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The effect of cholesterol on the adsorption of phenyltin compounds onto phosphatidylcholine and sphingomyelin liposome membranes

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Organometallic compounds are widely spread in the human environment sometimes, causing a substantial health risk. Their amphiphilic character enables them to intercalate and penetrate cell membranes, potentially affecting various vital cell functions. Compound adsorption onto the membrane depends on the compound properties, as well as on the membrane composition and state. When adsorbing onto the lipidic surface, phenyltins localize at areas where lipid bilayer organization is compatible with compound spatial requirements. The lipid bilayer is a dynamic and laterally nonuniform structure with complex local and global architecture correlated with a variety of cell functions. The selective binding of a toxic compound to selected membrane areas may, therefore, interfere with some types of cellular process. We present experimental results concerning phenyltin adsorption onto the lipid bilayer surface measured with the fluorescent probe fluorescein-PE. Model lipid bilayers were formed from lipid mixtures mimicking various plasma membrane regions. The adsorption of Ph_3SnCl and P_2SnCl_2 onto the phosphatidylcholine–cholesterol bilayer was qualitatively different from sphingomyelin–cholesterol. The results presented indicate that phenyltins are likely to accumulate in areas containing phosphatidylcholine, outside of lipid rafts.

KEYWORDS: adsorption; phenyltin; model membrane; fluorescein-PE; phosphatidylcholine; sphingomyelin; cholesterol; lipid rafts; fluorescence

INTRODUCTION

Organotin compounds are widely spread in the human environment due to a variety of biogeochemical and industrial applications.[1,2] The amphiphilic character of these compounds[3] enables them to partition into the lipid bilayer of cell membranes, hence potentially disrupting membrane organization and/or interfering with membrane-associated processes.[4–7] When approaching the cell from the extracellular environment, these compounds first encounter the outer surface of the plasma membrane. The outer plasma membrane layer in animal cells is often composed of about 100 kinds of lipid, mainly of phosphatidylcholine, sphingomyelin and cholesterol (which is also found in lysosome and endosome membranes).[8–10] These lipids are laterally nonuniformly distributed. Sphingomyelin and cholesterol form so called 'lipid rafts', whereas the remaining lipids remain in the bulk lipid bilayer. The amount of cholesterol in rafts reaches 35–40 mol%.[11–14] Cholesterol has a variety of effects on the physical properties of the lipid bilayer, including condensation and alteration in lateral lipid organization.[10,15–17] The lipid bilayer state may, in turn, affect the functions of integral proteins, and hence change the rate of membrane-associated metabolic processes.[18–23] As differing membrane domains contain different functional proteins, it is of importance in which domain the toxic

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compound is being accumulated. When a compound has a domain binding preference, its local concentration differs from that determined for the membrane as a whole. We have shown previously that phenyltin adsorption depends on phosphatidylcholine membrane organization (gel versus liquid-crystal state) and cholesterol content.[24,25] In addition, the available experimental data and molecular simulations indicate that diphenyltin and triphenyltin adsorb onto the lipid bilayer surface differently. Diphenyltin penetrates deep into the hydrophobic membrane interior, whereas triphenyltin is located within the interface region.[25,26] Besides altering lipid bilayer packing, both compounds introduce positive charges onto the membrane surface, which may interfere with processes that rely on electrostatic interactions. In this paper, we present data showing that phenyltins have reduced affinity towards membranes formed from raft-like lipid mixtures. This result suggests that phenyltins may accumulate in areas containing phosphatidylcholine, outside of the signal-transmitting rafts.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine, sphingomyelin and cholesterol were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Organotin compounds, namely diphenyltin dichloride ($(C_6H_5)_2SnCl_2$ or Ph_2SnCl_2) and triphenyltin chloride ($(C_6H_5)_3SnCl$ or Ph_3SnCl), were purchased from Alfa Products (Karlsruhe, Germany). Fluorescein-PE was obtained from Molecular Probes (Eugene, OR, USA). All remaining chemicals were of analytical grade.

