1 “Metal ion binding to phospholipid bilayers evaluated by microaffinity chromatography.” Eric E. Ross[†],
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6 7 **Abstract**

8 Group I and II ion binding to phospholipid membranes was evaluated by affinity chromatography
9 utilizing a new stationary phase system based on lipid bilayers supported within large-pore particles
10 composed of Stöber silica spheres. Using an inductively coupled plasma mass spectrometer for
11 detection, robust determination of binding selectivity within group II ions is achieved with capillary
12 columns containing nanomole quantities of lipid and using picomoles of metal analyte. Columns with a
13 unique lipid formulation can be prepared within three hours using a solvent-casting assembly method.
14 The observable thermotropic phase behavior of dipalmitoylphosphatidylcholine has a significant effect
15 on alkaline metal binding and demonstrates the dynamic nature of the supported bilayers. Of the group
16 I ions, only lithium exhibits retention with neutral phosphatidylcholine bilayer stationary phases. A
17 comparison of Stöber- based supports with two commercially available large-pore silicas reveals the
18 effect that particle structure has on analyte accessibility to the bilayer surface as evaluated by retention
19 per supported lipid mass.

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21
22 **Keywords.**

23 Zwitterionic ion chromatography, ICP-MS, affinity chromatography, phospholipid, Stöber silica, EggPC

1. Introduction

Metal ion interactions with lipid components of biomembranes are low affinity in nature. Nevertheless, the interaction plays a role in vesicle fusion [1-3], phospholipase activity [4], and physical properties of the lipid bilayer such as stiffness [5], phase transitions [6], lipid clustering [7-9], and surface charge [10]. The low binding affinity and its sensitivity to membrane electrostatics that are influenced by solution composition complicate the determination of association binding constants (K_{assoc}) of ions to lipid membranes. Indirect measurements of affinity must accurately model competing interactions or non-binding processes that can contribute to a measured parameter change. This may factor into the variability in K_{assoc} values determined by different techniques. A recent work by Szekely et al. cataloged many of the reported K_{assoc} values for cations with zwitterionic lipids which are major components of many biomembranes [11]. The values for calcium ion binding to dipalmitoylphosphatidylcholine, for example, vary from 1 to 190 in different studies.

The interactions between inorganic ions and zwitterionic surfactants have been studied by electrostatic ion chromatography, also called zwitterionic ion chromatography (ZIC)[12-17]. Most commonly, single-tail surfactants with zwitterionic headgroups are adsorbed onto alkylated porous silica to form a surfactant monolayer. The work of several research groups [15, 18-21] has led to an understanding of the mechanisms that give rise to the unique ion selectivity on different zwitterionic phases. While the development of ZIC has largely been for analytical separations, cation studies with monolayer films bearing phosphocholine headgroups [14, 18, 21] yield insight into interactions occurring at biological membranes. The biophysical relevance of the technique has been established through the characterization of anion binding to natural phosphatidylcholine lipids as reported by Hu and coworkers [19, 20]. To date however, no K_{assoc} values between metal ions and phosphatidylcholine lipids have been reported by ZIC studies which would be useful for a technical comparison between chromatographic and indirect techniques.

Although the single-tail surfactants used in several ZIC studies with cations bear resemblance to phosphatidylcholine lipids, there are important differences between them. Dual-tail membrane lipids contain phosphodiester linkages that contribute to membrane potential [22] and may have a secondary role in some metal cation interactions [23]. They also exhibit crystalline phase transitions that are known to be affected by metal binding [6]. This transition is associated with changes in, among other things, bilayer density, order within the lipid tails, and the rate of lateral diffusion, and it is affected by the presence of solid supports [24]. Although lipid monolayers on alkylated surfaces exhibit increased stability relative to lipid bilayers formed on hydrophilic ones [25], they have been shown to impact the phase transition temperature of adsorbed phospholipids in unexpected ways [26, 27]. For this reason, the present work is focused on the use of lipid bilayers to measure metal cation binding to phospholipids.

