Supporting Information For:

Modulation of Cu$^{2+}$ Binding to Sphingosine-1-Phosphate by Lipid Charge

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Materials and Methods

Materials

1-Palmitoyl-2-oleoyl-\textit{sn}-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-\textit{sn}-glycero-3-phospho-(1’-\textit{rac}-glycerol) (POPG), 1-palmitoyl-2-oleoyl-\textit{sn}-glycero-3-phospho-L-serine (POPS), and 1,2-dilauroyl-\textit{sn}-glycero-3-phosphoethanolamine (DLPE) were purchased from Avanti Polar Lipids (Alabaster, AL). D-erythro-sphingosine-1-phosphate (S1P) was purchased from Matreya, LLC. (State College, PA). 1,2-dihexadecanoyl-\textit{sn}-glycero-3- phosphoethanolamine, triethylammonium salt (TR-DHPE) and Lissamine rhodamine B sulfonyl chloride were purchased from Life Technologies (Grand Island, NY). Dow Corning Sylgard 184 Silicone Elastomer Kit, including poly(dimethylsiloxane) (PDMS), was purchased from Ellsworth Adhesives (Germantown, WI). Copper (II) chloride (99.999% trace metal basis), disodium phosphate (PBS), TRIS((hydroxymethyl)aminomethane (TRIS), sodium chloride, and isopropyl alcohol (IPA) were purchased from Sigma Aldrich (St. Louis, MO). Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}, 30% Solution) and BODIPY 581/591 NHS Ester (Lipid Peroxidation Sensor) were purchased from ThermoFisher Scientific (Waltham, MA).

Vesicle Preparation

Small unilamellar phospholipid vesicles (SUV) were prepared using the freeze-thaw/extrusion method, described as follows.\textsuperscript{1,2} POPC, a common zwitterionic filler lipid, POPG, a negatively charged lipid, and a fluorescent probe, TR-DHPE, were dissolved in chloroform, while S1P was suspended in methanol. The desired ratios of lipids were mixed, followed by removal of organic solvents by a stream of nitrogen gas (<2 ppm moisture N\textsubscript{2}); any remaining solvents were removed
by placing the lipids under vacuum for a minimum of 2 hours. The resulting lipid film was
rehydrated with PBS buffer (10 mM PBS, 100 mM NaCl, pH 7.4) to a concentration of 1 mg/ml,
unless otherwise noted. The suspensions were subjected to 10 freeze-thaw cycles using liquid
nitrogen and a warm bath, followed by extrusion (10 mL LIPEX Extruder, Northern Lipids Inc.,
Vancouver, Canada) 10 times through a 100 nm pore track-etched polycarbonate membrane
(Whatman, Florham Park, NJ). The resulting vesicles were sized by dynamic light scattering
(NanoBrook Omni, Brookhaven Instrument Corporation, Holtsville, NY) and found to be 120 nm
± 10 nm. The vesicles were stored at 4 °C for up to three months.

**Determining Cu²⁺:S1P Stoichiometry**

To determine Cu²⁺:S1P binding stoichiometry in a bulk solution assay, 20 mol % S1P vesicles
were made similarly to the procedure described above as describe above. Instead of hydrating with
PBS buffer, however, the lipid film was rehydrated in TRIS buffer (10 mM TRIS, 100 mM NaCl,
pH 7.4) with varying concentrations of CuCl₂. The Cu²⁺ was introduced at this step to obtain
uniform concentrations of CuCl₂ both inside and outside the vesicles. It should be noted that the
vesicles were hydrated in TRIS Buffer as copper precipitates out of solution above 100 µM in PBS
buffer. The total lipid density was 0.9 mg/mL, resulting in an S1P concentration of 262 µM.
Fluorescence measurements were collected on a QE-65000-FL Spectrometer with a DH-2000
Deuterium Tungsten Halogen light source using OceanOptics software. The excitation intensity
was centered at 575.953 nm and the fluorescence intensities were read at 610.613 nm. The scans
were recorded with a 5 second integration time, and 5 scans from 5 separate vesicle preparations
were averaged for each data point.
**Glass Cleaning**

