, D.A., Shaikh, Z.A., 1999. Cytotoxicity of cadmium and characteristics of its transport in cardiomyocytes. Toxicol. Appl. Pharmacol. 154, 59–66.

Lis, W.T., Parsegian, V.A., 1981. Adsorption of divalent cations to a variety of phosphatidylcholine bilayers. Biochemistry 20, 1771–1777.

Lis, L.J., Kauffman, J.W., Shriver, D.F., 1975. Effect of ions on phospholipid layer structure as indicated by Raman spectroscopy. Biochim. Biophys. Acta 406, 453–464.

Le, Mary T., Jennifer Litzenberger, K., Elmar Prenner, J., 2011. Book Chapter Biomimetic Model Membrane Systems Serve as Increasingly Valuable in Vitro Tools. In: George, Anne (Ed.), Advances in Biomimetics. InTech ISBN 978-953-307-191-6.

London, E., 2002. Insights into lipid raft structure and formation from experiments in model membranes. Curr. Opin. Struct. Biol. 12, 480–486.

Lorscheider, F., Vimy, M., Summers, A., 1995. Mercury exposure from silver tooth fillings: emerging evidence questions a traditional dental paradigm. J. Fed. Am. Soc. Exp. Biol. 9, 504–508.

Lou, M., Garay, R., Alda, J., 1991. Cadmium uptake through the anion exchanger in human red blood cells. J. Physiol. 443, 123–136.

MacDonald, R.C., MacDonald, R.I., Menco, B., Takeshita, K., Subbarao, N.K., Hu, L., 1991. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. Biochim. Biophys. Acta Biophys. Acta 1061, 297–303.

Madden, E.F., Fowler, B.A., 2000. Mechanisms of nephrotoxicity from metal combinations: a review. Drug Chem. Toxicol. 23, 1–12.

McRill, C., Boyer, L.V., Flood, T.J., Ortega, L., 2000. Mercury toxicity due to use of a cosmetic cream. J. Occup. Environ. Med. 42, 4.

Munro, S., 2003. Lipid rafts: elusive or illusive? Cell 115, 377–388.

Nakada, S., Inoue, K., Nojima, S., Imura, N., 1978. Change in permeability of liposomes caused by methyl-mercury and inorganic mercury. Chem. Biol. Interact. 22, 15–23.

Notarachille, G., Arnesano, F., Calò, V., Meleleo, D., 2014. Heavy metals toxicity: effect of cadmium ions on amyloid beta protein 1–42: possible implications for Alzheimer's disease. BioMetals 27, 371–388.

Ohvo-Rekilä, H., Ramstedt, B., Leppimäki, P., Peter Slotte, J., 2002. Cholesterol interactions with phospholipids in membranes. Prog. Lipid Res. 41, 66–97.

Olbrich, K., Rawicz, W., Needham, D., Evans, E., 2000. Water permeability and mechanical strength of polyunsaturated lipid bilayers. Biophys. J. 79, 321–327.

Olmo, R., Blanco, M.D., Teijón, C., Miguel del Socorro, J., Teijón, J.M., 2002. Studies of cadmium binding to hexokinase: structural and functional implications. J. Inorg. Biochem. 89, 107–114.

Pacyna, J., 1996. Toxicology of Metals, vol. 1. CRC Lewis Publishers, New York.

Parasassi, T., Gratton, E., 1995. Membrane lipid domains and dynamics as detected by Laurdan fluorescence. J. Fluoresc. 5, 59–69.

Parasassi, T., Krasnowska, E.K., Bagatolli, L., Gratton, E., 1998. Laurdan and prodan as polarity-sensitive fluorescent membrane probes. J. Fluoresc. 8, 365–373.

Park, J., Zheng, W., 2012. Human exposure and health effects of inorganic and elemental mercury. J. Prev. Med. Public Health 45, 344–352.

Park, J.D., Cherrington, N.J., Klaassen, C.D., 2002. Intestinal absorption of cadmium is associated with divalent metal transporter 1 in rats. Toxicol. Sci. 68, 288–294.

Peters, J.L., Perlstein, T.S., Perry, M.J., McNeely, E., Weuve, J., 2010. Cadmium exposure in association with history of stroke and heart failure. Environ. Res. 110, 199–206.