The structure of lipid films is sensitive to surface features of solid supports. Synthetic silica particles that contain a distribution of pores sizes and geometries have nanometer scale features [28-30]. Such features create defects and high curvature in lipid films that affect bilayer integrity [31], conformational order [32], and phase properties [33-35]. Stöber silica spheres are non-porous and dense [36], and lipid bilayers supported on them [37-40] are similar to those supported on smooth, planar glass surfaces. The latter is a venerable bilayer system widely used for studies of membranes

[41]. Stöber agglomerated particles (SAPs) were utilized in high performance size exclusion chromatography in the 1970s [42]. The smooth surface of Stöber spheres and the large, fully connected pores (>75 nm with 500 nm spheres) in SAPs suggest they are well-suited for hosting supported lipid bilayer stationary phases. In the present work, group I and II metal binding to natural zwitterionic lipids is evaluated using bilayers adsorbed on Stöber spheres. A capillary column format is described that requires several orders of magnitude less lipid than conventional scale HPLC columns.

2. Experimental Methods

A more detailed account of procedures, instrument schematics, and additional supporting data is in the Supplementary document.

2.1 Reagents.

Angstromsphere silica spheres (Stober spheres) with a diameter of 4 μm and 500 nm were purchased from Fiber Optic Center, Inc. (New Bedford, MA). The lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and L- α -phosphatidylcholine from chicken egg (Egg PC) were from Avanti Polar Lipids (Alabaster, AL). The emulsifying surfactant Kemelix 7475X was kindly donated by Croda Inc. (USA Headquarters, Edison, NJ). Commercial particles were obtained from two sources, a Viva silica column (5 μm , 300 $\text{\AA}$) from Restek Corp. (Bellefonte, PA) and two Nucleosil particles, 1000 and 4000 $\text{\AA}$ (10 μm spherical) from GFS Chemicals (Powell, OH). IAM.PC silica (10 μm spherical, 300 $\text{\AA}$) was from Regis Technologies (Morton Grove, IL). Deionized water (18.2 M Ω , Siemens Water Technologies, Munich, Germany) was used exclusively. A full list of chemicals is in Supplementary Section 1.1.

2.2 Formation of Stöber Agglomerated Particles (SAPs).

A water-in-oil emulsion procedure based on the work of Kim et al.[43] was used to prepare Stöber agglomerated particles (SAPs). The process is detailed in Supplementary Section 1.2. A scanning electron microscopy image of a SAP is shown in Figure 1. SAPs composed of 500 nm Stöber spheres (SAP₅₀₀) had a mean diameter of $11.5 \pm 3.3 \mu\text{m}$ as estimated from >100 spheres from three different SEM images.

2.3 Lipid bilayer formation.

Lipid bilayers were formed within SAPs by the rehydration of dried films deposited by solvent-casting with TRIS buffer. This procedure has been used to assemble bilayers on Stöber spheres [44], and is principally similar to the process that results in sphere-supported bilayers within colloidal crystals which are laterally mobile and accessible to soluble proteins [45]. The idealized structure of lipid-SAP particles is depicted in Figure 1. Stöber spheres have a surface area similar to smooth featureless spheres of the same diameter, measured by nitrogen adsorption to be 18-24 m²/g for 270 nm particles [46, 47]. The mass of lipid solvent-cast onto SAPs was based on the surface area of an equal mass of non-porous spheres. The process and calculation is detailed in Supplementary Section 1.3.

Vesicle fusion experiments used dispersions of small unilamellar vesicles (2.5 or 5.0 mg/mL) prepared by probe tip sonication (Fisher Scientific, model FB-120 with tip model CI-18) to fuse to silica particles within capillary columns. Turbid lipid suspensions were sonicated to clarity using 2 second on,

2 seconds off pulse cycles at 30% power for 10 minutes. Supplementary Section 1.3 contains distribution plots for the vesicles determined by dynamic light scattering (Corvus DLS01 instrument and software, Spokane, WA). The z-average hydrodynamic diameter of the vesicles was 80 nm. The mass of lipid adsorbed onto columns was evaluated by frontal analysis using the phosphorus-31 signal to detect the breakthrough volume of the vesicles.