24 × 40 mm No. 1.5 Glass Coverslips (Corning, Inc., Corning, NY) were cleaned in a boiling solution of one part 7X (MP Biomedicals, Solon, OH) and three parts 18 MΩ water (Barnstead Nanopure water-purification system, ThermoFisher Scientific, Waltham, MA). Once the solution was brought to a boil, where it was translucent, cleaning was conducted for approximately one hour. The coverslips were then rinsed in 18 MΩ water and dried by a stream of nitrogen gas (<2 ppm moisture N₂). The coverslips were boiled for a second hour, in a solution of 18 MΩ water. The glass coverslips were extensively rinsed in 18 MΩ water, blown dry (<2 ppm moisture N₂) and annealed at 540 °C for 5.5 hours.

**Microfluidic Fabrication**

Pictures were taken with MACO Orthochromatic Film (35 mm) (Structure Probe, Inc., West Chester, PA) of desired channel patterns and then developed. The film was used to make a chrome mask. The chrome mask was made by photolithography onto a low reflective AZ 1500 coated chrome film on a borosilicate slide (TELIC Company, Valencia, CA). After it was fabricated, the chrome mask was used to pattern SU-8 100 Negative Tone photoresist (MicroChem Corporation, Westborough, MA) coated on a 75 × 50 × 1 mm glass slide to form a microfluidic master. Once the master was fabricated, it was used to pattern a PDMS mold. To begin, PDMS was mixed in a mass ratio of 10 parts base to 1 part polymerizer, degassed under vacuum for one hour, and poured over the master. The master/PDMS system was baked at 60°C for two hours to accelerate the polymerization process. After two hours, the master/PDMS system was allowed to cool. Following this, the PDMS was separated from the master, resulting in a nascently formed PDMS mold. Using a 23 gauge beveled polished needle, holes were punched through the PDMS mold in order to access
the channels. Finally, a PDMS/glass device was assembled by oxygen plasma treatment of the PDMS mold and a piece of cleaned borosilicate glass for 30 seconds, and then pressing the two together. The device was then baked at 100°C for one minute.

**Supported Lipid Bilayer (SLB) Formation in a Microfluidic Device**

A 10 μL lipid solution was introduced into a PDMS/glass microfluidic channel and allowed to incubate for 10 minutes. After the incubation time was finished, excess vesicles were rinsed from the away using PBS buffer (10 mM PBS, 100 mM NaCl) at the desired pH. Buffer was introduced into the device by use of polytetrafluoroethylene (PTFE) tubing (ID = 0.66 mm) and gravity flow; the average flow rate was 300 μL/hour per channel. The device was rinsed with buffer until the signal was stabilized, which took approximately an hour. Buffer was continuously flowed through all channels and the used buffer was drained through an outlet to a waste container. The fluorescence intensity from the SLBs was monitored using a Nikon Eclipse Ti2-E inverted microscope coupled with an Andor iKon-M 934 CCD camera and a Lumencor SOLA SM Light Engine using a 4x objective.

**Binding Constant Measurements**

Copper chloride solutions were consecutively introduced into the SLB-coated microfluidic channels by going from low to high concentration. Solutions were changed by unplugging the PTFE tubing from the microfluidic device and draining the solution. The tubing was placed in the new solution and drawn through the tube using a syringe with a 23-gauge needle, then plugged back into the PDMS device through the punched holes. Once equilibrium was reached, a line scan for each set of experimental conditions was recorded and exported to Microsoft Excel, where the
background signal was removed. Referencing for each channel was done from conditions in the absence of Cu$^{2+}$, where the normalized fluorescence level was set equal to 1.0. After flowing a CuCl$_2$ solution through the channel, the resulting normalized fluorescence intensity values for all points were subtracted from 1.0 in order to obtain the ‘quenched fraction’. The quenched fraction values as a function of the copper concentration were then imported into Sigma Plot and fit to Langmuir Isotherms (equation S1) in order to determine the apparent equilibrium dissociation constant, $K_{D_{App}}$:

\[
\Delta F = \Delta F_{max} \times \frac{[Cu^{2+}]}{[Cu^{2+}] + K_{D_{App}}} \quad (\text{Eqn. S1})
\]

where $\Delta F$ is the quenched fraction for TR-DHPE, $\Delta F_{max}$ is the maximum fraction quenched at saturation and [Cu$^{2+}$] represents the bulk copper concentration at equilibrium.

