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Hg^{2+} and Cd^{2+} interact differently with biomimetic erythrocyte membranes

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Abstract In order to characterize the potentially deleterious effects of toxic Hg^{2+} and Cd^{2+} on lipid membranes, we have studied their binding to liposomes whose composition mimicked erythrocyte membranes. Fluorescence spectroscopy utilizing the concentration dependent quenching of Phen Green™ SK by Hg^{2+} and Cd^{2+} was found to be a sensitive tool to probe these interactions at metal concentrations $\leq$1 μM. We have systematically developed a metal binding affinity assay to screen for the interactions of Hg^{2+} or Cd^{2+} with certain lipid classes. A biomimetic liposome system was developed that contained four major lipid classes of erythrocyte membranes (zwitterionic lipids: phosphatidylcholine and phosphatidylethanolamine; negatively charged: phosphatidylserine and neutral: cholesterol). In contrast to Hg^{2+}, which preferentially bound to the negatively charged phosphatidylserine compared to the zwitterionic components, Cd^{2+} bound stronger to the two zwitterionic lipids. Thus, the observed distinct differences in the binding affinity of Hg^{2+} and Cd^{2+} for certain lipid classes together with their known effects on membrane properties represent an important first step toward a better understanding the role of these interactions in the chronic toxicity of these metals.

Keywords Toxic metals · Hg^{2+} · Cd^{2+} · Liposomes · Model systems · Membranes · Lipids · Erythrocytes · Fluorescence spectroscopy

Abbreviations

POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPE	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
POPS	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
Chol	Cholesterol
PGSK	Phen Green™ SK

Introduction

Toxic metals, such as Hg and Cd, are natural constituents of the earth's crust. Owing to the sun-driven hydrological cycle in conjunction with abiotic and biotic chemical weathering reactions at the surface of the earth, these and other toxic metals

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have been continuously mobilized from rocks and minerals and subsequently exchanged between various environmental compartments for millions of years (Gailer 2007). Consequently, all living organisms have been naturally exposed to background levels of Hg^{2+} and Cd^{2+}—mostly via food and drinking water—ever since life first evolved. The industrial revolution, however, represents the starting point for the ongoing large scale mobilization of additional amounts of these environmentally persistent pollutants from the earth's crust into the global environment (Gailer 2007). Therefore, the general population and particularly children are today unwittingly exposed to higher daily doses of Hg^{2+} and Cd^{2+} than ever before (Clarkson et al. 2003; Counter and Buchanan 2004; Gailer 2007). This, in turn, is of considerable public concern since the chronic exposure of humans to comparatively low doses (μg/day range) of Cd^{2+} is associated with cancer (Huff et al. 2007). Conversely, the chronic exposure of humans to Hg^{2+} is associated with cardiovascular disease (Kostka 1991; Virtanen et al. 2007) and in vitro studies suggest that this metal ion is immunotoxic to human T-cells at concentrations in the nanogram range (Shenker et al. 1992). Since the molecular basis for the chronic toxicity of these metals is not well understood (Gailer 2007), studies that aim to better characterize the interaction of Hg^{2+} or Cd^{2+} with potential in vivo molecular targets are needed in order to unravel all biochemical mechanisms that are involved in their toxic effects at the organ level. To this end, the vast majority of studies that have been conducted so far have focused on the individual interaction of each metal with plasma proteins (e.g., human serum albumin) (Lau and Sarkar 1979), transmembrane proteins in erythrocytes (e.g., the hexose transport protein) (Vansteveninck et al. 1965), intracellular sulfhydryl compounds (e.g., L-cysteine, L-glutathione (Rabenstein 1989) and proteins) and—more recently—reactive selenium metabolites (Gailer 2002). It has been known for a long time, however, that small concentrations of toxic metals can also produce appreciable changes of surface tension and surface charge in phospholipid bilayers (Passow et al. 1961) and can increase the permeability of liposomes (Nakada et al. 1978). Furthermore, Cd^{2+} and Hg^{2+} were found to change the membrane fluidity of hepatic microsomal membranes which is important since even minor changes can have pathological implications (Garcia et al. 2005). Moreover, Zn^{2+} has been demonstrated to bind and to reduce the fluidity of carp red blood cells without significantly affecting Na,K-ATPase activity (Akahori et al. 1999). Cu^{2+} binding was reported to induce membrane ordering thus affecting the fluidity but in addition a potential impact on the electric field was proposed and both effects could impact membrane proteins, in particular ion channels (Suwalski et al. 1998). Thus, the cell membrane itself must be considered as an important target for toxic heavy metals (see also Foulkes 1996). In addition, it has been shown that positively charged metal ions bind to lipids in red blood cells by extracting ^{45}Ca-labeled ghost membranes with organic solvents which resulted in 79% Ca bound to protein, 5% in the aqueous phase and 16% were recovered bound to lipids (Forstner and Manery 1971). Hence, the present study was aimed at the characterization of the interaction of Hg^{2+} and Cd^{2+} with biomimetic erythrocyte membrane models (Fig. 1).

The biological membrane of the mammalian erythrocyte is composed of five major lipid classes which include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), and cholesterol (Fig. 2) (Zachowski 1993). These amphipathic lipids are distributed asymmetrically in the inner and outer leaflet of the membrane (Zachowski 1993). At physiological pH, PC, PE, and SM are overall neutrally

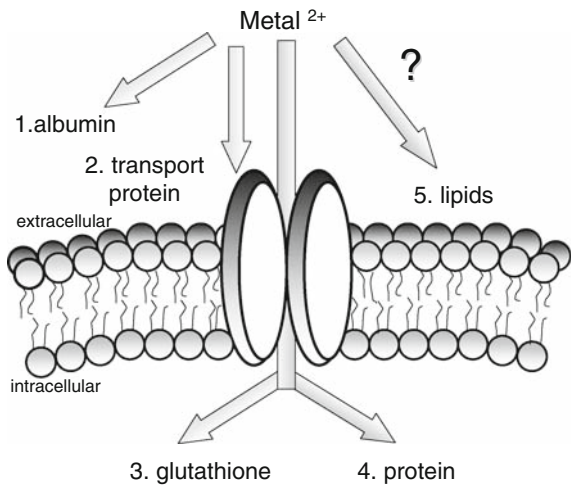

Fig. 1 Conceptual overview of potential toxic metal ligands/targets that could be involved in their chronic toxicity