2.4 Capillary column preparation, lipid-SAP and column characterization.

SAPs were packed into 150 μm i.d. fused silica capillaries (Polymicro Technologies, Phoenix, AZ) from aqueous slurries using a PC77 pressure injection cell (Next Advance, Averill Park, NY) and helium (100 to 500 psi). Frits were prepared from small plugs, nominally 1 mm long, of 4 μm Stöber spheres that were stabilized by exposure to silicon tetrachloride vapor (caution: silicon tetrachloride reacts violently with water).

The lipid:silica ratio of the SAPs was determined by thermal gravimetric analysis (TGA, Perkin Elmer TGA-7, Waltham MA) with approximately 15 mg of the slurry, air-dried overnight at 85 $^{\circ}\text{C}$. Post use, column void was determined by weighing it before and after drying to a constant mass at 85 $^{\circ}\text{C}$ (microbalance, Cahn Instruments Inc, Cerritos, CA), which was then used with the t_m to determine volumetric flow rate. The empty capillary mass (periodically reevaluated, typical value = 1.712 ± 0.011 mg/cm) was then used to determine the silica mass on the column to calculate the phase ratio. A Perkin-Elmer DSC-7 instrument was used to determine the phase transition temperatures of DPPC multilamellar vesicles and bilayers on SAP₅₀₀ particles.

2.5 Chromatography instrumentation.

Capillary columns were coupled to an Agilent 7700x ICP-MS (Agilent Technologies, Inc. Tokyo, Japan). A schematic of the system is in Supplementary Figure S3. The sub-microliter flow from the columns was coupled to flow from the ICP peristaltic pump for effective nebulization (Micromist nebulizer). Post-elution dead time was under three seconds. Metal ions, bromide, or phosphorous ions were simultaneously monitored in time-resolved analysis mode. Standard autotune-obtained parameters for no collision gas (group II ions) and H₂ collision gas (4.5 mL/min for group I ions) conditions were utilized. Supplementary Section 1.5 contains values for ICP-MS parameters.

A Series 1500 Lab Alliance pump (Scientific Systems, Inc. State College, PA) was used with two manual injection 6-port valves (360 μm fittings, Vici Valco Instrument Co. Inc. Houston, TX) linked in series. The first valve had a large volume injection loop (35 μL , approximately 30 minutes of flow) used for online changes to the mobile phase composition. All fittings (unions, reducers, tees) were from Vici Valco Instruments Co. Inc. (Houston, TX). A splitting tee with a 25 cm restriction capillary (50 μm i.d.) was used to reduce mobile phase flow rates from the pump. A 12 cm column heater and controller (Phoenix S&T, Chester, PA) were used in studies of Group II metal binding to DPPC between 30 and 50 $^{\circ}\text{C}$.

2.6 Chromatography methods.

Columns between 8 and 12 cm were used with volume flow rates of 400-600 nL/min resulting from a backpressure of 100-200 psi. Buffered aqueous mobile phases (15 mM) were used with or without 0.1

M NaCl. Tris-Tris-HCl was used at pH 7 and Tris-acetic acid was used for pH 5 and 6 mobile phases. Columns were conditioned for approximately 5 minutes prior to the collection of chromatograms. Over a two hour period and nine sequential injections, retention factors decreased slightly (6.5%) but ion selectivity remained constant (Supplementary Figure S4). The void marker for t_m was bromide, which has no detectable affinity for the silica or lipids in this system. When mobile phases contained sodium chloride, bromide co-eluted with the water void, evident from the dip in the sodium signal from the Na-free injection solvent. Though some studies have shown bromide ion to bind weakly to phospholipids (K_{assoc} values < 2) [48, 49], the interaction is evidently too weak to observe directly by this methodology.

Injections for zonal elution were performed by opening the injection valve for one second. At a typical flow of 600 nL/min, 5 pmol of ion are injected from a 0.5 mM metal solution. The variation in the volume injected by this method was approximately 10% (Supplementary Figure S4). Flow rates producing linear velocities under 0.1 cm/s were used. At higher rates, retention factors decreased for group II ions and the apparent plate height increased (Supplementary Figure S5).

