Supplementary Information

Vesicle aggregation by multivalent ligands: relating crosslinking ability to surface affinity.
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S.1. ITC measurements on the binding of membrane-bound Cu(1) to ligands 2, 3 and 4.

Ligand binding was monitored after dilution of the parent vesicle solution 1 in 10 to give a solution 2 mM in lipid. Heat flow to and from the sample was measured after each addition of an aliquot of ligands **2**, **3** or **4** (2 mM in histidine residues). All calorimetric measurements were repeated several times; shown in Figures S1 and S2 are some representative data.

Table S1. Thermodynamic data obtained at 298 K from the ITC titrations of Cu(**1**)/DSPC vesicles (0.1 mM Cu(**1**) and 2 mM in total lipid) with histidine-containing ligands **2**, **3** or **4**.

Ligand	K_{av} (M^{-1})	ΔH (kJ mol^{-1})	ΔS (J $mol^{-1} K^{-1}$)
2 $(His)_{39}$	$(5.6 \pm 1.6) \times 10^3$	-14 ± 3	25 ± 9
3 $(His)_{226}$	$(5.8 \pm 1.5) \times 10^3$	-9 ± 2	42 ± 9
4 AcHis	$(3.2 \pm 0.5) \times 10^3$	-6.6 ± 0.2	45 ± 2

Enthalpy changes due to non-specific vesicle-vesicle interactions during vesicle aggregation have been measured by other researchers and were found to be endothermic and small,[S1,S2] heat flows were dominated by the formation of specific crosslinking bonds. To try and estimate the contribution that vesicle-vesicle interactions make to the enthalpy change in our system, the dilution enthalpy of a concentrated DSPC vesicle suspension was measured. Addition of DSPC vesicles (800 nm diameter, 20 mM in lipid) in MOPS buffer at pH 7.4 to buffer solution revealed an exothermic enthalpy of dilution of -0.03 kJ mol^{-1} per phospholipid, equivalent to an endothermic enthalpy change of +0.6 kJ mol^{-1} per Cu(**1**) lipid for the reverse process; concentrating Cu(**1**)/DSPC vesicles (5 % mol/mol Cu(**1**)). Comparison to the enthalpy changes in Table S1 suggests heat flows resulting from bringing vesicles into proximity *via* aggregation should be a small contribution to the net ΔH.

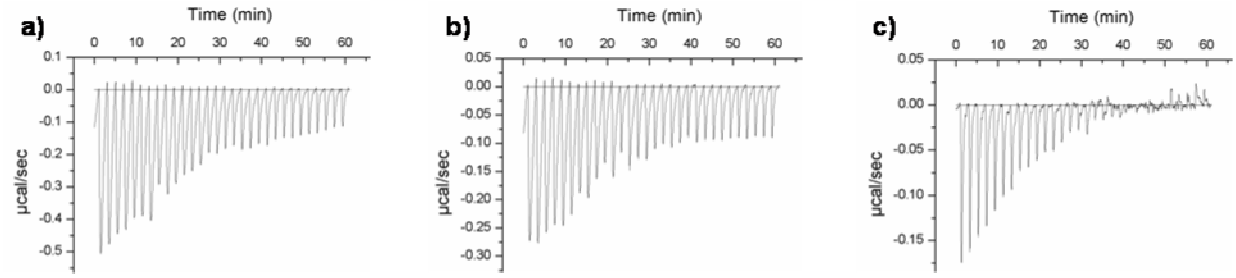

Figure S1. Representative ITC traces for the titration of DSPC vesicle suspensions containing 5% mol/mol Cu(**1**) (2 mM in lipid, 0.1 mM in Cu(**1**)) with L-histidine containing ligands ***without*** enthalpies of ligand dilution subtracted a) **2** $(His)_{39}$, b) **3** $(His)_{226}$ and c) acetyl histidine **4**.

