Vesicle aggregation by multivalent ligands: relating crosslinking ability to surface affinity†

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In an effort to improve the stability of our tissue-mimetic vesicle aggregates, we have investigated how increasing the valency of our multivalent crosslinking ligand, poly-L-histidine, affected both the extent of vesicle aggregation and the affinity of the multivalent ligand for the synthetic receptor Cu(**1**) embedded in the vesicle membranes. Although increasing ligand valency gave the anticipated increase in the size of the vesicle aggregates, isothermal calorimetric studies did not show the expected increase in the valence-corrected binding constant for the embedded receptors. To explain both observations, we have developed a simple new binding model that encompasses both multivalent binding to receptors on a single vesicle surface (intramembrane binding) and vesicle crosslinking (intermembrane binding).

Introduction

Nature uses cooperative multivalent binding between external ligands and membrane-bound receptors to mediate key cellular processes like adhesion and signaling. These processes are initiated by highly specific binding of multivalent ligands to receptors on the cell surface, resulting in the formation of multiple inter- and/or intramembrane links. Although the binding of most natural multivalent ligands to cell surface receptors is tightly controlled, immunoglobulins are an interesting class of natural multivalent ligands able to form both inter- and intramembrane links. For example, bivalent IgG can activate the immune response *via* two different binding modes; it can either bind two antigens on the same cell surface or agglutinate cells by crosslinking antigens on different surfaces.[1] Moreover, increasing the valency of these natural ligands improves both binding and crosslinking ability; the larger decavalent antibody IgM not only has high avidity for multivalent antigens like viruses but is a particularly strong agglutinating agent, the latter effect ascribed to the greater distance between the antigen binding sites.[1] This behaviour is not restricted to viral or cell surface antigens since IgG antibodies will also bind vesicle-embedded antigens in both modes.[2] Much like antibodies, many synthetic multivalent ligands have been found to bind cells in either mode,[3] although often in an unpredictable fashion.[3c] Understanding the relationship between these competing processes is important because developing multivalent ligands designed to crosslink cells could lead to drugs with new modes of action, for example drugs that agglutinate pathogens to form large aggregates, or drugs that form "cancer-nets" around solid tumors and prevent tumor growth.[3b,3c]

Understanding intermembrane (cell adhesion) or intramembrane binding (signaling) of natural multivalent ligands is difficult due to the complexity of biological systems. To circumvent this problem, vesicles with synthetic receptors embedded in their membranes have been employed as simple cell mimics and used to develop systems that emulate cell signaling[4] and cell adhesion.[5] In particular, mimicking cell adhesion is an area of much current interest due to the desirability of synthetic tissue-mimetic materials.[6] Several researchers have used multivalent chemical "glues" to aggregate receptor-doped vesicles; these "glues" have included tetravalent streptavidin (crosslinking biotin lipids),[7,8] iron(II) (crosslinking terpyridine lipids),[9] poly-L-arginine[10] and guanidinylated dendrimers (both crosslinking phosphate lipids).[11] We are also interested in the aggregation of vesicles by multivalent ligands, in particular determining how ligand valency affects the balance between intermembrane crosslinking and intramembrane ligand binding. Curiously, the effect of increasing ligand valency on the extent of vesicle aggregation has been little explored, though Sideratou *et al.* reported that doubling the valency of guanidinylated dendrimers from 32 to 64 gave an increase in vesicle crosslinking, even when compared on a per binding unit basis.[11]

In recent studies of vesicle adhesion, we used a highly multivalent ligand, a 39mer of histidine (**2**, Fig. 1), to crosslink distearoyl

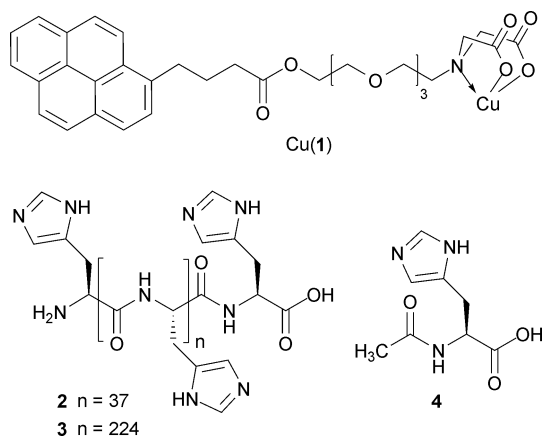

Fig. 1 Synthetic receptor Cu(**1**) and histidine-containing ligands **2**, **3** and **4**.