2.7 Data analysis.

From the ICP-MS software (Agilent MassHunter version B.01.01), raw count chromatograms were exported and fit with asymmetric peak functions using TableCurve 2D v.5.01 (Systat Software, Inc. San Jose, CA). Retention time was determined from the first statistical moment of the fit peak. For all data, chromatographic or otherwise, $n \geq 3$ and each data point used for statistics is from a separate column of independently prepared lipid-SAP materials unless noted.

The chromatographic retention factor (k) is related to the equilibrium partition constant, K_p , through the volume ratio of the stationary and mobile phases, $k = K_p(V_s/V_m)$. The volumes were determined by mass using a lipid and buffer densities of 1.01 g/cm³ and 1.0 g/cm³ respectively [50]. The relationship between K_p and K_{assoc} for an interaction having 1:1 binding stoichiometry in lipid bilayers is given by $K_p = K_{\text{assoc}}/V_L$, where V_L is the molar volume of the lipid phase [51].

3. Results and Discussion

3.1. Metal ion affinity for zwitterionic lipid bilayers with SAP particles.

Metal cation binding to phosphatidylcholine generally increases with ion charge [52, 53], but specific interactions [23] result in selectivity within charge groups. Binding affinity to EggPC liposomes measured by zeta potential is: group I ions $< \text{Ba}^{2+} < \text{Sr}^{2+} < \text{Mg}^{2+} < \text{Ca}^{2+}$ [53]. Calorimetry studies indicate that Li^+ binds more strongly than other group I ions [54]. The same affinity order is observed in the chromatographic traces (Figure 2A) and retention factor data (Figure 2B) on SAP₅₀₀ columns containing approximately 52 nanomoles of eggPC. The offset upper traces are for the bare silica SAPs which exhibits small but unselective affinity for these ions in contrast to eggPC.

Previously reported K_{assoc} values between phosphatidylcholine and group I ions are generally below 1 [11], and roughly 10-100 times smaller than those for group II ions. The retention factor for Ba^{2+} is only 0.3 (Figure 2B), thus it is not surprising that specific interactions with group I ions, apart from Li^+ , cannot be detected here. Li^+ exhibits small but distinct retention that is roughly a factor of 10 less than that for Ba^{2+} . Although the relative standard deviation for the very small retention factors of group I ions is large, confidence in the mean is reasonable from many trials (for this data, $n = 6$), and the trend is evident; EggPC bilayers reduce the retention of group I ions on bare silica by 50%. The residual

retention is interpreted as the fraction of silica that remains accessible to the ions, and it was used as a background correction in determining K_{assoc} values for other ions.

The apparent 1:1 K_{assoc} values for Ba, Sr, Mg, and Ca ions to EggPC are 7.3 ± 2 , 8.0 ± 2 , 30 ± 6 , and $59 \pm 12 \text{ M}^{-1}$ respectively, as per Section 2.7. These values are similar to those obtained by zeta potential measurements which are 10 ± 1 , 16 ± 2 , 30 ± 4 , and $42 \pm 5 \text{ M}^{-1}$ for the same series [53]. Those values were determined under low ionic strength conditions, but 0.1 M NaCl reduced the K_{assoc} value for Ca^{2+} less than ten percent. In this work, a greater distinction between Mg^{2+} and Ca^{2+} binding is observed. With SAP columns, the largest contributor to uncertainty is the variance in lipid mass on each column; the SAP lipid/silica ratio was $23 \text{ } \mu\text{g}/\text{mg} \pm 16.5\%$ from TGA data ($n = 6$ lipid-SAP preparations). The uncertainty in the retention factors and void volumes, the other parameters used in calculating K_{assoc} , are both smaller. The variance in K_{assoc} would likely be reduced by normalizing retention factors to the lipid content of each column, however the TGA and chromatography data were from different preparations of SAPs.