Measured $K_{D_{App}}$ values were corrected for the increase in surface potential ($\psi_0$) by employing the Gouy-Chapman Model (equation S2):

\[
\sigma^2 = 2 \cdot \varepsilon \cdot \varepsilon_0 \cdot k \cdot T \left( [Na^+] e^{-e\psi_0/kT} + [Cu^{2+}] e^{-2e\psi_0/kT} + [HPO_4^{2-}] e^{2e\psi_0/kT} + [Cl^-] e^{e\psi_0/kT} - [Na^+] - [Cu^{2+}] - [HPO_4^{2-}] - [Cl^-] \right) \quad (\text{Eqn. S2})
\]

where $\sigma$ is the surface charge, $\varepsilon$ is the relative permittivity of water (80.2), $\varepsilon_0$ is the permittivity of vacuum (8.85 × 10$^{-12}$ F/m), $kT$ is the Boltzmann constant times the temperature at which the experiments were performed (298 K), $e$ is the fundamental unit of charge (1.60 × 10$^{-19}$ C) and [ion] represents the concentrations of the respective ions in units of ions/m$^3$.

The surface charge, $\sigma$, is found by use of Eqn S3:

S6
\[ \sigma = \left( \frac{\chi_{\text{S1P}} \times \chi_{\text{S1P}} \times (\text{Cu}^{2+} : \text{S1P}^{-1})_{\text{Charge}}}{2} + \chi_{\text{TR}} \times \text{TR}_{\text{Charge}} \right) \times \frac{e}{S} \quad (\text{Eqn. S3}) \]

where \( \chi_{\text{S1P}} \) and \( \chi_{\text{TR-DHPE}} \) were the mole fractions of S1P and TR-DHPE, respectively, \( S1P \) was the fraction of deprotonated S1P, \( \text{Cu}^{2+} : \text{S1P}^{-1} \) Charge is -2, \( \text{TR}_{\text{Charge}} \) is -1, \( S \) is the lipid headgroup area (70 Å²) and \( e \) is fundamental unit charge (1.60 \times 10^{-19} \text{ C}). The factor of two in the denominator is present due to the binding stoichiometry between \( \text{Cu}^{2+} \) and S1P at \( K_{\text{Dapp}} \). As the surface charge is not attenuated at pH 7.4, \( S1P \) is -1, simplifying the equation 3 to be:

\[ \sigma = (\chi_{\text{S1P}} + \chi_{\text{TR}}) \times \frac{-e}{S} \quad (\text{Eqn. S4}) \]

Equation S2 can be used to solve for the surface potential, allowing for \( K_{\text{Dapp}} \) to be corrected for an increased surface potential with increasing mol % of S1P in the SLB:

\[ K_{\text{DInt}} = K_{\text{Dapp}} \times e^{-2e\varphi_0/kT} \quad (\text{Eqn. S5}) \]

The intrinsic equilibrium dissociation constant, \( K_{\text{DInt}} \), as well as the calculated surface charge (\( \sigma \)) and surface potential values (\( \varphi_0 \)) can be found in Table 2 for SLBs with 5.0 to 20 mol % S1P at pH 7.4.