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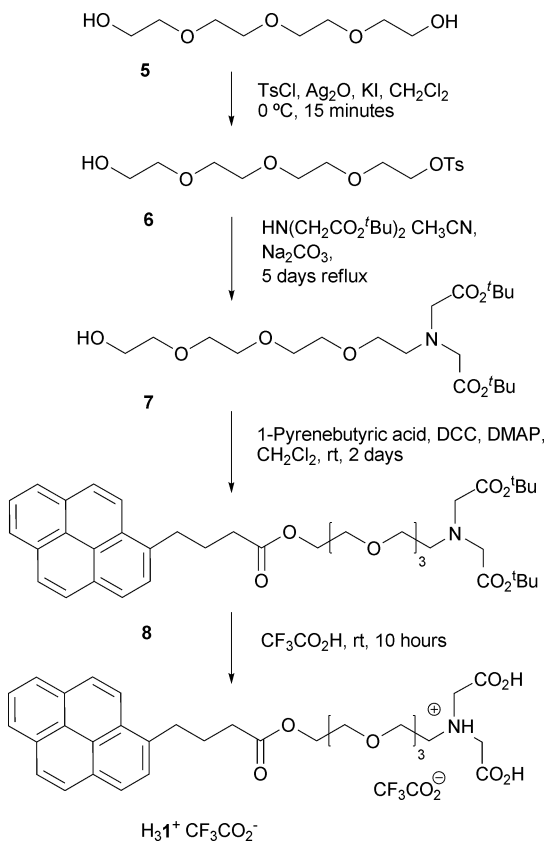
phosphatidylcholine (DSPC) vesicles doped with 5% mol/mol of a synthetic receptor Cu(**1**) (Fig. 1).[12] The relatively weak histidine–Cu(IDA) interaction, with a binding constant around 10^3 M^{-1}, was chosen because it is a good model of the weak interactions used in cell adhesion ($K \sim 10^4$ M^{-1}).[13] Addition of **2** to a suspension of Cu(**1**)-coated vesicles caused an increase in turbidity, and stable vesicle aggregates were observed by fluorescence microscopy, but these studies did not give any indication of the extent or strength of binding to Cu(**1**).[14] We then speculated that, as observed with immunoglobulins, increasing the valency of the multivalent glue would enhance both inter- and intramembrane binding; allowing us to use the former to increase the extent of vesicle aggregation and the latter to improve the stability of the resulting aggregates.

Presented here are our investigations into how changing ligand multivalency affected vesicle aggregation and the strength of binding to vesicle-embedded receptors. It was possible to change the valency of the poly-L-histidine ligand **2** easily, allowing binding strength and crosslinking ability to be correlated with the degree of multivalency. $(His)_{226}$ (**3**) and *N*-acetyl-L-histidine **4** are commercially available variants of **2**, which are longer and shorter respectively; furthermore, monovalent *N*-acetyl-L-histidine should be a binding, but non-crosslinking, control compound. As previously, fluorescence microscopy and changes in solution turbidity would reveal the ability of ligands **2**, **3**, and **4** to crosslink vesicles, whilst the binding affinity of the histidine ligands for the membrane-bound lipid Cu(**1**) could be determined by isothermal calorimetry (ITC), a technique widely used to study ligand binding to vesicle surfaces.[10,15] This ability to study crosslinking separately from all other binding events allowed us to elucidate the relationship between inter- and intramembrane binding.



Scheme 1 Synthesis of lipid H_2**1**.

Results and discussion

Synthesis of lipid $H_2$1 and Cu(1)/DSPC vesicles

The iminodiacetate-capped lipid H_2**1** has several features that make it ideal for these studies. The iminodiacetate groups chelate copper tightly to give receptor Cu(**1**) quantitatively,[16] and Cu(**1**) binds monovalent histidine ligands with affinities similar to those between selectins and monovalent carbohydrates.[13] The pyrene group is the membrane anchor, which also allows visualization of Cu(**1**)-vesicles by fluorescence microscopy and indicates any Cu(**1**) clustering in the membrane.