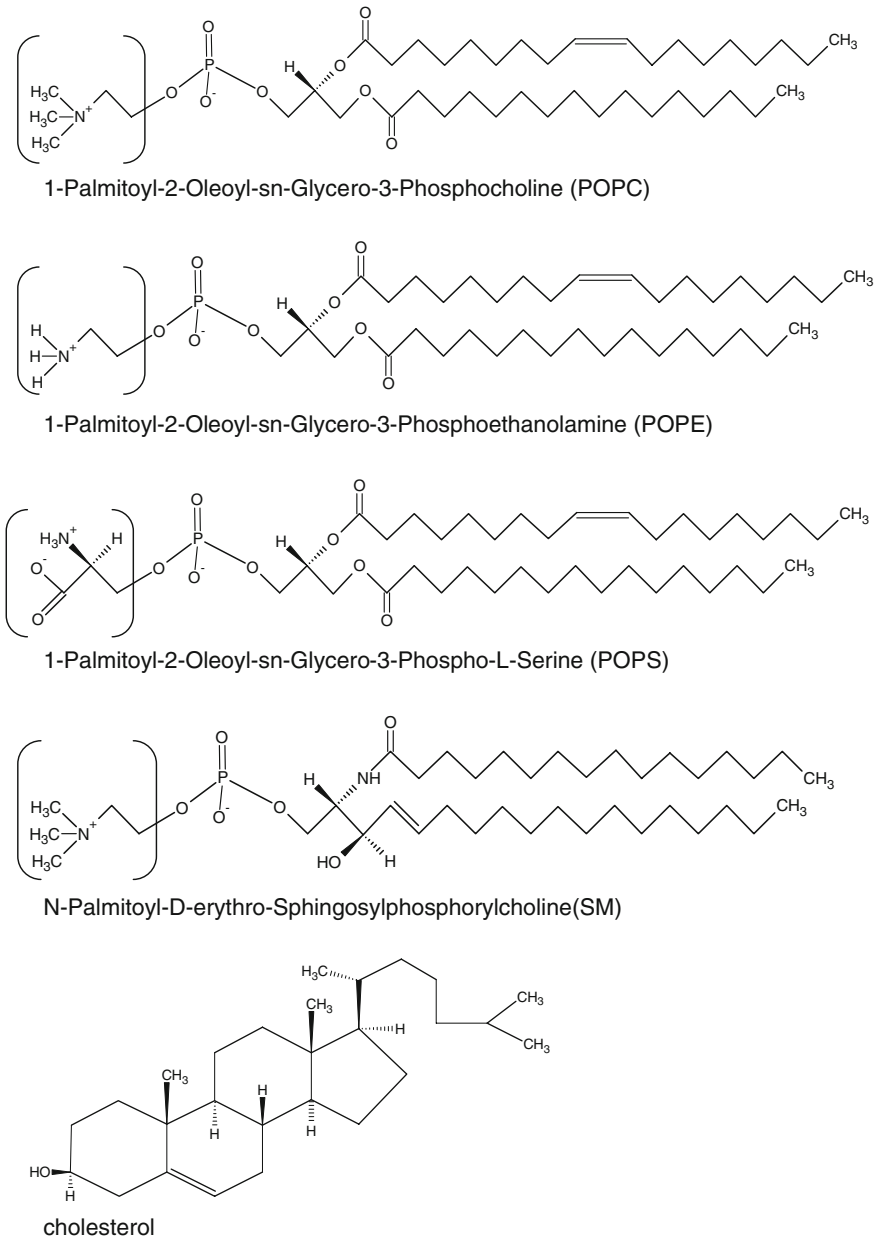

Fig. 2 Different lipid classes known to be present in human erythrocyte membranes. The headgroup region is indicated by the brackets (). Negatively charged: POPS; zwitterionic: POPC, POPE and SM; uncharged: chol

charged zwitterions, whereas PS is negatively charged and cholesterol is uncharged altogether.

With regard to Hg^{2+}, this metal ion has been demonstrated to increase the glucose permeability of egg PC/diacetyl phosphate/cholesterol liposomes (Nakada et al. 1978) and to disrupt the structure of model phospholipid bilayer membranes and erythrocytes at metal concentrations of 10 μM and 1 mM as evidenced by X-ray diffraction studies (Suwalsky et al. 2000). More recently, it was observed that Hg^{2+} concentrations as low as 1 μM stimulated the exposure of PS at the human erythrocyte cell surface and induced cell apoptosis (Eisele et al. 2006). Cd^{2+}, on the other hand, has been demonstrated by X-ray diffraction to interact with dipalmitoylphosphatidylcholine bilayers (Lis et al. 1981) and liposomes

containing PS (Bevan et al. 1983; Deleers et al. 1986). Moreover, Cd^{2+} has been demonstrated to decrease the fluidity of lipid headgroups and acyl chain regions in both human erythrocytes and dimyristoylphosphatidylcholine liposomes (Suwalsky et al. 2004), which was rationalized in terms of the electrostatic binding of Cd^{2+} to the phosphate groups of the lipids (Girault et al. 1998). Metal–lipid interactions have also been investigated by NMR spectroscopy and provided valuable insight into the binding of these metals to lipid headgroups (Delnomdedieu et al. 1992; Girault et al. 1996, 1998). In addition, it could be shown by X-ray diffraction that $HgCl_2$ binds to the lipid head group region in sciatic nerves (Kirschner and Ganser 1982). Both approaches, however, inherently require higher concentrations of lipids and metals (mM range).

In order to study metal–lipid binding to better understand the molecular toxicology of Hg^{2+} and Cd^{2+} in mammals, we used model liposomes which mimiced the erythrocyte membrane composition. Fluorescence spectroscopy was utilized to investigate the binding of Hg^{2+} and Cd^{2+} to lipid model systems because this highly sensitive technique allows for the analysis of analytes at nanomolar concentrations (Lakowicz 1999) and thus metal–lipid interactions could be probed at more toxicologically relevant metal concentrations (μM range). For this purpose we used the heavy-metal responsive dye PGSK which in aqueous solution is quenched by Hg^{2+} and Cd^{2+} in a concentration dependent manner. Based on this approach, we established a protocol that allowed us to perform such quenching experiments in the presence of biomimetic liposomes that was ultimately used to establish metal binding affinities to biological membranes. Hg^{2+} and Cd^{2+} were demonstrated to exhibit significantly different binding to different phospholipid headgroups.