Supporting Results and Figures
Figure S1: (a) Structures of TR-DHPE and (b) POPC.
**UV-Vis of Cu\(^{2+}\):Lipid Complexes** (Figure S2)

First, a desired lipid, initially suspended in an organic solution, was introduced into a scintillation vial and dried under a stream of flowing nitrogen gas (<2 ppm moisture N\(_2\)). Any remaining solvent was removed by placing the lipids under vacuum for a minimum of 2 hours. The lipid molecules were re-suspended in isopropyl alcohol to a concentration of 8 mM. An 8 mM CuCl\(_2\) solution was made separately in PBS buffer (10 mM PBS, 100 mM NaCl) at pH 7.4. To perform an experiment, 200 \(\mu\)L of the lipid vesicle solution was added to 50 \(\mu\)L of the Cu\(^{2+}\) buffer solution in a cuvette. This was placed into an Agilent 8453 G1103A UV-Vis Spectrophotometer. Data was recorded using UV-Vis Chem Station Software. Figure S2 shows the emission spectra of TR-DHPE (black) and the absorbance spectra of the resulting Cu\(^{2+}\):lipid complexes for POPS (red), S1P (green), POPE (magenta) and POPA (blue).

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**Determining Zeta Potential and Size of S1P Vesicles** (Table S1)

![Graph showing normalized intensity vs. wavelength for TR Emission, Cu\(^{2+}\):PS, Cu\(^{2+}\):S1P, Cu\(^{2+}\):PE, and Cu\(^{2+}\):PA.]

*Figure S2:* An emission spectrum of TR-DHPE (black) with absorbance spectra for Cu\(^{2+}\):POPS (red), Cu\(^{2+}\):S1P (green), Cu\(^{2+}\):POPE (magenta) and Cu\(^{2+}\):POPA (blue). Other than for the Cu\(^{2+}\):PS complex, there is poor overlap between the emission of TR-DHPE and the absorbance of the lipid complexes.
Vesicles were prepared using the freeze-thaw/sonication method. Similar to the procedure above, POPC and S1P were added to a 24 mL scintillation vial. The desired ratios of lipids were mixed, followed by removal of the organic solvents by a stream of nitrogen gas (<2 ppm moisture N2). Any remaining solvent was removed by placing the lipids under vacuum for a minimum of 2 hours. The resulting lipid film was rehydrated with TRIS buffer (10 mM TRIS, 100 mM NaCl, pH 7.4) with varying concentrations of CuCl2. Cu²⁺ was introduced at this step to obtain uniform concentrations of CuCl2 both inside and outside the vesicles. The total lipid concentration was 1.0 mg/mL, resulting in an S1P concentration of 262 μM. The vesicles were placed in a cuvette and both zeta potential and particle size information was obtained using a NanoBrook Omni instrument (Brookhaven Instrument Corporation, Holtsville, NY). It should be noted that vesicles aggregation was observed under certainly conditions. For example, upon the addition of 100 μM CuCl2, a second size population arose from the aggregation of vesicles. As such, two numbers are reported in the size column at 100 μM CuCl2 and above. The first represents the initial vesicle size, while the second one reports the size after addition of CuCl2.
In order to determine if the bilayers used in these studies were two-dimensionally fluid at room temperature (23 °C), fluorescence recovery after photobleaching (FRAP) measurements were performed. FRAP experiments were performed on a Nikon Eclipse TE-2000-U inverted microscope through a 10X objective. A 568 nm line from a Stabilite 2018 2.5 W mixed Ar+/Kr+ laser (Spectra Physics, Mountain View, CA) was employed to bleach TR-DHPE in the S1P bilayers. The diameter of the spot was measured to be 22 μm, and the fluorescence intensity in the bleached area was monitored over time with images were taken every three seconds. Fluorescence recovery over time (Figure S3) was used in order to determine the mobile fraction and diffusion coefficients of TR-DHPE. All bilayers in these experiments were found to have diffusion coefficients of 1.2 ± .2 μm²/s and mobile fractions ranging from 94 to 98%.