The monotosylation of tetraethylene glycol by *para*-toluenesulfonyl chloride, a key step in the synthesis of lipid H_2**1** (Scheme 1), was carried out in the presence of freshly prepared silver(I) oxide and potassium iodide, a method used to desymmetrise a range of diols.[17a] The monofunctionalised tosylate then required heating at reflux for an extended period with di(*tert*-butyl)-protected iminodiacetic acid in acetonitrile to displace the tosylate and afford the protected binding group **7**.[17b] The fluorescent membrane anchor was then appended to **7** through dicyclohexylcarbodiimide-mediated esterification with 1-pyrenebutyric acid. Although neat trifluoroacetic acid was required to remove the *tert*-butyl protecting groups from **8**, which limited the transferability of this methodology to other syntheses, this protecting group proved to be adequate for the synthesis of lipid H_2**1**. The *tert*-butyl protecting groups were removed quantitatively within 10 hours to afford lipid H_2**1** as the trifluoroacetic acid salt; provided water was strictly excluded, the ester linkage in H_2**1** remained intact. This salt was suitable for further use, but free-base H_2**1** was obtained by reverse-phase chromatography on octadecyl-functionalised silica gel, eluting with acetone–water (60 : 40).

To solely examine the effect of changing the ligand valency on vesicle binding and aggregation, the membrane loading of receptor Cu(**1**) was fixed at 5% mol/mol, a membrane concentration known to give vesicle aggregation with ligand **2**.[12] Unilamellar vesicles (approx. 800 nm diameter, 20 mM lipid in 20 mM MOPS buffer, 100 mM NaCl, pH 7.4) composed of DSPC doped with H_2**1** were prepared by extrusion of a phospholipid suspension through 800 nm pore size polycarbonate membranes at 60 °C, a temperature above the phase transition temperature of DSPC. The fluorescence spectra of these vesicle suspensions showed only pyrene monomer emission, indicating dispersal of lipid H_2**1** over the vesicle surface. Lipid H_2**1** in DSPC bilayers chelates copper(II) with an average binding constant of 4×10^7 M^{-1},[12] so addition of one equivalent of Cu(II) to H_2**1** at millimolar concentrations gave the amine-binding synthetic receptor Cu(**1**) quantitatively. This addition of copper(II) to H_2**1**/DSPC vesicles gave no significant increase in solution turbidity, and fluorescence microscopy confirmed no vesicle aggregation during formation of Cu(**1**)/DSPC vesicles.[18] Titration of copper(II) into H_2**1**/DSPC vesicles (0.1 μM lipid H_2**1**) has shown that the entire cohort of lipid H_2**1** is available for complexation, and flip-flop between bilayer leaflets is rapid ($t_{1/2} < 30$ s).[12]

In the turbidity and ITC studies, the key comparison is between ligands **2** and **3**, which will show whether increasing poly-L-histidine valency has given a commensurate increase in crosslinking efficiency and affinity for membrane-bound Cu(**1**). The monomeric ligand **4** should not crosslink vesicles, but it will give the affinity of a solution-phase histidine group for membrane-embedded Cu(**1**). This value will be an approximation of the equilibrium constant of the first binding event (K_1) that leads to immobilization of poly-L-histidine on the vesicle surface.

Vesicle aggregation by poly-L-histidine

The 39mer of histidine, $(His)_{39}$ **2** (~14 nm extended length), had previously been shown to aggregate Cu(**1**)/DSPC vesicles, so the same conditions were employed to test the ability of $(His)_{226}$ **3** (~82 nm extended length) and *N*-acetyl-L-histidine **4** to aggregate 800 nm diameter vesicles. Titration of *N*-acetyl-L-histidine **4** (2 mM) into suspensions of Cu(**1**)/DSPC vesicles (2 mM in lipid, 0.1 mM in Cu(**1**)) gave little change in turbidity, as monitored at 700 nm in the UV-visible spectrum, showing that the morphology of the vesicles remained unchanged (Fig. 2). Fluorescence microscopy showed only individual vesicles and no aggregates. As observed previously, titration of the shorter poly-L-histidine **2** into Cu(**1**)/DSPC vesicles resulted in a strong increase in turbidity (Fig. 2), which started to plateau after 0.125 equivalents of **2** had been added (5 histidine residues per Cu(**1**)). In comparison, titration of the longer poly-L-histidine **3** into Cu(**1**)/DSPC vesicles resulted in a stronger increase in turbidity earlier in the titration (Fig. 2) which peaked at 0.005 equivalents of **3** (1.1 His residues per Cu(**1**)). Receptor Cu(**1**) was always required for aggregation to occur; no aggregation was observed when any of ligands **2**, **3** or **4** were mixed with undoped DSPC vesicle suspensions.