Prenner, E., Honsek, G., Hönig, D., Möbius, D., Lohner, K., 2007. Imaging of the domain organization in sphingomyelin and phosphatidylcholine monolayers. Chem. Phys. Lipids 145, 106–118.

Prozialeck, W.C., Edwards, J.R., Woods, J.M., 2006. The vascular endothelium as a target of cadmium toxicity. Life Sci. 79, 1493–1506.

Rabenstein, D.L., Isab, A.A., 1982. A proton nuclear magnetic resonance study of the interaction of mercury with intact human erythrocytes. Biochim. Biophys. Acta 721, 4229–4232.

Rabenstein, D.L., Isab, A.A., Kadima, W., Mohanakrishnan, P., 1983. A proton nuclear magnetic resonance study of the interaction of cadmium with human erythrocytes. Biochim. Biophys. Acta 762, 531–541.

Rabenstein, D., 1989. Metal complexes of glutathione and their biological significance. Glutathione Chem. Biochem. Med. Asp. 3, 147–186.

Rajan, S., Kang, S., Gutowsky, H.S., Oldfieldf, E., 1981. Phosphorus nuclear magnetic resonance study of membrane structure. J. Biol. Chem. 256, 1160–1166.

Repetto, M., Semprine, J., Boveris, A., 1985. Lipid peroxidation: chemical mechanism, biological implications and analytical determination. J. Free Radical Biol. Med. 1, 3–30.

Rinia, H.A., Snel, M.M.E., van der Eerden, J.P.J.M., de Kruijff, B., 2001. Visualizing detergent resistant domains in model membranes with atomic force microscopy. FEBS Lett. 501, 92–96.

Sallsten, G., Thoren, J., BarregArd, L., Schutz, A., Skarping, G., 1996. Long-term use of nicotine chewing gum and mercury exposure from dental amalgam fillings. J. Dent. Res. 75, 594–598.

Santillan-Medrano, J., Jurinak, J., 1975. The chemistry of lead and cadmium in soil: solid phase formation. Soil Sci. Soc. Am. J. 39, 851–856.

Satarug, S., Garrett, S.H., Sens, M.A., Sens, D.A., 2010. Cadmium, environmental exposure, and health outcomes. Environ. Health Perspect. 118, 228–269.

Savage, D.F., Stroud, R.M., 2007. Structural basis of aquaporin inhibition by mercury. J. Mol. Biol. 368, 607–617.

Schwartz, G., Reis, I., 2000. Is cadmium a cause of human pancreatic cancer? Cancer Epidemiol Biomarkers Prev. 9, 139–145.

Seelig, J., 1978. ^{31}P nuclear magnetic resonance and the head group structure of phospholipids in membranes. Biochim. Biophys. Acta 515, 105–140.

Sheetz, M.P., Singer, S.J., 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. Proc. Natl. Acad. Sci. U. S. A. 71, 4457–4461.

Shinada, M., Muto, H., Takizawa, Y., 1991. In vitro interaction of selected phospholipid species with mercuric chloride using Fourier transform H-NMR. Bull. Environ. Contam. Toxicol. 47, 350–354.

Simons, K., Ehehalt, R., 2002. Cholesterol, lipid rafts, and disease. J. Clin. Invest. 110, 597–603.

Simons, K., Gerl, M.J., 2010. Revitalizing membrane rafts: new tools and insights. Nat. Rev. Mol. Cell Biol. 11, 688–699.

Simons, K., Toomre, D., 2000. Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. 1, 31–39.

Sin, K.W., Tsang, H.F., 2003. Large-scale mercury exposure due to a cream cosmetic: community-wide case series. Hong Kong Med. J. 9, 329–334.

Sklar, L.A., Miljanich, G.P., Dratz, E.A., 1979. Phospholipid lateral phase separation and the partition of cis-parinaric acid and trans-parinaric acid among aqueous, solid lipid, and fluid lipid phases. Biochemistry 18, 1707–1716.

Skurnik, D., Ruimy, R., Ready, D., Ruppe, E., Bernède-Bauduin, C., Djossou, F., Guillemot, D., Pier, G.B., Andremont, A., 2010. Is exposure to mercury a driving force for the carriage of antibiotic resistance genes? J. Med. Microbiol. 59, 804–807.