The mobile fraction and diffusion coefficients were determined by diving the fluorescence intensity from the bleached spot in each time frame by the fluorescence intensity of the spot prior to bleaching in order to correct the images for differences in light intensity. The percent recovery of the fluorescence intensity as a function of time, F(t), was determined by equation S6:

| Cu²⁺ Added (μM) | ζ Potential (mV) | Size (nm)   |
|-----------------|------------------|-------------|
| 0               | -19.4 ± 0.7      | 128 ± 0.3   |
| 25              | -19.7 ± 0.9      | 124 ± 0.7   |
| 50              | -20.9 ± 0.6      | 148 ± 0.6   |
| 75              | -20.4 ± 0.8      | 155 ± 0.9   |
| 100             | -13.9 ± 0.8      | 150 ± 1.0   |
| 150             | -10.4 ± 0.5      | 150 ± 1.0   |
| 200             | -7.51 ± 0.5      | 150 ± 1.0   |

Fluorescence Recovery After Photobleaching (FRAP) (Figure S3)
\[ F(t) = \frac{F_t - F_0}{1 - F_0} \quad \text{(Eqn. S6)} \]

where \( F_t \) is the normalized light intensity of the bleach spot at time \( t \), and \( F_0 \) is the normalized intensity of the spot at \( t = 0 \) s. The resulting recovery data was then plotted as a function of time and fit to a single exponential:

\[ F(t) = a \left( 1 - e^{-bt} \right) \quad \text{(Eqn. S7)} \]

where \( a \) is the mobile fraction of the bilayer and \( b \) is a value employed in equation S8 to calculate the diffusion coefficient, \( D \), by equation S9.

\[ \tau_{1/2} = \frac{\ln(2)}{b} \quad D = \frac{w^2}{4\tau_{1/2}} \times \gamma \quad \text{(Eqns. S8 & S9)} \]

where \( w \) is the diameter of the laser beam and \( \gamma \) is the correction factor with a value of 0.88.\(^3\)
Figure S3: (a) Fluorescence images of a 10 mol % S1P bilayer with 0.5 mol% TR-DHPE and 89.5 mol% POPC just after bleaching (left) and 10 minutes after bleaching (right). The red circle in the images highlights the bleached area. (b) Corresponding FRAP recovery curve that was used to calculate the diffusion coefficient and the mobile fraction of the bilayer.
Investigating S1P under a 100X Oil Objective (Figure S4)

Bilayers were investigated in the absence and presence of Cu$^{2+}$ under a 100x oil immersion objective to see if the addition of the metal ions induced domain formation. Specifically, we examined 20 mol % S1P, 79.5 mol % POPC and 0.5 mol % TR-DHPE SLBs under high magnification to ensure that the bilayers were domain free. SLBs were formed by incubating 100 μL of 1 mg/mL lipid vesicles in a PDMS well on a cleaned borosilicate glass slide for 10-minutes. Excess vesicles were rinsed from the device using 18 mΩ water, followed by scratching the surface of the glass slide with an 18-guage needle. The slide was then rinsed with 18 MΩ water or 25 μM Cu$^{2+}$/PBS Buffer (10 mM PBS, 100 mM NaCl at pH 7.4), respectively, to rinse off any vesicles that were detached during the scratching process. The surface was scratched in order to provide a point of contrast and to aid in focusing the image. The resulting images are shown in Figure S4. As can be seen, no domains were visible in the absence, or presence, of Cu$^{2+}$.

Figure S4: 100X Oil Objective images for SLBs composed 20 mol % S1P, 79.5 mol % POPC and 0.5 mol % TR rinsed with (a) 18 MΩ water and (b) 25 μM Cu$^{2+}$/PBS buffer (10 mM PBS, 100 mM NaCl at pH 7.4), respectively. The scale bar in each image is 20 μM.
**Amine Deprotonation of S1P at pH 9.4 (Table S2)**