Liposome preparation and fluorescence measurements

Small unilamellar vesicles (SUVs) were prepared as described elsewhere.[27] In brief, the lipid (egg phosphatidylcholine, sphingomyelin and cholesterol) with an appropiate amount of fluorescent probe were mixed in chloroform. After removing solvent, the dry lipid film was hydrated with 140 mM NaCl with phosphorane buffer at pH 7.4 (PBS). The sample was then vortexed to obtain a milky suspension of multilamellar vesicles and sonicated for 10 min with a 20 kHz sonicator (equipped with titanium probe) in order to obtain small vesicles. The resulting small vesicle suspension was centrifuged to remove titanium particles. The final concentration of lipid was 1.0 mg ml^{-1} (1.3 mM). Organotin compound (Ph_2SnCl_2 or Ph_3SnCl) was added gradually from a concentrated methanol solution (2×10^{-3} M) to the vesicle suspension (at increasing total lipid concentration in the sample from 0.04 mM to 0.24 mM, or at constant lipid concentration equal to 0.16 mM) in a thermoregulated cuvette holder (the concentration of methanol never exceeded 2%). The concentration of organometallics varied in the range of 0–80 μM. Fluorescence intensity was measured before (F_0) and after (F_i) each addition of organotin compound at excitation (λ_{EX}) and emission (λ_{EM}) wavelengths: 495 nm and 520 nm respectively. The relative change in fluorescence intensity, as presented in all figures, is the fraction calculated as $(F_i - F_0)/F_0$. Steady-state fluorescence measurements were carried out on a fluorimeter (Kontron Instruments, Switzerland). Fluorescence intensities were corrected for the inner filter and dilution effects.[28] Each experiment was repeated twice.

RESULTS AND DISCUSSION

Hydrophilic fluorescein dye, covalently bound to the phosphatidylethanolamine lipid molecule, is located at the vicinity of the membrane surface.[29] This location, combined with its pH sensitivity, makes it a very useful tool for measuring local proton concentration.[29,30] Surface pH depends on the electrostatic field generated by membrane charges associated with the surface.[30,31] The adsorption of charged molecules onto the lipid bilayer, i.e. phenyltins, alters the local pH, hence changing the fluorescein-PE fluorescence intensity.[32] This change can be used to evaluate the amount of cationic compound adsorbed onto the membrane surface. The character of adsorption depends on underlying molecular processes, including membrane inhomogeneity and the aggregation of the adsorbing compound in the aqueous and membrane phases. Such complex processes should be reflected in the adsorption curves. Figure 1 shows the relative change in fluorescein-PE fluorescence intensity upon sample titration with Ph_2SnCl_2. Two cases are presented: when the model membranes are formed from only egg phosphatidylcholine or from sphingomyelin. Fluorescence changes are proportional to the amount of the compound added, with no signs of saturation in the concentration range studied, i.e. up to 80 μM. The extent of the relative fluorescence change depends on the lipid concentration in the expected manner. Namely, when the amount of lipid increases (which means that the total surface of liposomes increases), the fluorescence change is reduced (Fig. 1C). In addition, the character of this dependence on the amount of diphenyltin added does differ between phosphatidylcholine and sphingomyelin membranes—albeit that the absolute value of fluorescence increase is higher for the phosphatidylcholine bilayer, indicating greater diphenyltin adsorption. Plots presented in Fig. 1A and B are practically linear for all quantities of lipid in the sample. This indicates that the quantity of Ph_2SnCl_2 used is well accommodated within the lipid bilayer without additional effects. This result agrees with literature data showing that Ph_2SnCl_2 behaves like a simple detergent, intercalating within the lipid bilayer hydrocarbon chain region.[25]

The results of similar experiments for Ph_3SnCl are presented in Fig. 2. In this case, the adsorption pattern shows signs of saturation, especially for sphingomyelin vesicles. Saturation levels depend on the amount of lipid, suggesting that there are only limited amounts of binding sites for