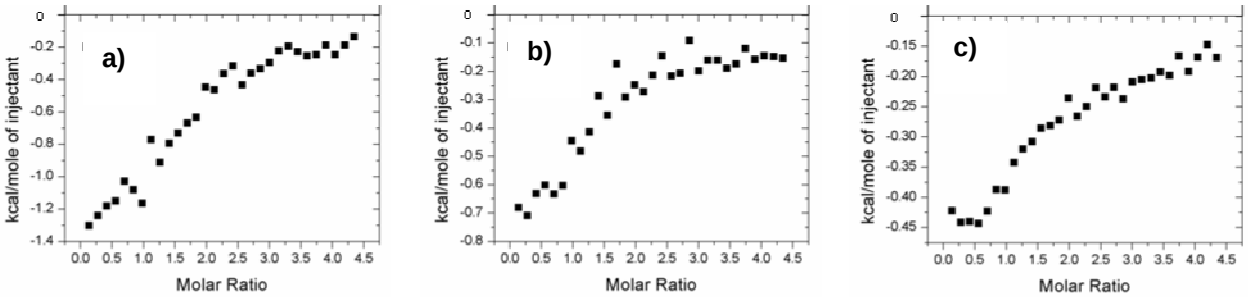

Figure S2. Representative ITC data for the titration of DSPC vesicle suspensions containing 5% mol/mol Cu(**1**) (2 mM in lipid, 0.1 mM in Cu(**1**)) with L-histidine containing ligands ***with*** enthalpies of ligand dilution subtracted a) **2** $(His)_{39}$, b) **3** $(His)_{226}$ and c) acetyl histidine **4**.

Curve fits to the subtracted data were obtained using the One Site binding isotherm in Origin® with the number of equivalent binding sites n = 1. In cases of weak binding the value of "n" is difficult to determine from the shape of the titration curve and may give unrealistic values during fitting. In these cases it is better to fix n = 1; the insignificance of any potential second binding constant was evident during the analysis of the cumulative heat release data. Nonetheless fitting the data using floating "n" in the One Site binding isotherm in Origin® gave values of K that were the same within error as those found with "n" fixed at 1 (see Table S2).

S.2. The relationship between individual microscopic binding constants and the valence-corrected binding constant.

Calculating the binding constant on a per binding site or valence-corrected basis greatly simplifies the analysis of multivalent binding for highly valent species, and affords the geometric mean binding constant. The valence-corrected free energy change from a multivalent ligand interacting with n receptors is:[S3]

$$\Delta G_{av} = \frac{1}{n}\left(\Delta G_1 + \Delta G_2 + \Delta G_3 + ... + \Delta G_n\right)$$

Thus $K_{av} = (K_1 K_2 K_3 ..... K_n)^{1/n}$ where each K_i is a microscopic binding constant.

It can be shown that K_{av} is the value calculated when determining a valence-corrected binding constant using the One Site binding model in the Origin™ curve-fitting package accompanying a Microcal VP-ITC. This package uses the fractional occupancy of sites, θ, to calculate K. The expression used to calculate K from θ and the concentration of receptor [R] in the Single Set of Identical Sites Model in Origin™ is given by:[S4]

$$K = \frac{\theta}{(1-\theta)[\mathrm{R}]} \quad \text{or} \quad \theta = \frac{[\mathrm{R}]K}{1+[\mathrm{R}]K} \qquad \textbf{Equation S1.}$$

This expression is equivalent to the relationship between θ and K_{av} for an n-valent ligand.[S5]

$$\theta = \frac{r}{n} = \frac{[\mathrm{R}]}{(K_d)+[\mathrm{R}]} = \frac{[\mathrm{R}]}{\left(\frac{1}{K_{av}}\right)+[\mathrm{R}]} = \frac{[\mathrm{R}]K_{av}}{1+[\mathrm{R}]K_{av}} \qquad \textbf{Equation S2.}$$

Thus the valence-corrected value of K calculated using the Single Set of Identical Sites Model in Origin™ is actually better described as K_{av}, the geometric mean of all the microscopic binding constants K_1……K_n. Strictly speaking, Equation S2 is only true if all binding sites are identical, but a good fit to our data was obtained using this model presumably because of the high degree of multivalency in our ligands and the similarity between the individual affinities of the different binding sites. Indeed many other workers have used this valence-corrected approach to assess average binding constants by ITC.[S6]

S.3. Comparison of ITC binding constants obtained using different methods to fit the ITC binding.