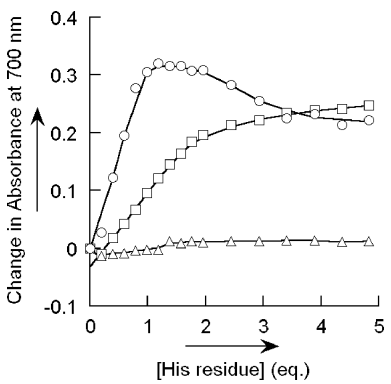


Fig. 2 The change in solution turbidity resulting from aggregation of Cu(**1**)/DSPC vesicles (5% mol/mol Cu(**1**) and 2 mM total lipid) due to addition of histidine-containing ligands **2** (□), **3** (○) or **4** (△). The lines are smooth curve fits to guide the eye.

The median effective concentration (EC_{50}) values for aggregation reveal the effect of multivalency; the concentration of **2** required to give a 50% turbidity response was 3.3 μM (0.13 mM histidine residues) whilst the concentration of **3** that gave a 50% response was only 0.25 μM (0.055 mM histidine residues). Furthermore, even when compared on a valence-corrected basis the longer poly-L-histidine **3** is over twice as effective at aggregating Cu(**1**)/DSPC vesicles compared to **2**.

Inspection of the turbid mixtures by fluorescence microscopy showed that addition of 2.5 μM **2** or 0.45 μM **3** (one eq. His residues in each case) gave large clusters of intact aggregated vesicles (Fig. 3).[19] There was a wide distribution of aggregate sizes in each sample, but for $(His)_{39}$ the average cross-sectional area of the aggregates was 99 ± 33 μm^2, whilst larger aggregates were observed for $(His)_{226}$ with an average cross-sectional area of 180 ± 81 μm^2. The larger size of the aggregates observed in mixtures of **3** and Cu(**1**)/DSPC vesicles correlates well with the higher turbidity of these mixtures.[6b,11,14]

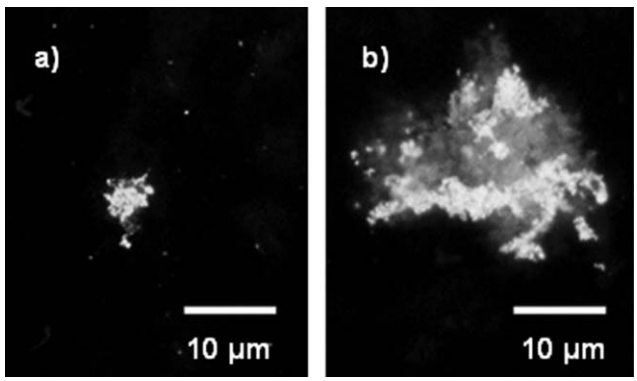


Fig. 3 Representative fluorescence micrographs showing vesicle aggregates caused by addition of (a) 2.5 μM ligand **2** or (b) 0.45 μM **3** to a suspension of Cu(**1**)/DSPC vesicles (5% mol/mol Cu(**1**) and 2 mM in total lipid).

It was tempting to conclude that the greater ability of **3** to aggregate vesicles was due to stronger cooperative binding of this more multivalent ligand to membrane-embedded Cu(**1**). However, this would be misleading since changes in turbidity do not reflect the strength and extent of intramembrane binding within a single vesicle. Therefore, to measure affinity for Cu(**1**) we used ITC to measure heat flow to and from Cu(**1**)/DSPC suspensions during the addition of **2**, **3** or **4**, which allowed us to evaluate their respective valence-corrected binding constants to membrane-bound Cu(**1**).