Ion selectivity (α) is not, in principle, sensitive to minor variation in the lipid mass on the column. Whereas K_{assoc} for Sr^{2+} and Ba^{2+} are statistically undistinguished when values from different EggPC SAP preparations are compared, Ba^{2+} always elutes prior to Sr^{2+} ($\alpha_{\text{Sr}/\text{Ba}} = 1.1$). The ion selectivity values evaluated from individual EggPC SAP columns had percent relative standard deviation values less than 4% ($n = 5$) with $\alpha_{\text{Ca}/\text{X}}$ equal to 1.99, 7.44, and 8.11 for $\text{X} = \text{Mg}$, Sr , and Ba ions respectively, allowing the preferential affinity of EggPC for Sr^{2+} over Ba^{2+} to be confirmed. It should be noted that uncertainty in the fraction of lipids that are accessible to ions and the influence of the support on bilayer structure affect the K_{assoc} value. These are factors endemic to studies with supported lipid films. Therefore, it is not suggested that the individual values of K_{assoc} determined here are more accurate than those from other methods, but that the similarity of the values supports a case for the utility of the system which can provide a relatively quick evaluation of apparent affinity and binding selectivity under a variety of conditions.

3.2. Comparisons to ZIC studies.

Separation of metal cations is achieved with phosphocholine-type surfactants in ZIC. A mechanism factoring Donnan membrane ion exclusion and the chaotropic character of ions explains the effect of counterions on retention and selectivity [18]. Not surprisingly, elution order on the surfactants in ZIC and on lipids with identical zwitterionic headgroups in the present work is the same. A partial list of the series reported by Cook et. al [18] with N-tetradecylphosphocholine is $\text{K}^+ < \text{Li}^+ < \text{Ti}^+ < \text{Ba}^{2+} < \text{Co}^{2+} < \text{Ca}^{2+}$, and Hu et. al [14] showed that Mg^{2+} eluted between Ba^{2+} and Ca^{2+} on a similar surfactant; this is also the order observed with EggPC bilayers in the present work (a chromatogram with Ti^+ and Co^{2+} is in Supplementary Figure S6).

ZIC studies with phosphocholine surfactants have largely used pure water as the eluent where cations elute as ion-pairs [14, 16, 21]. Here, the presence of electrolyte was required to reduce ion interaction with the silica; on bare and EggPC SAP columns no ions elute within 30 minutes in a pure water mobile phase. Thus, differences in elution conditions must be considered when comparing the lipid studies here with ZIC results for surfactants. In ZIC, the distinction between the retention of Ca^{2+} and Mg^{2+} thiocyanate ion-pairs was relatively small compared to the factor of two difference for

unpaired ions on EggPC SAP columns [14, 16, 21]. Further, ZIC studies show less distinct Li^+ affinity over other group I ions for a phosphocholine surfactant phase [18]. Here, specific retention of K^+ cannot be measured, while Li^+ retention indicates that it binds the lipid with at least several times greater affinity. Several factors likely contribute to the observational difference. One, K^+ genuinely binds phosphatidylcholine lipids more weakly than Li^+ . Several studies have shown distinctive lithium binding to [55], and effect on [56, 57], lipid bilayers relative to other alkali ions. Two, the uncertainty in the very small retention factors coupled with the small background retention on the bare SAP particles obscures the specific retention of non- Li^+ group I metals. Finally, the phase ratio of the lipid SAP columns is roughly 10 times less than those used in ZIC which have contained, for example, about 0.7 mmol of surfactant on 25 cm x 4.6 mm id columns packed with high surface area particles ($\sim 300 \text{ m}^2/\text{g}$) [14].

In ZIC, the effect of electrolyte concentration, pH, and counter anion on cation retention was used to model mechanisms for separations on phosphocholine-type surfactants [18]. Sodium chloride increased the retention of those cations with greater affinity than Na^+ for the zwitterionic phase by decreasing the ionic self-association of the zwitterions. Increasing electrolyte concentration had little effect on cation retention because neither sodium nor chloride ions bind strongly enough to significantly alter the overall charge of the membrane. In contrast, 0.1 M NaCl decreases ion retention on EggPC

SAPS. Without it in the buffered mobile phase the retention factors increase by a factor of 7.2 for Ba^{2+} and Sr^{2+} , and 5.5 for Mg^{2+} . The increased retention made Ca^{2+} analysis difficult due to signal noise. On EggPC, the decreased ion retention in the presence of NaCl likely arises from two factors, Na^+ competition for binding sites, and screening of electrostatic interactions. Weaker binding ions (Ba^{2+} and Sr^{2+}) are affected to a greater degree than Mg^{2+} , which is consistent with the effect of competitor cations in ZIC studies [18].