Due to the 1:2 binding of Cu²⁺–S1P, the equilibrium dissociation constant at pH 9.4 should be corrected for the presence of doubly deprotonated phosphate in the SLB (S1P²⁻). Under these conditions, the surface charge can be determined by employing a modified version of the surface charge equation (Eqn. S3) to account for the fractions of S1P that are -1 charge (doubly deprotonated) and -2 charge:

\[
\sigma = \left( [S1P^2-] \times \chi_{S1P} \times (S1P^2-_{\text{charge}}) \right) + \left[ \frac{S1P^- \times \chi_{S1P} \times (Cu^{2+}:S1P^-_{\text{charge}})}{2} \right] + \left[ \chi_{TR} \times TR_{\text{charge}} \right] \times \frac{e}{S}
\]

where \( \chi_{S1P} \) and \( \chi_{TR-DHPE} \) are the mole fractions of S1P and TR-DHPE, respectively. \( S1P^- \) is the fraction of deprotonated S1P\(^-\), \( Cu^{2+} \)–S1P\(^-\)\(_{\text{charge}}\) is -2 due to the 1:2 binding. \( S1P^2- \) is the fraction of deprotonated S1P\(^2-\), \( S1P^2-\)\(_{\text{charge}}\) is -2. The charge on TR-DHPE is -1. \( S \) is the lipid headgroup area (70 Å\(^2\)) and \( e \) is fundamental unit of charge (1.60 \( \times \) 10\(^{-19} \) C). The factor of two in the denominator is present due to the binding stoichiometry between Cu\(^{2+}\)–S1P at \( K_{D_{\text{app}}} \). Equation S10 simplifies to:

\[
\sigma = ([S1P^2- \times \chi_{S1P} \times 2] + [(S1P^-) \times \chi_{S1P}] + \chi_{TR}) \times \frac{-e}{S}
\]

Prior to binding at pH 9.4, approaching pKa₃ of S1P, 26% of S1P is triply deprotonated to have a -2 charge. This leads to the bilayer having a surface potential of -34 mV. This differs from pH 7.4 where all S1P has a charge of just -1. The surface potential of the SLB is calculated to be -28 mV.
for a 10 mol % S1P at pH 7.4. When correcting $K_{D,app}$ for the surface potential, one must do so for the charge after Cu$^{2+}$ binds. Once Cu$^{2+}$ binds to the triply deprotonated S1P (S1P$^{2-}$), S1P resumes its -1 charge due to binding to both the amine and phosphate, as shown in Figure 1a.

Due to the definition of $K_{D,app}$ being when 50 % of all binding sites are occupied, there are four scenarios for Cu$^{2+}$–S1P binding at pH 9.4. The first is where Cu$^{2+}$ binds only to S1P$^{1-}$, leaving all S1P$^{2-}$ unbound (26 % of total S1P is -2 charge in the bilayer). The second scenario would be where half of the S1P$^{2-}$ sites are occupied, reducing the amount of S1P$^{2-}$ in the SLB to be 13 %. Third, with 50 mol % of sites being occupied at $K_{D,app}$, 25 % of those sites can be S1P$^{2-}$, with the remaining 25 % being S1P$^{1-}$, lessening the amount of triply deprotonated S1P to be 1%. Lastly, Cu$^{2+}$ could first bind to all triply deprotonated S1P, and then the remainder of occupied sites would be S1P$^{1-}$. This would result in no S1P$^{2-}$ in the SLB once copper binds, as the resulting Cu$^{2+}$–S1P complex reverts to a -1 charge. Under these four possibilities for bound Cu$^{2+}$, the surface potential would range from -34 mV to -28 mV, for the first and last scenario, respectively. Moreover, $K_{D,int}$ may vary between $4.0 \times 10^{-6}$ M and $6.3 \times 10^{-6}$ M (Table S2).