Table S2. Comparison of ITC binding constants obtained using different methods to fit the ITC binding.

Method	*Ligand*		
	2 $(His)_{39}$	**3** $(His)_{226}$	**4** AcHis
K_{av} (L mol^{-1}) Origin® with the heats of ligand dilution subtracted and "*n*" = 1.	$(5.6 \pm 1.6) \times 10^3$	$(5.8 \pm 1.5) \times 10^3$	$(3.2 \pm 0.5) \times 10^3$
K_{av} (L mol^{-1}) Origin® with the heats of ligand interaction with undoped DSPC vesicles subtracted	$(4.5 \pm 1.5) \times 10^3$	$(5.4 \pm 1.6) \times 10^3$	$(2.7 \pm 0.4) \times 10^3$
K_{av} (L mol^{-1}) Dynafit using a 1:1 binding model	$(7.2 \pm 2.6) \times 10^3$	$(7.0 \pm 1.4) \times 10^3$	$(2.5 \pm 0.2) \times 10^3$
K_{av} (L mol^{-1}) Origin® with the heats of ligand dilution subtracted and "*n*" floating	$(9.4 \pm 2.3) \times 10^3$	$(8.7 \pm 2.3) \times 10^3$	$(3.0 \pm 0.5) \times 10^3$

S.4. Comparison of the valence-corrected turbidimetric and ITC data obtained for the binding of membrane-bound Cu(1) to ligands 2, 3 and 4 respectively.

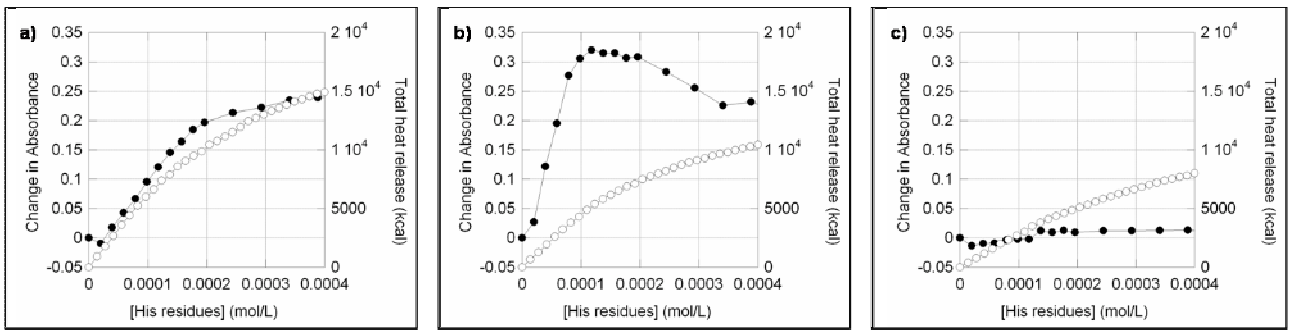

Figure S3. Plots showing the lack of correlation between turbidity change (due to vesicle aggregation) and cumulative heat release (due to the binding of histidine containing ligands to the surface of Cu(**1**)/DSPC vesicles). Data is shown for titration of vesicles (5 % mol/mol Cu(**1**) and 2 mM in total lipid) with ligands a) **2**, b) **3** and c) **4**.

S.5. Each observed binding event is composed of two microscopic binding events; inter- and intra- membrane binding.