Figure 3 shows that cation binding to EggPC decreased with the pH of the mobile phase. Hu et al. showed that anion binding to the natural lipid dimyristoylphosphatidylcholine was sensitive to factors that affected the surface potential of the membrane including mobile phase pH [20]. As noted by Zhou et al. [58], the effect of pH on membrane electrostatics has received little experimental attention. In that work, significant changes in both the zeta potential and intramembrane dipole potential of phospholipid vesicles over a pH range of 3-9 occurred, largely attributed to hydroxide interactions with the lipids. Therefore, the increased metal interaction with EggPC at higher pH values in this work may be related to these interfacial and intramembrane electrostatic factors, though changes in the mechanical properties of the lipid bilayer or how it interacts with the support silica cannot be ruled out.

Figure 4 contains the group II ion retention data on EggPC SAPs with different counter anions to the 0.1 M Na salt at pH 7. Exchanging Cl^- for ClO_4^- or SCN^- anions approximately doubled cation retention, while I^- , NO_3^- , and Br^- had a lesser effect. This effect matches the anion affinity series for zwitterionic phospholipids determined chromatographically [20], and is consistent with the effect of perchlorate on cation retention relative to less chaotropic ions in ZIC studies with phosphocholine-type surfactants [18]. Adsorption of anions decreases the surface potential of the EggPC bilayers which increases the affinity for cations for the lipid phase. In summary, ion retention in the present system appears to share mechanistic elements with retention on phosphocholine-type surfactants. Cation binding to the EggPC bilayers appears more sensitive to elevated salt concentrations in the mobile phase than binding to single-tail phosphocholine surfactants suggesting a greater electrostatic factor in the interaction.

3.3. Temperature dependence of ion binding.

In liposomes, DPPC bilayers undergo a cooperative chain-melting transition at 41°C (the T_m) that is shifted to lower temperature when the lipids are supported on silica colloids [59]. This transition is observed in the Group II ion binding to DPPC bilayers over the range of 30 to 50 °C (Figure 5). The sharp reduction in retention between 37.5 and 40 °C coincides with the endothermic phase transition observed by calorimetry at approximately 38 °C on the SAPs (Supplementary Figure S9). The decrease in retention is similar to the decrease in Ca^{2+} affinity for DPPC liposomes across the phase transition as measured by electrophoretic mobility studies (a factor of 2.4 versus 2.3) [53]. Lipid unsaturation has also been shown to reduce cation affinity [11, 53]. At 45 °C, Mg^{2+} binding to DPPC is 50% stronger than to EggPC which contains approximately 50% unsaturated lipids (based on fatty acid composition information available at vendor website).

The temperature dependence of chromatographic retention factors is often evaluated by a linear van't Hoff plot to determine the thermodynamic contributions to adsorption free energy, though there is some debate over the accuracy of such an approach [60] and there is often significant disagreement to the values found by calorimetry [61]. The data indicate that ion binding is exothermic in both phases, and that the enthalpy is temperature dependent within several degrees of the transition as evident by the curvature in the plots (Supplementary Figure S11). Calorimetric studies on liposomes show group I and II ion binding to PC lipids to be endothermic and driven by entropy [52, 54]. Therefore the solid support clearly influences the energetics of ion binding to the lipids, likely by restricting conformational changes accompanying binding that contribute to the enthalpy in liposomes. Substrate influences on supported bilayer properties are not unexpected, silica affects lipid lateral diffusion rate and its temperature dependence [62], the T_m , and the formation of lipid domains and certain phases [24, 63].