| Scenario                                      | 0% Bound to S1P$^{2-}$ | ½ of S1P$^{2-}$ Sites Occupied | ½ of all sites are S1P$^{2-}$ | Binds to S1P$^{2-}$, then S1P$^{1-}$ |
|-----------------------------------------------|------------------------|---------------------------------|-------------------------------|--------------------------------------|
| % S1P$^{2-}$ in SLB                           | 26 %                   | 13 %                            | 1 %                           | 0 %                                  |
| % S1P$^{1-}$ in SLB                           | 74 %                   | 87 %                            | 99 %                          | 100 %                                |
| $\sigma$ (V/cm$^2$)                           | $-3.0 \times 10^{-2}$  | $-2.7 \times 10^{-2}$           | $-2.4 \times 10^{-2}$         | $-2.4 \times 10^{-2}$               |
| $\psi$ (mV)                                   | -34                    | -31                             | -28                           | -28                                  |
| $K_{D,int}$ (M)                               | $6.3 \times 10^{-6}$   | $5.0 \times 10^{-6}$            | $4.0 \times 10^{-6}$          | $4.0 \times 10^{-6}$                |
Determining Particle Dispersity of PBS Buffer Solutions Containing Cu\(^{2+}\) (Table S3)

Buffer containing 10 mM PBS and 100 mM NaCl was prepared and adjusted to pH 7.4 and pH 9.4. Next, CuCl\(_2\) was introduced to make solutions ranging from 0.01 to 100 μM Cu\(^{2+}\). The solutions were sized by dynamic light scattering (NanoBrook Omni, Brookhaven Instrument Corporation, Holtsville, NY) in order to find the polydispersity. By comparison of the samples to that with 0 μM Cu\(^{2+}\), we may gather information regarding the formation of Cu(OH)\(_2\) at the respective pH values. The dispersity for the Cu\(^{2+}\) solutions at pH 7.4, as seen in Table S2, do not vary from that of buffer. Conversely, due to the K\(_{SP}\) of Cu(OH)\(_2\), there is already deviation in the particle dispersity at pH 9.4 when 15 μM Cu\(^{2+}\) is added to the buffer. This indicates the formation of Cu(OH)\(_2\) aggregates, which is why the Cu\(^{2+}\) titrations were not performed above 10 μM Cu\(^{2+}\) for the experiments performed at pH 9.4.

### Table S3: Particle Dispersity of PBS Buffer as a Function of Cu\(^{2+}\) Concentration

| Cu\(^{2+}\) Added (μM) | pH 7.4     | pH 9.4     |
|------------------------|------------|------------|
| 0                      | 0.40 ± 0.05| 0.45 ± 0.02|
| 1 × 10\(^{-2}\)       | 0.37 ± 0.03| 0.45 ± 0.08|
| 1 × 10\(^{-1}\)       | 0.42 ± 0.01| 0.47 ± 0.07|
| 1                      | 0.46 ± 0.02| 0.43 ± 0.03|
| 10                     | 0.47 ± 0.03| 0.43 ± 0.05|
| 15                     | 0.46 ± 0.05| 1.30 ± 0.72|
| 100                    | 0.53 ± 0.04| 3.80 ± 2.37|
Figure S5: Quenched fraction of SLBs containing 10 mol% S1P, 89.5 mol% POPC and 0.5 mol% TR-DHPE at three different pH values vs. Cu^{2+} concentration, expanded to include data up to 100 μM for pH 5.4.
Investigation of Cu\(^{2+}\) Binding to POPA (Figures S6 and S7)

To monitor Cu\(^{2+}\) binding to POPA, Figure 4a, a different pH sensitive dye was employed, POPE-ortho-rhodamine B. TR-DHPE couldn’t be used due to the shift in the Cu\(^{2+}\)--PA absorbance curve. Figure S2 shows the emission spectra of TR-DHPE (black) and the absorbance spectra of Cu\(^{2+}\):lipid complexes with PS (red), S1P (green), PE (magenta) and PA (blue). For PS, S1P and PE, the amine allows for transfer of electron density to Cu\(^{2+}\). For PA, without an amine, the peak position is red shifted compared to the others as it only contains a hard acid, phosphate. Thus, TR-DHPE is not an applicable dye for this system.