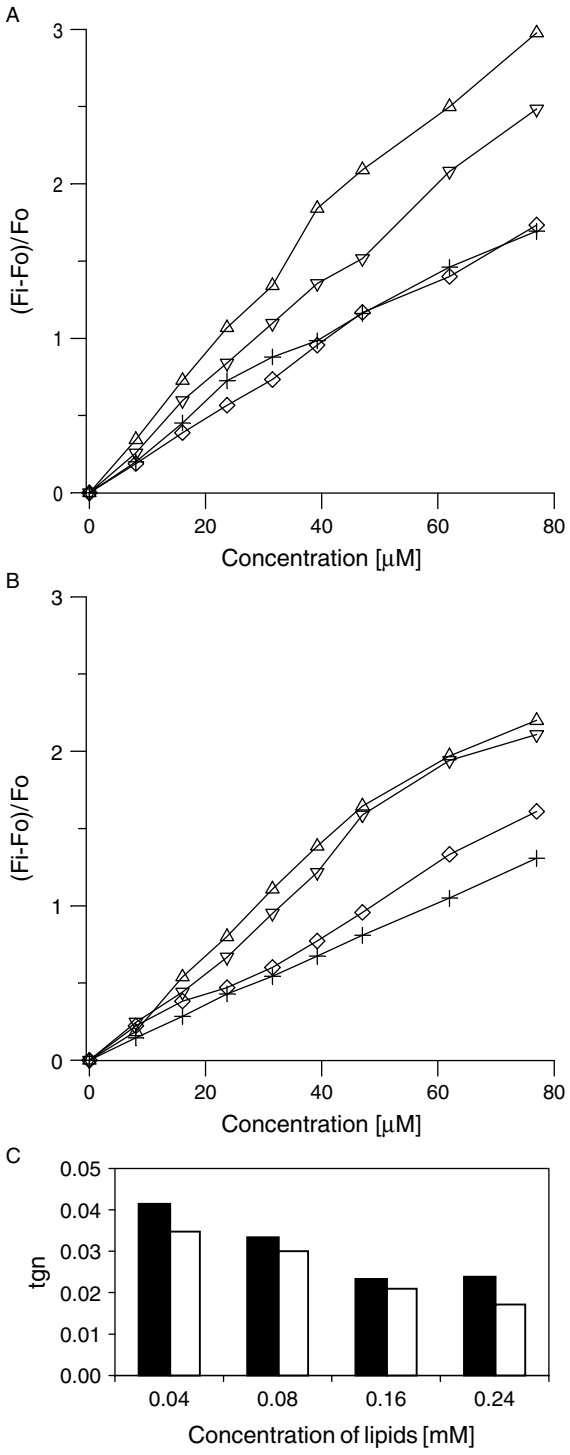

Figure 1. Relative change in fluorescein-PE fluorescence intensity in liposomes formed from egg phosphatidylcholine (A) and sphingomyelin (B) upon adding Ph_2SnCl_2. Samples contained various amounts of lipid: 0.04 mM (triangles), 0.08 mM (reversed triangles), 0.16 mM (rhombuses) and 0.24 mM (crosses). (C) Slopes (tangents) as functions of lipid concentration, calculated from the curves presented in (A) and (B); black and white bars are for phosphatidylcholine and sphingomyelin membranes, respectively.