3.4. Vesicle fusion assembly on large Stöber spheres.

Vesicle fusion is a well-characterized method of self-assembly that generates single (non-multilayer) bilayers on planar [64] and colloidal silica surfaces [37, 39, 65]. The presence of multilayer stacks would hinder ion access to the supported lipids and ion retention, relative to column lipid mass, would be reduced. Vesicle fusion was used to prepare columns with single lipid bilayer films supported on Stöber silica spheres. When EggPC vesicles were injected on bare SAP_{500} columns however, the amount of lipid retained was highly variable and generally 40% less than that deposited by solvent casting. The variability is likely due to SAP_{500} pores clogging with unfused vesicles which have a mean hydrodynamic diameter comparable to the SAP pores but a more heterogeneous distribution. Columns packed with solid 4 μm Stöber spheres create much larger pores (minimally 600 nm). Here, vesicle fusion resulted in 2.6 ± 0.4 μg of lipid retained per mg of silica. Solvent-casting on SAP_{500} generated a lipid:silica ratio of 23.9 ± 3.8 $\mu\text{g}/\text{mg}$ which is larger by a factor of 9.1. The ratio agrees well with the 8:1 surface area ratio of SAP_{500} :4 μm spheres.

The retention factor for Mg^{2+} is 11.2 times greater on solvent-cast SAPs relative to the vesicle-fused bilayers which exceeds the 9.1 factor difference in the lipid:silica ratio. This indicates that ions have relatively greater access to the solvent-cast lipids on the SAPs. It is possible that bilayers formed by

vesicle fusion have greater and more uniform conformation to the silica surface which could reduce ion access to the leaflet adjacent to the silica. The general agreement of these values with the relationship predicted by surface area provides a margin of validation for the lipid mass used in the solvent casting procedure, and some support for the depiction of colloid-supported lipid bilayers on the SAPs in Figure 1. It is consistent with protein adsorption and fluorescence image data from other studies where solvent-casting lipids within packed beds or crystals of Stöber spheres appears to generate single, supported bilayers [44, 45].

3.5. Alternative silica particles and IAM.PC silica.

Mg²⁺ binding to EggPC bilayers on other large-pore silica particles was evaluated for comparison to SAP particles. A covalent lipid-mimic phase widely used for lipophilicity measurements, IAM.PC silica, was also evaluated. Synthesized silica particles contain a distribution of pore sizes and an irregular surface structure that makes the actual surface area suitable for bilayer support less predictable than for smooth Stöber spheres. Several masses of lipid were solvent-cast onto each commercial particle based on the surface areas reported by the manufacturers. For Viva 300 Å this was between 113 and 117 m²/g, and for Nucleosil 1000 and 4000 Å, it was 25 and 10 m²/g respectively. For comparison, the pore size of SAP₅₀₀ particles is nominally ≥ 750 Å, defined geometrically by the close-packed spheres.

Figure 6 shows the Mg²⁺-EggPC binding data for the different silicas, normalized to SAP₅₀₀ particles (grey bars). In parentheses below the silica type is the mass of lipid added to the particles relative to silica. Mg²⁺ retention comparable to SAPs occurs only when significantly more lipid is solvent-cast onto the synthetic silica particles.

Retention factors normalized for the lipid mass on each column type (determined by TGA) is shown by the white bars (Figure 6) which provides a comparative measure of how accessible the supported lipid is to Mg²⁺, presuming that the binding strength is not affected by silica type. The mass-normalized retention factors for Mg²⁺ on the synthetic silicas were much lower than for the SAP₅₀₀ columns which indicates that the lipids on these particles are not as ion accessible.

The mass-normalized retention data for the Viva and Nucleosil particles trend in opposite directions as the amount of lipid added to the silica is increased. On Viva particles, which have the largest surface area, the data indicate that lipid accessibility increases with added lipid. This can be explained as the saturation of a population of small pores (< 10 nm) that host lipid layers that are less accessible to Mg²⁺. Capillary condensation may result in lipid being favorably deposited within smaller pores after solvent-casting. With increased lipid mass, the fraction of lipid occupying larger, more accessible pores increases. The trend for the Nucleosil silica is the opposite and more difficult to rationalize, with lipid accessibility decreasing as lipid mass is increased. This silica has a similar surface area to SAPs, but the pores are larger and differently structured (scanning electron microscopy images are in Supplementary Figure S12). These pores may result in solvent-casted lipid films being less uniformly distributed, and consequently increasing the formation of multi-layer or liposome structures upon rehydration that reduce Mg²⁺ access to the lipid.