Measurement of binding by isothermal calorimetry

Isothermal calorimetry has been used to study a range of binding events at phospholipid bilayer surfaces,[10,15] but only rarely used to study the formation of vesicle aggregates. Vesicles in suspension do not normally aggregate because of repulsion between the hydrated bilayer surfaces (often referred to as the "hydration force");[15b,20] a specific crosslinking interaction is necessary for vesicles to aggregate. Furthermore, this repulsion between vesicle surfaces usually requires a spacer group to be present to distance the vesicle surface from the crosslinking groups.[5b,15c,21] In our system a tetra-ethylene glycol spacer was incorporated into lipid H_2**1**; we found the copper(II) complex of *N*-nonyliminodiacetate embedded at 5% mol/mol in DSPC vesicles was unable to mediate vesicle adhesion by poly-L-histidine. The tetra-ethylene glycol linker minimises unfavourable vesicle–vesicle interactions, allowing ΔG for the formation of crosslinking bonds to overcome the unfavourable ΔG for vesicle aggregation. This vesicle–vesicle repulsion will weaken the crosslinking interactions (represented by the binding constant K_i^{inter}) relative to the solution control (K_1) (*vide infra*, Fig. 4).

The ITC analysis conditions were identical to the conditions used for the turbidimetric analyses. Thus solutions of ligands **2**, **3** and **4** were made up in MOPS buffer (20 mM) at pH 7.4 with each solution 2 mM in histidine residues. Titration of these solutions into vesicle-free buffer showed the respective heats of dilution were small ($\sim$0 kJ mol^{-1} for **2**, < -0.4 kJ mol^{-1} for **3**, and < -0.9 kJ mol^{-1} for **4**) and fitting of the ITC data showed no measurable binding. To check for non-specific binding to the surface of DSPC vesicles, these three ligand solutions were also titrated isothermally into undoped DSPC vesicles under the same conditions. In each case little heat release was observed ($\sim$0 kJ mol^{-1} for **2**, < -0.5 kJ mol^{-1} for **3**, and < -0.8 kJ mol^{-1} for **4**) and there was no measurable binding. In conjunction with the absence of turbidity changes, these data suggested that none of **2**, **3** or **4** associated with undoped DSPC vesicles. These solutions of ligands **2**, **3** and **4** were then titrated into suspensions of 800 nm diameter DSPC vesicles doped with 5% mol/mol Cu(**1**) (0.1 mM bulk concentration of vesicle-embedded Cu(**1**)). In each case significant heat release was observed upon addition of the histidine-containing ligands to the vesicle suspensions, with the lowest enthalpy change observed during the titration of **4** into Cu(**1**)/DSPC vesicles.

Analyzing the ITC data on a per ligand basis for these highly multivalent poly-L-histidine ligands was impractical, so instead the binding isotherms were analysed on a per binding unit (valence-corrected) basis.[22,23] This afforded the average binding constants K_{av}, each of which is the geometric mean of the individual microscopic binding constants.[24,25] Since Cu(**1**) should have two free sites for coordinating histidine, the refined ITC data with the respective heats of ligand dilution subtracted was fitted using both the one-site and two-site binding models in the Origin® curve-fitting package accompanying the Microcal VP-ITC. Both binding models were found to fit the data, but the second binding constant in the two-site model was found to be redundant. These binding constants were then checked by fitting the cumulative heat release to Cu(IDA) + (His) and Cu(IDA) + 2(His) binding isotherms.[26] The 1 + 1 model gave the best fit to all the data sets and gave similar values of K_{av} as those obtained with Origin®. If the data from the control titrations of undoped DSPC vesicles with ligands **2**, **3** or **4** were subtracted from the Cu(**1**)/DSPC vesicle titration data, the calculated binding constants were the same within error.[25]

For ligands **2**, **3** or **4** complexing to Cu(**1**) we found the measured affinities per histidine residue lay in a small range between 3 and 7×10^3 M^{-1}. In all cases, binding of histidine-containing ligands **2**, **3** or **4** to membrane-embedded Cu(**1**) was exothermic (-7 to -14 kJ mol^{-1}) and entropically favourable (25 to 45 J mol^{-1} K^{-1}).[25] Table 1 shows the striking similarity between the valence-corrected affinities of the different ligands for Cu(**1**) embedded in DSPC vesicles, quite at odds with the wide variation observed in the turbidimetric data.[25] Indeed, there is little correlation with the turbidimetric data: *N*-acetyl-L-histidine, which does not aggregate vesicles, bound to Cu(**1**) only slightly more weakly than the multivalent ligands, whilst short poly-L-histidine **2** bound with the same affinity (within error) as the more strongly aggregating and higher valency poly-L-histidine **3**.