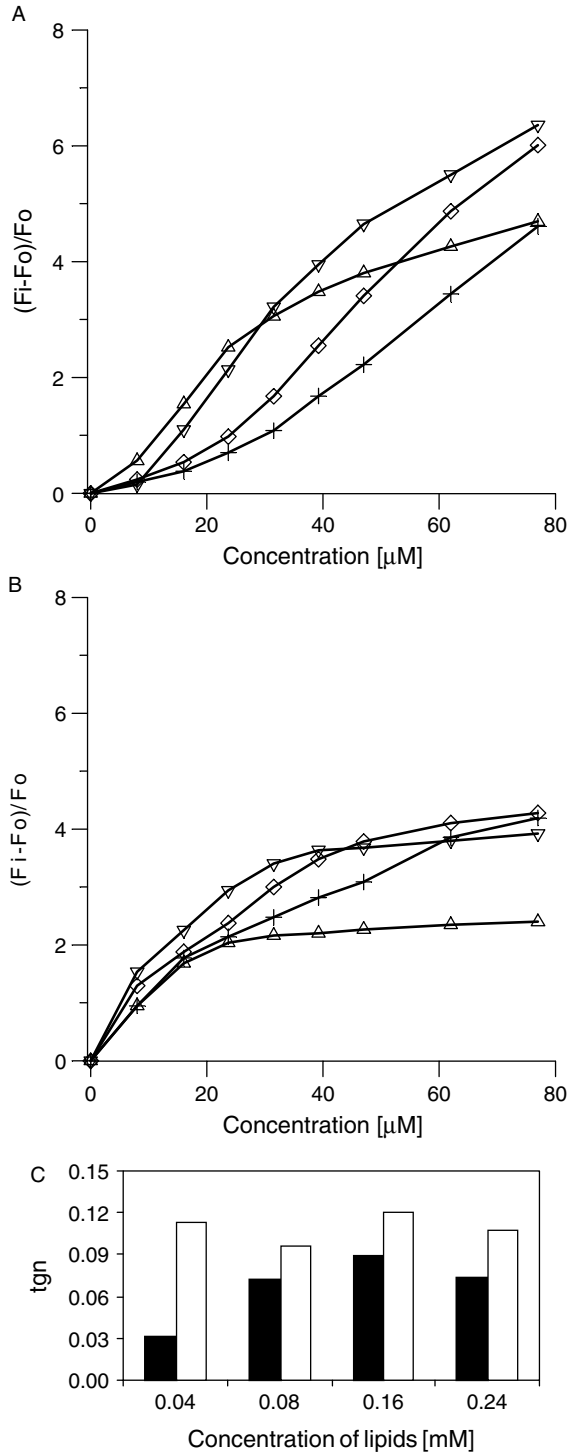

Figure 2. Relative change in fluorescein-PE fluorescence intensity in liposomes formed from egg phosphatidylcholine (A) and sphingomyelin (B) upon the addition of Ph_3SnCl. Samples contained various amount of lipids: 0.04 mM (triangles), 0.08 mM (reversed triangles), 0.16 mM (rhombuses) and 0.24 mM (crosses). (C) Slopes (tangents) as functions of lipid concentration, calculated from the plots in (A) and (B); black and white bars are for phosphatidylcholine and sphingomyelin membranes respectively.

Ph_3SnCl on the lipid surface or that the surface pH reaches a value above the probe's detection level (the local pH is too high). In addition, there are qualitative differences between both groups of binding curves. The compound that binds to sphingomyelin liposomes has a simple saturation character, whereas binding curves for phosphatidylcholine liposomes have an evident sigmoidal character, indicating some sort of cooperativity (not observed in the case of Ph_2SnCl_2). The shape of the plots depends strongly (see particularly in Fig. 2A) on lipid concentration in the sample. Such a dependence implies that the relative amount of the adsorbing compound and the membrane surface area may change the character of the interaction.

The slopes of the respective curves are presented in Fig. 2C. Their values are now much less informative, since the adsorption process is complex and requires detailed studies, i.e. any quantitative analysis needs to be performed based on a simulation of Ph_3SnCl adsorption onto the phosphatidylcholine surface. If the relative change in fluorescence can be correlated with the amount of compound bound, as shown in Figs 1 and 2, then the extent to which both Ph_2SnCl_2 and Ph_3SnCl are bound is similar for pure egg phosphatidylcholine and sphingomyelin membranes. The differences observed between the two compounds can be explained by their location within the lipid bilayer. The two flexible phenyl rings of Ph_2SnCl_2 adapt to the organized lipid bilayer interior. However, bulky Ph_3SnCl cannot penetrate much further than the glycerol level.[6,7,25] The saturation character of the titration curves reflects the limited membrane capacity for compound accommodation.

The presence of cholesterol in the lipid bilayer should decrease the free volume of the membrane[14,16,33] and, therefore, reduce the adsorption of phenyltins. Figures 3 and 4 show the effect of cholesterol on phenyltin adsorption onto the egg phosphatidylcholine–cholesterol and sphingomyelin–cholesterol mixtures. The presence of cholesterol in the egg phosphatidylcholine bilayer increases Ph_2SnCl_2 adsorption, as indicated by an elevated relative fluorescence change (Fig. 3A). The character of the binding isotherm is not altered much by the presence of cholesterol, except at very high concentrations (then the plots show signs of saturation). Binding curves for the sphingomyelin–cholesterol mixture are qualitatively different (Fig. 4B), especially when the amount of cholesterol is higher than 10 mol%. The extent of adsorption drops significantly by a factor of about three. These results show that the behaviour of these two membrane types is qualitatively different when the amount of cholesterol rises. Adsorption patterns suggest that cholesterol loosens the lipid packing in the egg phosphatidylcholine membrane, enhancing penetration by the amphiphilic compound. In the case of sphingomyelin, the bilayer is tightly packed, preventing the penetration of the Ph_2SnCl_2 phenyl ring into the hydrocarbon chain region.