On the IAM.PC particles, no retention of Ba²⁺ or Sr²⁺, and very slight retention of Mg²⁺ and Ca²⁺ (k < 0.05) was observed. Bromide was slightly retained (k = 0.18) however, suggesting that it can access residual, unreacted propylamine groups that are used to couple the lipid monomers to the silica surface [66]. Incomplete shielding of these groups has been previously reported by Escher et al. to affect

prediction of membrane partitioning for some ionizable species [67]. With metal ions, it is unclear whether the lack of retention on these materials is an issue of electrostatic repulsion or differences in lipid structure.

4. Conclusion

Association binding constants for group II metals to a zwitterionic lipid were determined using a new particle system for supported lipid bilayers in a capillary ion chromatography format. The results compliment previous metal ion studies with ZIC, showing that the same cation affinity rank is shared between single-tail phosphocholine surfactants and phosphatidylcholine lipids, and providing K_{assoc} values for comparison to those from non-chromatographic techniques. Good agreement in this regard, both quantitatively in the general values of K_{assoc} , and qualitatively in the effects that lipid phase and structure have on lipid-metal affinity, provides evidence that the system is useful for evaluating parameters that influence low-affinity and phase-sensitive binding events at dynamic lipid bilayers. Under some conditions, ion affinity to the bare silica may obscure very weak ion-lipid interactions. Compared to two alternative large pore synthetic silicas, solvent-cast lipids on SAPs were more accessible to metal ions. The present work demonstrates a viable platform for conducting microscale lipid-bilayer-based ion affinity chromatography studies. Application of the system to the study of ion binding to bilayer hosted ionophores is under investigation.

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Notes

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Figure Captions

Figure 1. Scanning electron microscopy image of a single SAP₅₀₀ (left) and a depiction of the lipid bilayers supported on constituent colloids of the SAPs packed within a fritted fused silica capillary (right).

Figure 2. Chromatograms for group II ions on bare silica (top traces) and EggPC SAP₅₀₀ columns (bottom traces) with 0.1 M NaCl in a pH 7 mobile phase (A). The t_m marker is bromide which elutes with the water void. Peaks are 1 = Ba²⁺, 2 = Sr²⁺, 3 = Mg²⁺, 4 = Ca²⁺, and the signal for each ion has been scaled for presentation. (B) A graph of retention factors for group II (top graph) and I (bottom graph) ions to both bare (gray bars) and EggPC (white bars) coated SAPs, conditions are the same as in (A).

Figure 3. Retention factors for group II ions at pH 5, 6, and 7 corrected for retention observed on bare silica SAPs. Ba²⁺ and Sr²⁺ cannot be distinguished due to symbol overlap. Diamonds = Ba²⁺, squares = Sr²⁺, triangles = Mg²⁺, circles = Ca²⁺.

Figure 4. Graph depicting the effect of the sodium counter anion (0.1 M NaX) on Ba²⁺ (light grey), Sr²⁺ (white), and Mg²⁺ (dark grey) retention on EggPC-SAP₅₀₀ columns.

Figure 5. The temperature dependence of Ba²⁺, Sr²⁺, and Mg²⁺ retention on DPPC. Mg ion retention to EggPC, which does not undergo a phase transition, is also shown. Diamonds = Ba²⁺, squares = Sr²⁺, triangles = Mg²⁺, open circles = Mg²⁺ on EggPC.

Figure 6. Graph of retention data for Mg²⁺ on silica supports evaluated in this work. Grey bars are k values normalized to SAP₅₀₀ k , and white bars are k values normalized to the lipid mass on the column type. The mass of lipid added to the particles by solvent-casting, and the percent of the lipid actually retained after the hydration and washing steps are provided in rows below the graph.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.