Sorensen, E.M., Acosta, D., Nealon, D.G., 1985. Effects of cadmium and calcium on the fluidity of plasma membranes. Toxicol. Lett. 25, 319–326.

Stohs, S., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radical Biol. Med. 18, 321–336.

Summers, A., Wireman, J.O.Y., Vimy, M.J., Lorscheider, F.L., Marshall, B., Levy, S.B., Bennett, S.A.M., Billard, L., 1993. Mercury released from dental silver fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. Antimicrob. Agents Chemother. 37, 825–834.

Summers, A.O., 2002. Generally overlooked fundamentals of bacterial genetics and ecology. Clin. Infect. Dis. 34, S85–S92.

Sundaralingam, M., Jensen, L.H., 1965. Crystal and molecular structure of a phospholipid component: -agr-glycerophosphorylcholine gadmium ghloride trihydrate. Science 150, 1035–1036.

Suwalsky, M., Ungerer, B., Villena, F., Cuevas, F., Sotomayor, C., 2000. HgCl$_2$ disrupts the structure of the human erythrocyte membrane and model phospholipid bilayers. J. Inorg. Biochem. 81, 267–273.

Suwalsky, M., Villena, F., Norris, B., Cuevas, F., Sotomayor, C.P., 2004. Cadmium-induced changes in the membrane of human erythrocytes and molecular models. J. Inorg. Biochem. 98, 1061–1066.

Tchounwou, P.B., Ayensu, W.K., Ninashvili, N., Sutton, D., 2003. Environmental exposure to mercury and its toxicopathologic implications for public health. Environ. Toxicol. 18, 149–175.

Trisak, S., Doumgdee, P., Rode, B., 1990. Binding of zinc and cadmium to human serum albumin. Int. J. Biochem. 22, 977–981.

Tristram-Nagle, S., Kim, D.J., Akhunzada, N., Kucerka, N., Mathai, J.C., Katsaras, J., Zeidel, M., Nagle, J.F., 2010. Structure and water permeability of fully hydrated diphytanoylPC. Chem. Phys. Lipids 163, 630–637.

Tsuzuki, Y., Yamada, T., 1979. INhibitory actions of mercury compounds against glucose-6-phosphate dehydrogenase from yeast. J. Toxicol. Sci. 4, 105–113.

Valko, M., Morris, H., Cronin, M., 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem. 12, 1161–1208.

Vincent, P., Blackburn, C., 1958. The effects of heavy metal ions on the human erythrocyte. Aust. J. Exp. Biol. Med. Sci. 36, 471–478.

Virtanen, J.K., Rissanen, T.H., Voutilainen, S., Tuomainen, T.-P., 2007. Mercury as a risk factor for cardiovascular diseases. J. Nutr. Biochem. 18, 75–85.

Walter, a, Gutknecht, J., 1986. Permeability of small nonelectrolytes through lipid bilayer membranes. J. Membr. Biol. 90, 207–217.

Webb, M.S., Steponkus, P.L., 1993. Dehydration-induced lamellar-to-hexagonal-II phase transitions in DOPE/DOPC mixtures. Biochim. Biophys. Acta 1145, 93–104.

Weed, R., Eber, J., Rothstein, A., 1962. Interaction of mercury with human erythrocytes. J. Gen. Physiol. 45, 395–410.

Wells, A.F., 2012. Structural Inorganic Chemistry. Oxford University Press.

World Health Organization, 1991. Inorganic Mercury, in Environmental Health Criteria, vol. 118. World Health Organization, Geneva.

World Health Organization, 1992. Cadmium, in Environmental Health Criteria, vol. 134. World Health Organization, Geneva.

Yabuuchi, H., O'Brien, J.S., 1968. Positional distribution of fatty acids in glycerophosphatides of bovine gray matter. J. Lipid Res. 9, 65–67.

Yawata, Y., 2003. Cell Membrane: The Red Blood Cell as a Model. Wiley-VCH.

Zachowski, A., 1993. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem. J. 294, 1–14.

Zalups, R.K., 2000. Molecular interactions with mercury in the kidney. Pharmacol. Rev. 52, 113–143.