Materials and methods

Chemicals

1-Palmitoyl-2-oleoyl-*sn*-Glycero-3-phosphocholine (POPC); 1-palmitoyl-2-oleoyl-*sn*-Glycero-3-phosphoethanolamine (POPE); 1-palmitoyl-2-oleoyl-*sn*-Glycero-3-[phospho-L-serine] (POPS); and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). $HgCl_2$ and $CdCl_2$ were purchased from Sigma–Aldrich (Oakville, ON). The Phen Green™ SK (PGSK) dye consists of a transition metal chelator (phenanthroline) and an attached fluorophore (fluorescein) and was purchased from Invitrogen (Burlington, ON). Since $HgCl_2$ has been previously shown to interact with organic buffers like Tris and HEPES (Delnomdedieu et al. 1992) which we confirmed in preliminary experiments, a 20 mM phosphate buffer was used throughout this study. At the $CdCl_2$ concentration employed in this study the quenching of the dye by Cd^{2+} resulted in a linear Stern–Volmer plot. All solutions (liposomes, metal, and the PGSK dye) were prepared in 20 mM phosphate buffer at pH 7.4.

Liposome preparation

Five liposome preparations were screened for interactions with Hg^{2+} and Cd^{2+}: 100% POPC; 85% POPC–15% POPE; 85% POPC–15% POPS; 85% POPC–15% cholesterol; and 35% POPC–35% cholesterol–15% POPE–15% POPS. The majority of the outer erythrocyte leaflet is composed of PC (65–75%), whereas only a small percentage of PE and PS are present (Zachowski 1993). When the dried lipid films are hydrated to form liposomes, the lipids in all mixtures (e.g., 85% PC–15% PE or 85% PC–15% PS) will redistribute between both leaflets, and thus the lipid concentration of the minor components (PE, PS) on the outside of the resulting liposomes was a good mimic of the actual PE and PS concentrations in the outer erythrocyte membrane leaflet. Furthermore, cholesterol was also incorporated into the model systems as it is known to be an integral constituent of both the outer and inner leaflet of erythrocyte membranes, making up ~40% of the total lipid content (Block 1985). The liposomes were prepared using the ethanol injection method (Kremer et al. 1977). In brief, lipids (3.0 mg) were dissolved in 2.0 ml chloroform/methanol (v/v = 2:1). Aliquots of the lipid solution were dried down with argon and left in the vacuum overnight to remove remaining solvent. Twenty-five microliters of ethanol were then added to dissolve the lipids in each aliquot and the obtained solution was then injected into 5.0 ml phosphate buffer and was stirred for one hour using a magnetic

stirrer (300 rpm). This method is known to produce small unilamellar liposomes (Kremer et al. 1977).

Liposome characterization

Dynamic light scattering is also known as Photon Correlation Spectroscopy and can be used to determine the size of particles to ensure that a homogeneous distribution of liposomes is obtained (Berne 2000). The hydrodynamic radii of the small unilamellar liposomes were determined using a DynaPro Titan MicroSampler, a dynamic light scattering system (Wyatt Technologies Co., Santa Barbara, CA). Because of signal detector overload, all measurements were carried out with 10% laser power. We calculated the number of available liposomes in the systems, which allowed us to define the actual ratios between metal, liposomes and the PGSK dye. Using the hydrodynamic radii (derived from light scattering), the surface area of a sphere was calculated. Assuming that the average lipid headgroup is ~ 75 Å^2, the number of lipids/liposome was calculated. Lipids on the inner leaflet were not considered as binding sites and thus we divided the lipid concentration by two to obtain the number of liposomes in the cuvette to calculate the ratios of liposomes, metals and the PGSK dye (see Table 2).

Experimental

In order to develop a protocol for the study of metal–lipid interactions using the PGSK dye, it was necessary to characterize the mutual interactions of all components in the system, namely liposomes, metal and dye. Initially the respective binary interactions (Fig. 3a, b) were studied and subsequently extended to the ternary system (Fig. 3c–e). These investigations revealed that the order of addition of these components impacted the results.

All measurements were carried out in a 1 cm pathlength quartz cuvette at room temperature using a fiber-optic fluorimeter (Ocean Optics, Dunedin, FL) with a blue LED which excites at a wavelength centered at 450 nm. The final concentrations of the PGSK dye, liposomes, and metal were 2.0, 100, and 1.0 μM, respectively. The data presented are mean values and the error bars represent the standard deviation of three

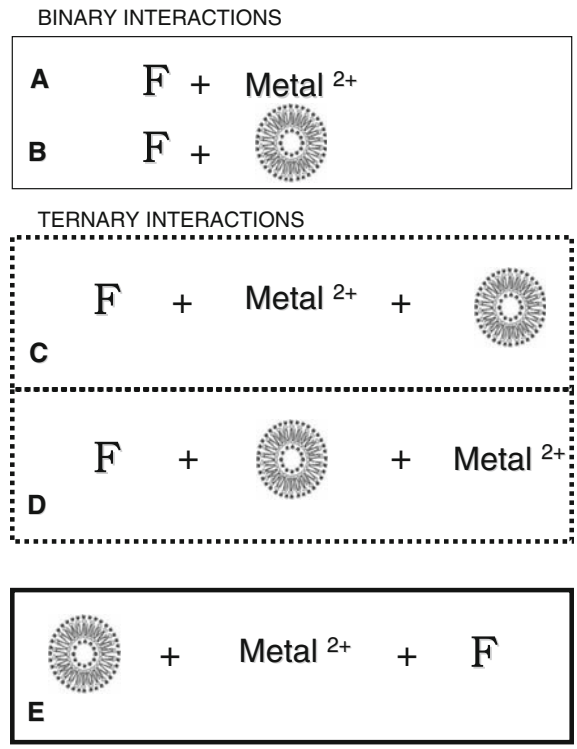

Fig. 3 Overview of interactions between PGSK dye, metals and liposomes that were investigated to develop the final metal binding assay to membranes F = PGSK dye; Metal^{2+} = Hg^{2+} or Cd^{2+} = liposomes (the order in the cartoon from the left to the right represents the sequence of additions)

experiments. Based on these systematic investigations, a final assay was developed.