Figure 4A shows the relative change in fluorescence of the fluorescent probe incorporated in lipid bilayers formed from egg phosphatidylcholine mixed with various amounts

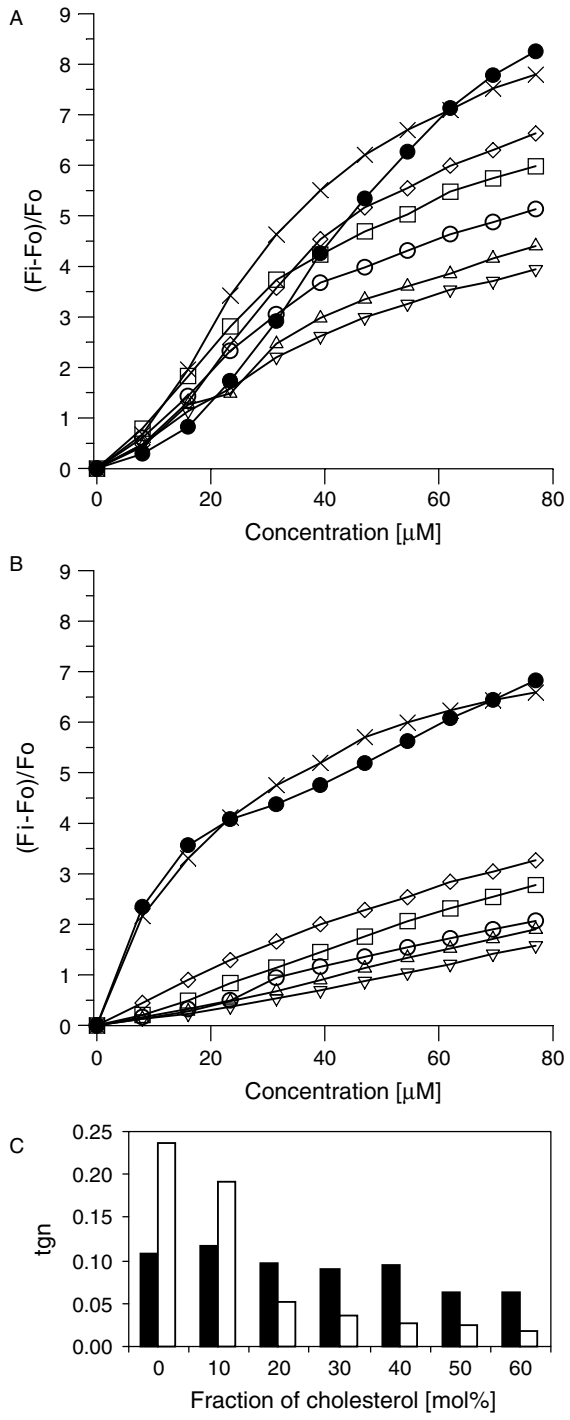

Figure 3. Change in fluorescein-PE fluorescence intensity in egg phosphatidylcholine (A) and sphingomyelin (B) membranes (0.16 mM) upon the adsorption of Ph_2SnCl_2 as a function of various quantities of cholesterol, i.e. 0 mol% (filled circles), 10 mol% (crosses), 20 mol% (rhombuses), 30 mol% (squares), 40 mol% (open circles), 50 mol% (triangles) and 60 mol% (reversed triangles). (C) Slopes (tangents) as functions of cholesterol fraction in the membrane, calculated from the plots in (A) and (B); black and white bars are for phosphatidylcholine and sphingomyelin membranes respectively.