The synthesis and separation of ortho-rhodamine B conjugated POPE (oRB-POPE) was reported in a previous publication. As copper binds to the phosphate headgroup of POPA, the second phosphate group is deprotonated, shifting the pH of the system and turning the fluorescence off, Figure S6. However, Cu\(^{2+}\) is able to bind directly with ORB. In order to investigate Cu\(^{2+}\) binding solely to POPA, a Cu\(^{2+}\) titration with a 0 mol% POPA SLB was measured and subtracted from the quenched fractions with 10 mol % POPA SLBs (Figure S7). The data points from the difference were then fit to a Langmuir Isotherm, Equation S1, in order to determine the apparent equilibrium dissociation constant, and plotted in Figure 5b. The equilibrium dissociation constant for Cu\(^{2+}\) to POPA was determined to be 100 \mu M.
**Figure S6**: The structural change upon protonating and deprotonating oLRB. Upon decreasing the pH, the dye shows a turn-on fluorescence response. The left-hand-side depicts the "off" state and the right-hand-side depicts the "on" state.

**Figure S7**: Titration curve representing the fluorescence quenching with increasing CuCl$_2$ concentrations for SLBs containing 0.5 mol % ORB, 0 or 10 mol % POPA, and 89.5 or 99.5 mol % POPC, respectively. The red data points (0 mol % POPA) represent the titration curve for a POPC SLB in which Cu$^{2+}$ binds directly with ORB. The blue data points represent an SLB that has Cu$^{2+}$ binding to both POPA and ORB. By taking the difference between these two curves, the titration curve for 10 mol % POPA, Figure 5b, could be determined.
Subtraction of Collisional Quenching for POPG Bilayers (Figures S8 and S9)

At high concentrations of Cu$^{2+}$, for example the titration for POPG (Figure S8a), the fluorescence quenching of the SLBs has two causes. First, quenching occurs due to the formation of Cu$^{2+}$–S1P complexes. The second source is due to the collisional quenching that occurs when Cu$^{2+}$ directly interacts with TR-DHPE. In order to determine quenching of TR-DHPE that results from the formation of Cu$^{2+}$–S1P complexes, the collisional quenching must be subtracted off from the overall quenching. To achieve this, a Cu$^{2+}$ titration with a 0 mol% POPG SLB was performed. As seen in Figure S8b, linear quenching was found as indicated by the black points and the corresponding linear fit. Next, the experiment was repeated with 10 mol% POPG and the data look almost the same. The blue triangles at the bottom of the figure show the subtraction of one from the other.

Figure S9 plots quenched response of an SLB containing 10 mol% POPG (red data points) compared to that of 10 mol% S1P (black data points). The 10 mol% POPG data points resulted in very little quenching of TR-DHPE in the concentration range investigated for S1P.
Figure S8: (a) Structure of POPG. (b) Titration curve representing fluorescence quenching with increasing CuCl$_2$ concentrations. The data for a 0 mol% POPG bilayer, black data points (behind red points in many cases), were fit to a straight line. The red data points (0 mol% + 10 mol% POPG) represent the titration curve for the POPG SLB. By obtaining the difference between the two curves, the titration curve for 10 mol% POPG (blue data points) was found.
Figure S9: Quenched response of an SLB containing 10 mol% POPG (red data points) compared to that of 10 mol% S1P (black data points). The experiments were conducted at pH 7.4. For S1P, a buffer containing 10 mM PBS and 100 mM NaCl was used. For POPG, 10 mM TRIS and 100 mM NaCl was used. CuCl$_2$ was introduced into the respective buffer systems. The points and error bars represent data points and standard deviations based on 10 measurements, respectively. The data is plotted as a fraction of dye quenched by Cu$^{2+}$ (one minus the normalized fluorescence).
Figure S10: (a) $K_{D\text{App}}$ values and (b) $K_{D\text{Int}}$ values plotted as a function of mol\% S1P (bottom x-axis) and S1P surface density (top x-axis). The line in each plot is the best fit to the two-step bivalent binding at interfaces.
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