Quenching of PGSK dye by Hg^{2+} or Cd^{2+}

Since the emission wavelength of the dye did not change upon the addition of metal, the intensity at the emission maximum was used. The total volume in the cuvette was 1.4 ml, which contained 2.0 μM PGSK dye and 0.00, 0.25, 0.50, 0.75, and 1.00 μM of Hg^{2+} or Cd^{2+}. The PGSK dye was incubated with the metal for 10 min (Fig. 3a; section "Liposome preparation"). In order to quantitate the quenching effect, the decrease in fluorescence intensity due to the addition of toxic metal was analyzed by the Stern–Volmer equation:

$$F_o/F_q = 1 + K_{SV}[Q] \quad (1)$$

where F_o and F_q correspond to the observed fluorescence in the absence and the presence of a quencher.

K_{SV} is the Stern–Volmer quenching constant, and [Q] is the quencher concentration (Lakowicz 1999).

Interaction of PGSK dye with liposomes

Two micromoles of the PGSK dye were mixed with 100 μM of liposomes in a cuvette (Fig. 3b). The fluorescence intensity of the dye was monitored after a 10-min incubation time with the different liposome models (see "Liposome preparation" section).

PGSK dye quenching by metals in presence of liposomes

The effect of liposomes on metal–dye interactions was determined using the Carey Eclipse Fluorescence Spectrophotometer (Varian Inc., Palo Alto, CA) because this instrument allowed for the recording of kinetic data. Excitation of the PGSK dye at 506 nm (slit widths 5.0 nm) resulted in a fluorescence emission maximum at 532 nm.

Since the major component in all investigated membrane models was POPC, this lipid was used in this control experiment (see Fig. 3b). The cuvette contained 100 μM liposomes and 2.0 μM of the PGSK dye (total volume: 2.1 ml) to which Hg^{2+} or Cd^{2+} was added in aliquots of 0.53 μl until a final metal concentration of 1.0 μM was achieved. In total, there was less than 1.0% sample dilution. Thus, no further corrections of the fluorescence intensity were performed.

Once it was established that an incubation time of 10 min was sufficient for binding of these metal to the PGSK dye, all further experiments were carried out with this time interval using the fiber-optic fluorimeter.

Finally, the impact of the sequence of addition of the three components, namely metal, liposomes, and the PGSK dye was investigated. The final concentrations of PGSK dye, liposomes, and metal were 2.0, 100, and 1.0 μM, respectively. At first dye and metal were incubated before the model membranes were added (Fig. 3c). Subsequently, the PGSK dye was incubated with liposomes to check for changes in emission wavelength or intensity before the metal was added (Fig. 3d). Finally, the affinity of each metal for the liposomes was investigated by initial mutual incubation before the dye was added (Fig. 3e). The latter protocol was then used to study the metal binding affinity to all model membrane systems in this study.

Results

Liposomes consisting of hydrated lipid bilayers are ideal model systems to investigate the interaction of metals with biological membranes and were prepared using the ethanol injection method. Dynamic light scattering showed that the prepared liposomes were uniform in size (109 ± 8 nm) (Table 1) and thus any impact of size and curvature on the later observed binding affinities could be excluded. Nevertheless, minor changes in particle diameter resulted in somewhat different overall surface areas of the liposome preparations and changed the ratios of metal to dye and liposomes as indicated in Table 2. However, if one considers the total surface area of these particles (3.8×10^6 Å^2), it is obvious that all bound dye and/or metal ions only cover a small fraction of the overall surface area and thus these differences in ratios will not impact the overall observations. On average there were ~2,050 PGSK dye molecules/liposome and ~1,020 metal ions/liposome (Table 2).

Quenching of PGSK dye by Hg^{2+}or Cd^{2+}

The fluorescence intensity maximum at 532 nm of the unquenched dye in buffer remained stable for at least an hour. In order to quantify the quenching effect of Hg^{2+} and Cd^{2+} on the PGSK dye, the fluorescence emission spectrum was recorded in the absence and presence of each metal. PGSK dye–metal interactions were characterized by using

Table 1 Hydrodynamic radii of the different liposome preparations

Lipid mixture	Hydrodynamic radii (nm)
100% POPC	50.5
85% POPC–15% POPE	51.1
85% POPC–15% POPS	58.8
85% POPC–15% cholesterol	55.3
35% POPC–35% cholesterol–15% POPE–15% POPS	57.7

Table 2 Estimated ratios of metal, dye, and liposomes

Lipid mixture	PGSK dye/liposome	Metal/liposome	PGSK dye/metal
100% POPC	1,709	855	2 to 1
85% POPC–15% POPE	1,747	873	2 to 1
85% POPC–15% POPS	2,515	1,257	2 to 1
85% POPC–15% cholesterol	2,043	1,022	2 to 1
35% PC–35% cholesterol–15% PE–15% PS	2,243	1,121	2 to 1

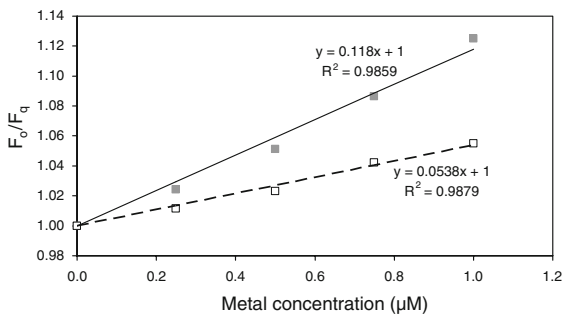

Fig. 4 Stern–Volmer plot for the quenching of 2.0 μM PGSK dye Hg^{2+} (■) and Cd^{2+} (□)

Stern–Volmer plots of F_o/F_q vs. [Q]. The fluorescence intensities at the emission maximum wavelength were used to plot the ratio of the initial fluorescence, F_o (PGSK only), to the fluorescence measured after the metal addition, F_q, with increasing metal concentrations. The fluorescence quenching ratio increased in proportion to the concentration of Hg^{2+} and Cd^{2+} in the buffer. Hg^{2+} was a twofold more effective quencher than Cd^{2+} (Fig. 4).

Interaction of PGSK dye with liposomes

To characterize the impact of model membranes on the fluorescence emission of the PGSK dye (2.0 μM), 100 μM POPC liposomes were added. The fluorescence intensity decreased slightly to 99.7% of the initial value (Fig. 5). In contrast, the other liposome systems affected the PGSK dye in a more pronounced fashion and decreased the fluorescence intensity in the following order: PE (−7.7%) > cholesterol (−5.1%) > PC/PE/PS/cholesterol (−4.3%) > PS (−2.9%). Thus the PGSK dye emission is impacted differently by the various lipid systems and this needs to be taken into account for the development of a suitable assay to study metal–lipid interactions.