If we assume K_i^{inter} is less than K_1 for all $i > 1$, then at low values of χ, $K_{av} < K_1$. By making a few assumptions, it is possible to show this mathematically:

- Each binding unit on the multivalent ligand can exist in three configurations: unbound, bound to receptor R through an intermembrane link or bound to receptor R through an intramembrane link. Therefore the partition function is:[S7]

$$\Xi = 1 + K_i^{\text{inter}}[\text{R}] + K_i^{\text{intra}}[\text{R}]$$

Since $\overline{N} = \frac{[\text{R}]}{\Xi}\frac{\partial \Xi}{\partial [\text{R}]}$, then:

$$\overline{N} = \frac{[\text{R}](K_i^{\text{inter}} + K_i^{\text{intra}})}{1 + (K_i^{\text{inter}} + K_i^{\text{intra}})[\text{R}]} \qquad \textbf{Equation S3}.$$

which is equivalent to the binding isotherm for single site binding of a ligand which can only bind in one mode: $\overline{N} = \frac{K_i[\text{R}]}{1 + K_i[\text{R}]}$

where $K_i = K_i^{\text{inter}} + K_i^{\text{intra}}$

thus $K_i^{\text{obs}} = K_i^{\text{inter}} + \kappa_i \chi$ and $\frac{K_i^{\text{obs}}}{K_1} = \frac{K_i^{\text{inter}}}{K_1} + \frac{\kappa_i}{K_1}\chi$ **Equation S4**.

S.6. Increasing ligand valency may not change K_{av} significantly for a highly multivalent ligand

- The average (geometric mean) binding constant for a multivalent ligand is given by:

$$K_{av}^n = \sqrt[n]{K_1 K_2^{\text{obs}} \ldots K_n^{\text{obs}}} \quad \text{so} \quad K_{av}^n = K_1 \sqrt[n]{\frac{K_2^{\text{obs}}}{K_1} \ldots \frac{K_n^{\text{obs}}}{K_1}} \qquad \textbf{Equation S5}.$$

Combining Eqns. S4 and S5 gives:

$$K_{av}^n = K_1 \sqrt[n]{\left(\frac{K_2^{\text{inter}}}{K_1} + \frac{\kappa_2}{K_1}\chi\right) \ldots \left(\frac{K_n^{\text{inter}}}{K_1} + \frac{\kappa_n}{K_1}\chi\right)} \qquad \textbf{Equation S6}.$$

Since $K_i^{\text{inter}} < K_1$ for all $2 \le i \le n$, then as $\chi \to 0$, then:

$$\left(\frac{K_i^{\text{inter}}}{K_1} + \frac{\kappa_i}{K_1}\chi\right) < 1$$

for all $2 \le i \le n$. Therefore $K_{av}{}^{\text{n}} < K_1$ at low values of χ.

Furthermore, provided that we assumed all K_i are approximately the same for $i > 1$ (as implied from the ITC data we obtained from our multivalent system), then for high values of n there will be little change in the affinity of the multivalent ligand for the surface as the valency increases, i.e. $K_{av}^n \approx K_{av}^{n+1}$.

Since $K_{av}^n = K_1 \sqrt[n]{\frac{K_2^{obs}}{K_1} \ldots \frac{K_n^{obs}}{K_1}}$ then $\frac{K_{av}^n}{K_1} = \sqrt[n]{\frac{K_2^{obs}}{K_1} \ldots \frac{K_n^{obs}}{K_1}}$

Since we have assumed all K_i are approximately the same as K_2^{obs} then:

$$\frac{K_{av}^n}{K_1} \approx \left(\frac{K_2^{obs}}{K_1}\right)^{\frac{n}{n+1}} \qquad \textbf{Equation S7.}$$

For large values of n then $\frac{n}{n+1} \to 1$, so $K_{av}^n \approx K_2^{obs}$.

Furthermore, increasing the value of n will not significantly change $K_{av}{}^{n}$ as $\frac{n}{n+1}$ will still approximate one, i.e. for $K_{av}{}^{n+1}$, $\frac{n+1}{n+2} \rightarrow 1$

Supplementary References

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