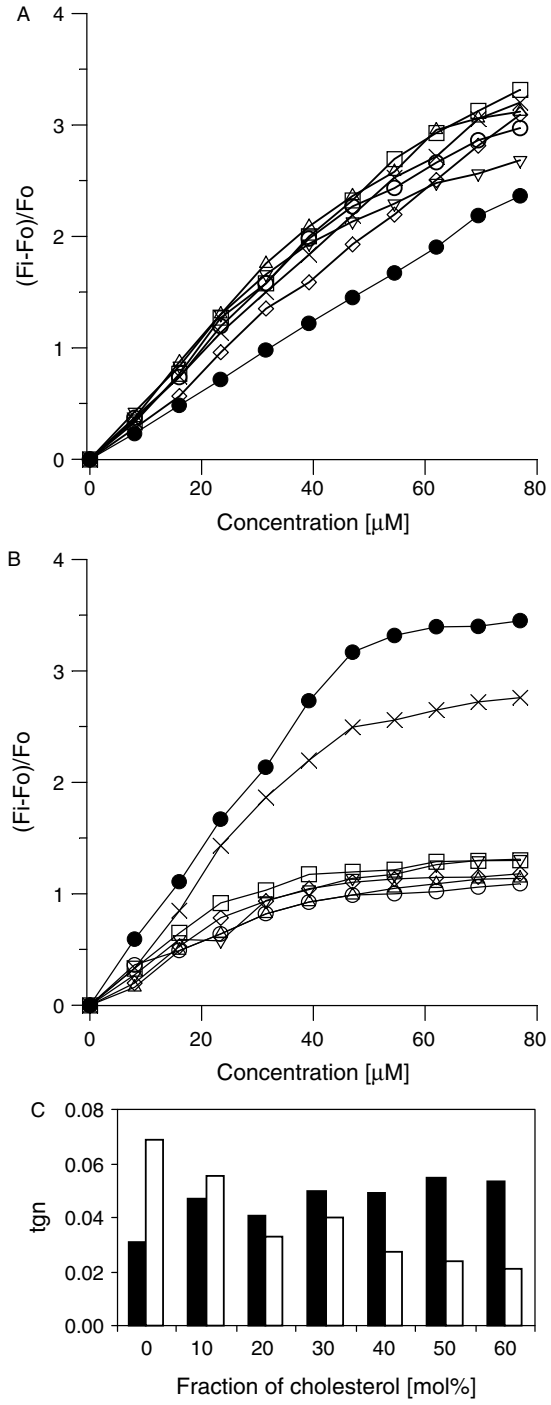

Figure 4. The adsorption of Ph_3SnCl onto the egg phosphatidylcholine (A) and sphingomyelin (B) lipid bilayers (0.16 mM) as a function of the amount of cholesterol in the bilayer, i.e. 0 mol% (filled circles), 10 mol% (crosses), 20 mol% (rhombuses), 30 mol% (squares), 40 mol% (circles), 50 mol% (triangles) and 60 mol% (reversed triangles). (C) Slopes (tangents) as functions of cholesterol fraction in the membrane, calculated from the plots in (A) and (B); black and white bars are for phosphatidylcholine and sphingomyelin membranes respectively.

of cholesterol when titrated with Ph_3SnCl. The binding pattern is quantitatively different than that of Ph_2SnCl_2. The initially sigmoidal curve changes its character with rising cholesterol amount and becomes similar to that observed for sphingomyelin. Plots flatten when the amount of cholesterol in the bilayer rises. This is accompanied by a reduction in the amount of bound compounds at higher concentrations (above 60 μM). At lower Ph_3SnCl concentration, the relative fluorescence change rises with increasing cholesterol amount. This effect may reflect a reduction in the postulated cooperativity and a limited amount of available binding sites. Ph_3SnCl binding to the sphingomyelin bilayer is similar to that of Ph_2SnCl_2, putting aside that the drop in the adsorption is larger (by an order of magnitude). Such differences between compounds are likely due to variations in their location within the lipid bilayer (Ph_2SnCl_2 penetrates the hydrophobic membrane interior, whereas Ph_3SnCl adsorbs within the headgroup region).

The observed dependence of binding patterns on the composition of the lipid bilayer may have an important effect on phenyltin toxic potency. It has been shown previously that a variety of cell functions depend on plasma membrane topology. Rafts are involved in lateral protein sorting, which in turn affects a variety of cell functions.[34] Rafts were detected by utilizing their relative solubility by Triton X100, i.e. by measuring the adsorption of detergent molecules into different membrane fractions.[11] Phenyltins (amphiphilic molecules), when adsorbing onto the lipid bilayer, may also partition differently at distinct locations on the plasma membrane. Adsorption will also depend on the properties of the phenyltin molecule itself. As presented elsewhere,[25] the Ph_3SnCl located at the surface adsorbs even when the membrane is in the gel state, albeit to a lesser extent. Ph_2SnCl_2; on the other hand, it is not able to penetrate the well-packed hydrocarbon chain region at similar concentrations.[25] The difference in each compound's preference towards different lipid bilayer regions may imply that they have a binding preference towards certain lateral lipid structures. The association of diphenyltin with lipid bilayers formed from egg phosphatidylcholine and sphingomyelin does not differ significantly. The variations observed in Ph_3SnCl binding are interesting; nonetheless, again, no drastic selectivity is observed. However, adsorption patterns for both compounds become qualitatively and quantitatively different when cholesterol is added to the lipid bilayers. At moderate amounts of cholesterol, the adsorption of both the compounds studied onto liposomes formed from sphingomyelin is drastically reduced (see Figs 3C and 4C). No such situation is observed when the membrane is formed from phosphatidylcholine. In this case, even a significant cholesterol content does not prevent compound binding. Based on the data presented, it can be postulated that when phenyltins adsorb onto the plasma membrane they are preferentially located outside the raft regions.

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