PGSK dye quenching by metals in the presence of liposomes

In order to develop an assay to study the binding of metal ions to lipid membranes it was necessary to determine the extent of metal quenching in the presence of lipid model systems (Fig. 3c). Thus, we

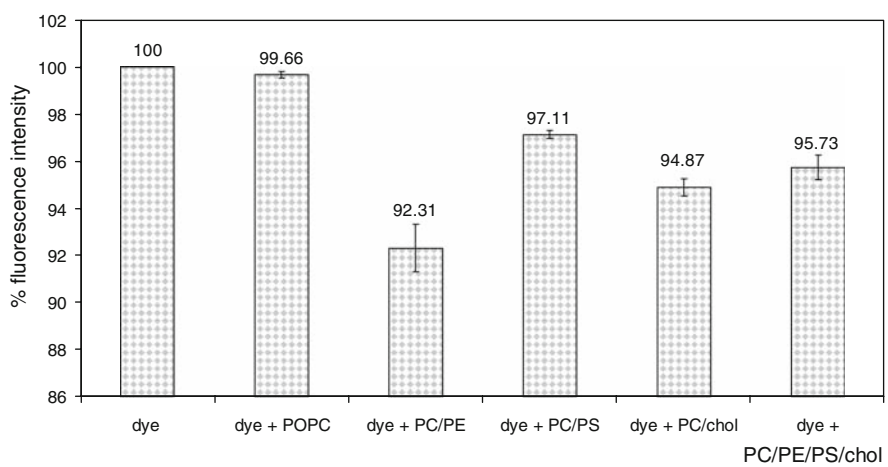

Fig. 5 Fluorescence quenching of 2.0 μM PGSK by 100 μM of the investigated liposome models

investigated the quenching of the PGSK dye after incubation with liposomes (100% POPC) for 10 min. After the consecutive addition of metal aliquots (Fig. 6, additions indicated by arrows) an initial rapid decrease in fluorescence intensity was observed and the resulting reading remained constant over a 10-min period. This indicates rapid metal binding to the PGSK dye and—more importantly—demonstrates the feasibility to observe metal quenching in the presence of liposomes. Even though the extent of dye quenching by Cd^{2+} was somewhat less pronounced, it showed the same general behavior as Hg^{2+} (Fig. 6).

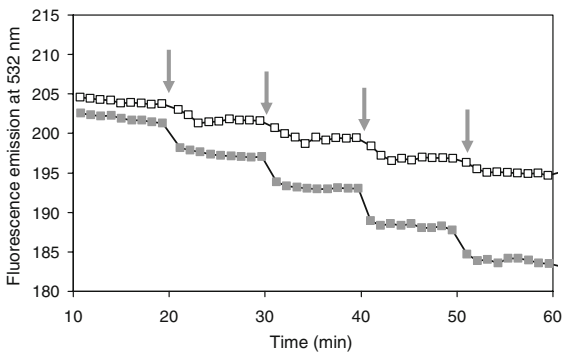

Fig. 6 Fluorescence quenching of 2.0 μM PGSK in the presence of 100 μM POPC liposomes by Hg^{2+} (■) and Cd^{2+} (□). Arrows indicate each addition of metal aliquots (final metal concentrations: 0.25, 0.50, 0.75, and 1.00 μM)

In order to quantify the extent of metal quenching in the ternary mixture, it was necessary to characterize the impact of POPC liposomes on the interaction of the dye with the metal. Thus, we incubated 1.0 μM metal with 2.0 μM PGSK dye for 10 min (Fig. 7A) before liposomes were added and incubated for another 10 min (Fig. 7B). When we compared the metal quenching in the presence of POPC model systems to a solution without, differences in the overall quenching effect were observed. In solution, 1 μM Hg^{2+} reduced the PGSK fluorescence to 88.9% (Fig. 7A) versus 92.6% in the presence of POPC membranes (Fig. 7B). Based on these data and their respective error bars, the observed difference between Hg^{2+} quenching in the presence or absence of POPC liposomes was not very pronounced, but should not be completely neglected either. In contrast, Cd^{2+} did not reveal a significant difference in quenching for both experiments (Fig. 7A, B). In order to detect a potential impact of the sequence of how the three components were added, the PGSK dye and liposomes were incubated first before the metals were added (see scheme in Fig. 3d). As seen in Fig. 7C, the overall quenching was significantly reduced for Hg^{2+} (99.7% vs. 92.6% in Fig. 7B) as the PGSK dye intensity was increased. Again, a less dramatic change for Cd^{2+} was evident. The other membrane systems showed less impact for Hg^{2+} and Cd^{2+}

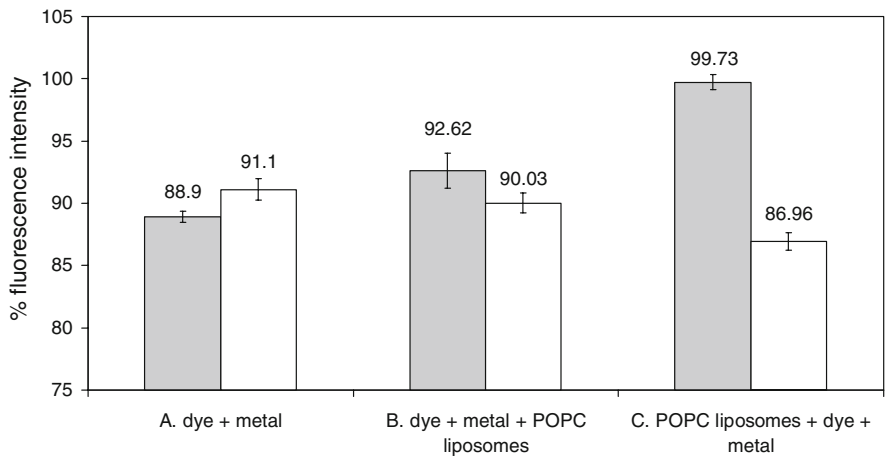

Fig. 7 Effect of the presence of liposomes and sequence addition on metal-induced dye quenching; (**a**) Fluorescence intensity after 10-min incubation of 2.0 μM PGSK dye and 1.0 μM of Hg^{2+} (■) and Cd^{2+} (□); (**b**) Fluorescence intensity after a 10-min incubation of 2.0 μM PGSK, 1.0 μM of Hg^{2+} (■) and Cd^{2+} (□), and 100 μM POPC liposomes; (**c**) Fluorescence intensity after 10-min incubation of 100 μM POPC liposomes, 2.0 μM PGSK, and 1.0 μM of Hg^{2+} (■) and Cd^{2+} (□)

binding (data not shown), but it was clearly evident that the sequence of addition affected the results.

Comparing the larger surface area of the liposomes (see Table 2) to the size of the metal, ~2 million Cd^{2+} ions would be necessary to saturate the surface of one POPC model systems, whereas the experimentally used metal concentrations resulted in ~850 metal ions/liposome. Since the PGSK dye concentration was twice that of the respective metal, it is evident that large parts of the liposome surface were not affected by metal or dye binding. On the other hand, the impact of the lipid composition of each membrane system (see Fig. 5) on the overall quenching of PGSK dye by metals had to be considered. In order to allow a comparison of metal quenching for the individual lipid model membrane systems we normalized the quenching data to 100% fluorescence intensity for the PGSK dye bound to the membrane blank prior to metal quenching (using values from Fig. 5).

To establish the sequence of addition for the final assay, the following rationale was applied. Conceptually, in a biological setting, metals such as Hg^{2+} and Cd^{2+} will bind to a membrane target. Once equilibrium is reached, an analytical probe (e.g. a fluorescent dye) can be added to determine the extent of binding. Since the control experiments allowed us to normalize and quantify our data, the following protocol was used to assess the metal affinity for liposomes in general and certain lipid classes in particular: various membrane systems were incubated with metals for 10 min before the dye was added for another 10-min incubation.

Different lipid affinity of Hg^{2+} and Cd^{2+}

Since these metals have been previously demonstrated to exhibit an affinity for a variety of lipids (Delnomdedieu et al. 1992; Girault et al. 1996, 1998), and this binding equilibrium is established fast (Fig. 6), only the remaining fraction of the metal ions in solution will be available for fluorophore quenching. The difference between the quenching of the PGSK dye by these ions in solution and the observed quenching after the incubation of liposomes with the metals was used as a measure for the relative binding affinity of Hg^{2+} and Cd^{2+} for the various lipid classes. Any reduction in quenching in the presence of model systems indicated an interaction

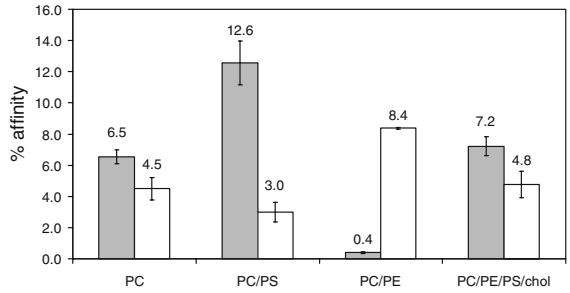

Fig. 8 Different binding affinity of Hg^{2+} (■) and Cd^{2+} (□) to various biomimetic liposomes (%) using the optimized binding protocol. Liposome composition: PC = 100%POPC; PC/PS = 85% PC/15% PS; PC/PE = 85% PC/15% PE; PC/PE/PS/chol = 35% PC/35% chol/15% PS/15% PE

with lipids. Since Hg^{2+} is a stronger quencher for the PGSK dye compared to Cd^{2+} (Fig. 4), we used the respective correction factor and multiplied the Cd^{2+} data in section "Liposome preparation" to allow direct comparison of the results on the same scale. In order to illustrate the metal–lipid affinity, we plotted the difference between metal quenching in solution to quenching after the metal–liposome incubation, which is a measure of metal affinity for the lipids. Less quenching means more interactions of the metals with lipids and vice versa. In Fig. 8, these differences are quantified and plotted as relative binding affinity of Hg^{2+} and Cd^{2+} for the various liposome systems. Hg^{2+} was found to preferentially bind to: PS (12.6%) > 35% PC–35% cholesterol–15% PS–15% PE (7.2%) > PC (6.5%) ≫ PE (0.4%) ⋙ cholesterol (0%), whereas Cd^{2+} preferentially bound to: PE (8.4%) > 35% PC–35% cholesterol–15% PS–15% PE (4.8%) > PC (4.5%) > PS (3.0%) ⋙ cholesterol (0%). Hence, Hg^{2+} was four-times more effective to bind to PS than Cd^{2+}. There was also a 21-fold higher affinity of Cd^{2+} over Hg^{2+} to bind to PE. Neither Hg^{2+} nor Cd^{2+} bound to the cholesterol-containing liposome system at all (data not included in Fig. 8).

Discussion

The chronic exposure of certain populations to increased dietary levels of toxic metals is of much public concern. Despite extensive research into the toxicology of Hg^{2+} and Cd^{2+}, the molecular mechanism(s) of their chronic toxicity remain elusive.

After absorption from the gastrointestinal tract into the bloodstream, one of the first cell types that these toxic metals will encounter in vivo are erythrocytes. Hence, one feasible mechanism of chronic metal toxicity is the interaction of these metals with erythrocyte membranes. In fact, it has been proposed that the hydrolytic cleavage of the vinyl–ether linkage in plasmalogens by methyl mercury could be involved in its mechanism of neurotoxicity (Segall and Wood 1974).

Consequently, we have systematically studied the binding of Hg^{2+} and Cd^{2+} to biomimetic liposomes which contained the major lipid classes of mammalian erythrocyte membranes (Zachowski 1993) (Fig. 2). The outer erythrocyte leaflet is composed of SM (>85%) and PC (65–75%) (Zachowski 1993). Since toxic metals will target erythroctyes from the outside and PC and SM share the same choline headgroup, we selected PC as the major lipid constituent in our liposome models (85%). PE and PS are minor components of the outer erythrocyte membrane leaflet, but have been shown by others to be potential metal binding sites (Suwalsky et al. 2000). In order to avoid an impact of liposome size on the metal binding affinity, we determined the radii of the liposome preparations by dynamic light scattering and found a uniform size distribution and an average diameter of 109 ± 8 nm (Table 1). This distribution was sufficiently small to exclude liposome size as a determining factor in the analysis of our experimental findings.

Bioassay development to assess metal–lipid binding affinity

In order to investigate the binding affinity of Hg^{2+} and Cd^{2+} to liposomes containing different lipid classes we utilized fluorescence spectroscopy and the commercially available dye PGSK, which is known to be quenched by metals ions in solution (Shingles et al. 2004). To this end, it was necessary to characterize PGSK interactions with metals (Fig. 3a) and the effect of liposomes (Fig. 3b). Furthermore, the dye was twofold more sensitive to quenching by Hg^{2+} than Cd^{2+} (Fig. 4).

The first step was to elucidate if liposomes of different lipid composition will affect the PGSK dye. Figure 5 illustrates the fluorescence intensity at the emission maximum in the presence of liposomes without any metal ions. The various lipid models reduced the observed fluorescence intensity to a different degree and this fact had to be considered. Although the reduction in dye emission prior to metal binding was only small for POPC, the cholesterol containing systems as well as the negatively charged PS system showed a more significantly reduced intensity (3–5%) and the zwitterionic ethanolamine headgroup showed an even larger reduction (8%). Although we do not have a mechanistic explanation for this observation, these changes are relevant for the interpretation of the three-component mixture. We therefore normalized the fluorescence intensity for each membrane system prior to the addition of metals.

The second step was to establish the kinetics of metal binding to liposomes. Since POPC was the main lipid component of our membrane models, we used this lipid system for the respective control experiments. Moreover, it was advantageous that this model system did not show a strong impact on the PGSK fluorescence emission (see Fig. 5). When metals were added to POPC liposomes an immediate decrease in the fluorescence intensity (dye quenching by metals) was observed for both Hg^{2+} and Cd^{2+} (Fig. 6), although the latter displayed a smaller overall quenching ability (Fig. 4). This rapid initial response did not significantly change over time and after 10 min a plateau was reached. Thus, all further incubations were carried out using this time interval. The arrows in Fig. 6 indicate the addition of successive metal ion aliquots to the solution. These experiments showed that it was possible to observe PGSK dye quenching by metals in the presence of model lipid systems within 10 min.

The third step was to establish if the sequence of addition of the three components (metal, dye, liposomes) would affect the results. In Fig. 7, PGSK quenching by metals in solution (Fig. 7A) was compared to ternary mixtures with a different sequence of addition of the components (Fig. 7B, C). The three-component mixtures showed reduced quenching, as indicated by the observed higher fluorescence intensities compared to the solution. The changes for Cd^{2+} induced quenching were moderate and the absolute observed values were just outside the experimentally determined standard deviations. In contrast, PGSK quenching by Hg^{2+} showed a strong dependence on the sequence of addition.

When dye and metal were incubated first, the quenching after addition of Hg^{2+} was only moderately reduced which indicated a stronger affinity of the metal for the dye versus the liposomes. Conversely, when the dye was incubated with POPC model systems first, its fluorescence intensity was not changed (see also Fig. 5), but the ability of subsequently added Hg^{2+} to quench the dye was significantly reduced (almost 100% of fluorescence emission was observed).

Building on the aforementioned control experiments and with the ultimate goal to mimic a biological relevant metal–lipid binding event, we incubated liposomes and metal for 10 min before the PGSK dye was added (see Fig. 3e). A reduction of the dye quenching effect by metal ions in the presence of liposomes (compared to quenching in their absence) indicated that only a fraction of the metals interacted with the lipid membrane.

Assessment of metal binding affinity to liposomes

The most important finding of our investigations was the fact that Hg^{2+} and Cd^{2+} exhibited different binding affinities towards various liposomes of different composition (Fig. 8). Studies into the binding of Hg^{2+} to POPC model systems or binary lipid mixtures revealed that this metal had the strongest binding affinity for the negatively charged PS (12.6%), followed by 6.5% for the pure zwitterionic PC, 0.4% for zwitterionic PE containing liposomes and no binding for the cholesterol containing lipid mixture (85% PC/15% chol). Although PS and PE each have a primary amine on their polar heads, the percent binding affinity of Hg^{2+} to PS was 32-fold higher compared to PE, which suggests that the net negative charge of the PS headgroup plays an important role in Hg^{2+}–lipid binding via electrostatic interactions. These results are relevant with regard to the observation that the addition of 1 μM Hg^{2+} to erythrocytes exposed PS at the cell surface (Eisele et al. 2006). The headgroup of the overall zwitterionic PE ($^{+}H_3N–CH_2–CH_2$), is smaller and able to undergo hydrogen bonding which is known to result in tighter packing of such membranes (Suwalsky et al. 2000). These facts may also explain the stronger binding of Hg^{2+} to PC versus PE-containing model systems (Suwalsky et al. 2000). In pure PC liposomes the quaternary ammonium group of PC must also be considered as an important binding site for Hg^{2+} (Lis et al. 1981; Suwalsky et al. 2000). The latter observation may be an important contributing factor as to why the membrane leakage of intracellular contents was observed when egg lecithin (predominantly PC) liposomes were exposed to 10^{-7} M of Hg^{2+} which resulted in an increased release of the encapsulated glucose marker (Nakada et al. 1978).

The low binding affinity of Hg^{2+} to PE in this study contrasts with previous work in which complexation of PE and PS (but not with PC systems) by Hg^{2+} was reported using NMR spectroscopy (Delnomdedieu et al. 1992). Moreover, Hg^{2+} induced precipitation of liposomes in the order PE > PS > PC has been observed (Girault et al. 1996). In contrast, a study by Suwalsky et al. (2000) using model PC and PE liposomes and X-ray diffraction demonstrated a stronger effect of Hg^{2+} on the membrane structure on PC over PE containing membranes. The authors were able to demonstrate an effect of 10 μM metal on PC systems which is significantly lower than the metal concentration used in the NMR spectroscopy studies (Delnomdedieu et al. 1989; Girault et al. 1996). Our investigations extend these studies to lower metal concentrations (≤1 μM). Thus, these observed differences clearly indicate that the overall metal concentrations and the respective metal–lipid ratios strongly affect the experimental results. The fact that no affinity of Hg^{2+} was observed for liposomes containing equivalent amounts of cholesterol (15% vs. 15% of PS or PE as discussed above) strongly indicates a role of lipid packing and membrane fluidity as a consequence of metal binding. The presence of cholesterol in model systems is known to increase van der Waals interactions between adjacent lipid hydrocarbon chains leading to a tighter packing of acyl chains (condensing effect) (Bhattacharya and Haldar 2000). This may explain why Hg^{2+} was unable to interact with the investigated cholesterol containing liposomes.

The most relevant biomimetic model membrane in this study mimicked erythrocyte membranes and contained all four important lipid classes (35% PC/35% chol/15% PS/15% PE). According to our Hg^{2+} binding data (Fig. 8) this biomimetic membrane contains two putative strong binding sites (PS and PC) and a weak one (PE). The cholesterol content was more than twice as high as in the binary mixture

to more closely mimic the erythrocyte lipid composition (Zachowski 1993). Less tight packing of PC and PS membranes may facilitate the access of the metals to the lipid phosphate groups, which have been previously identified as a binding site by NMR spectroscopy (Girault et al. 1998). We have demonstrated that the differences of molar lipid ratios in binary mixtures can have dramatic effects on lipid packing and the lateral membrane organization (Prenner et al. 2007). Although the cholesterol content in the biomimetic liposomes was more than double compared to the binary (PC/chol) mixture, an appreciable lipid binding affinity of Hg^{2+} was still observed (7.2%). Hg^{2+} binding to erythrocytes has been shown before by electron microscopy (Hg^{2+} concentration in low mM range) (Suwalsky et al. 2004) and by NMR spectroscopy (metal concentration in the 100 mM range) (Delnomdedieu et al. 1992). Therefore, the developed biomimetic system is a useful model to study metal–lipid interactions by fluorescence spectroscopy in vitro.

In order to reveal potential differences between the binding of Hg^{2+} and Cd^{2+} to liposomes, the above mentioned experiments were extended to Cd^{2+} which has a similar ionic radius as Ca^{2+} (Suwalsky et al. 2004). Cd^{2+} has been previously shown to interact with lipids similarly to Ca^{2+} (Girault et al. 1998) and both ions contributed to the externalization of PS in other cell types, inducing apoptosis (Watjen et al. 2002; Coonse et al. 2007; Lee et al. 2007; Sarang et al. 2007). In contrast to our Hg^{2+} results, Cd^{2+} demonstrated preferential interactions to PE containing liposomes with a binding affinity of 8.4 versus 0.45% for Hg^{2+} (Fig. 8). PC and PS represented other potential binding sites with binding affinities of 4.5 and 3.0%, respectively. Since Ca^{2+} has been demonstrated to bind to the phosphate moiety in lipids and thus to bridge adjacent headgroups (Vandijck et al. 1978), it has been proposed that Cd^{2+} interactions with lipids could be similarly electrostatic in nature with phosphate groups as binding sites (Girault et al. 1998). Interestingly, our results also revealed differences between Cd^{2+} interactions with PE, PC, and PS lipid headgroups that cannot be exclusively attributed to its binding to the lipid phosphate groups (Fig. 8). Cd^{2+} bound to PS the least strongly, which may be due to the presence of the COO^- group. Our results suggest that electrostatic interactions may be involved in Cd^{2+}–lipid interactions, but it was observed that Cd^{2+} binds preferentially to zwitterionic lipid headgroups (PC, PE) over negatively charged systems (PS). At present, the reason for this observation is not understood, but it can be speculated that both the charge and the size and the packing of the lipid headgroups may be important factors in these metal–lipid interactions.

Previous NMR spectroscopy studies reported a >200-fold increase of the partitioning coefficient of Cd^{2+} for negatively charged membranes (e.g., PS) (Girault et al. 1998) over zwitterionic systems (PC and PE). In contrast, X-ray diffraction studies revealed that 10 μM Cd^{2+} affected PC membranes and this metal was shown to demolish bilayer structures at a concentration of 1 mM (Suwalsky et al. 2004). These authors also reported smaller effects of Cd^{2+} on PE (Suwalsky et al. 2004), which may reflect the fact that PE membranes are more rigid than the respective PC membranes. Cd^{2+} has been shown to induce peak broadening in ^{31}P-NMR spectra for the negatively charged membranes, such as PS (Girault et al. 1998). The preferential binding of Cd^{2+} to the PE headgroup (Fig. 8) is of potential toxicological relevance because this metal is known to induce hexagonal phases in egg-PE at 24°C and this significant change in membrane structure resulted in the leakage of cellular contents (Allen et al. 1990). Moreover, we did not observe any Cd^{2+} affinity for the cholesterol-containing binary liposomes. In the biomimetic membrane, the relative proportions of PE and PS (15%) were relatively the same as in the simpler binary systems, but Cd^{2+} binding affinity was decreased (4.8%) compared to the binary PE-containing system (8.4%). The presence of cholesterol decreased the amount of Cd^{2+} binding to all potential binding sites (PC, PE, and PS headgroups). These observations are consistent with previous studies which demonstrated that Cd^{2+} will induce lipid disordering in isolated erythrocyte membranes above ~100 μM (Suwalsky et al. 2004).

A comparison of Hg^{2+} and Cd^{2+} binding data to the complex 4 component system (35% PC–35% chol–15% PS–15% PE) which mimiced the erythrocyte membranes the best, clearly demonstrated that Hg^{2+} has a higher binding affinity than Cd^{2+} (7.2% vs. 4.8%; Fig. 8). Our data, obtained at 1 μM metal ion concentration reflect their binding to lipid membranes. Studies are underway to investigate the impact of metal binding on membrane fluidity and

related changes in lipid packing and membrane phase behavior at the metal concentrations that were used in this study.

Conclusion

Fluorescence spectroscopy was demonstrated to be a suitable and sensitive technique to investigate the potentially deleterious interactions of Hg^{2+} and Cd^{2+} with various model lipid systems at metal concentrations ≤ 1 μM. The results showed that Hg^{2+} preferentially bound to negatively charged PS liposomes followed by zwitterionic lipid headgroups, such as PC and PE-containing model systems. Conversely, Cd^{2+} bound preferably to zwitterionic lipid headgroups (PC, PE) over negatively charged systems (PS). The complex biomimetic four component lipid model system of erythrocytes was found to be a suitable model to investigate metal–lipid in vitro. These liposomes represent an important prerequiste to elucidate the binding of metals to biological membranes. In summary, toxic Hg^{2+} and Cd^{2+} displayed a distinct difference in binding affinity towards certain lipid classes that are present in erythrocyte membranes. In view of the known adverse effects of these metals on membrane fluidity, the binding of Hg^{2+} and Cd^{2+} to lipid membranes is potentially involved in their chronic health effects.

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