Table 1 Binding constants 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}
2 $(His)_{39}$	$(5.6 \pm 1.6) \times 10^3$
3 $(His)_{226}$	$(5.8 \pm 1.5) \times 10^3$
4 AcHis	$(3.2 \pm 0.5) \times 10^3$

Analysis and binding model

Several different theoretical frameworks have been developed to explain the binding of multivalent ligands to membrane-bound receptors. However, these frameworks have regarded multivalent ligands either as "stickers" that crosslink vesicles[2b] or chelating ligands that bind solely to a single vesicle surface.[27] We propose here a model that accommodates both inter- and intramembrane binding of multivalent ligands, and can be used to predict and control the extent of vesicle crosslinking by multivalent ligands.

The binding of multivalent ligands to membrane-bound receptors is often considered to be "cooperative" because the binding constant, calculated using the bulk concentrations of the binding species, is higher than that calculated for binding to analogous receptors that are not membrane-bound. This apparent binding enhancement actually largely stems from the concentrating effect of embedding receptors within a vesicle membrane.[28,29] The effective molarity of a membrane-bound receptor correlates with the percentage loading of receptor in the membrane, χ, and it has been demonstrated that as χ increases intramembrane binding becomes increasingly favourable.[28,29] Whether or not this is regarded as true cooperativity depends upon the definition used; some definitions of cooperativity include concentrating effects caused by linking binding units together or embedding them in a membrane,[30] whilst others exclude such concentrating effects.[31] The lower polarity of the interface can also enhance binding to membrane-embedded receptors, but our monovalent control **4** has a value of K(**4**) (3×10^3 M^{-1}) that lies within the range observed for the binding of histidine ligands to Cu(IDA) complexes in solution (1×10^3 M^{-1} to 6.3×10^3 M^{-1}).[32]

The per unit binding constant for $(His)_{39}$ **2** to Cu(**1**) in DSPC bilayers, K_{av}(**2**) $= 6 \times 10^3$ M^{-1}, is only slightly greater than that of *N*-acetyl-L-histidine. Similarly, with the longer analogue $(His)_{226}$ **3**, the per unit binding constant for membrane-embedded Cu(**1**), K_{av}(**3**) $= 6 \times 10^3$ M^{-1} is, within error, the same as that observed for **2**. The similarity between these three values suggests that following immobilization of poly-L-histidine on the vesicle surface, the strength of which can be estimated from K(**4**), subsequent binding of histidine residues to Cu(**1**) in the membrane is only slightly more favourable than the initial binding interaction. It would appear that despite the very high effective concentration of Cu(**1**) in the vesicle membrane (5% mol/mol corresponds to a local concentration of 0.06 M), intramembrane binding is actually quite weak. However, such relatively weak intramembrane binding is essential for vesicle aggregation to occur in our system; it allows intermembrane binding (crosslinking) to compete with intramembrane binding (chelation) of the poly-L-histidine.

It may be that molecular constraints in our system inhibit intramembrane binding and promote vesicle aggregation; for example, poly-L-histidine may adopt conformations that hinder coordination of all histidine residues to embedded Cu(**1**) and/or the gel-phase DSPC membrane may prevent intramembrane clustering of Cu(**1**) in response to binding.[33] However, multivalent

ligand-mediated vesicle aggregation has been observed in other systems that use highly flexible ligands and/or receptors embedded in fluid-phase membranes, two factors that should promote intramembrane coordination over vesicle aggregation.[7–11,34] This suggests there must be other factors that affect the balance between inter- and intramembrane binding.

To understand this interplay between inter- and intramembrane binding, we have expanded upon a published binding model[28] to develop a simple new model for the binding of multivalent ligands to vesicle-bound receptors that also accounts for vesicle crosslinking. Fig. 4 shows the first binding event, represented by K_1, and the two subsequent binding possibilities; intermembrane binding (K_2^{inter}) and intramembrane binding (K_2^{intra}).

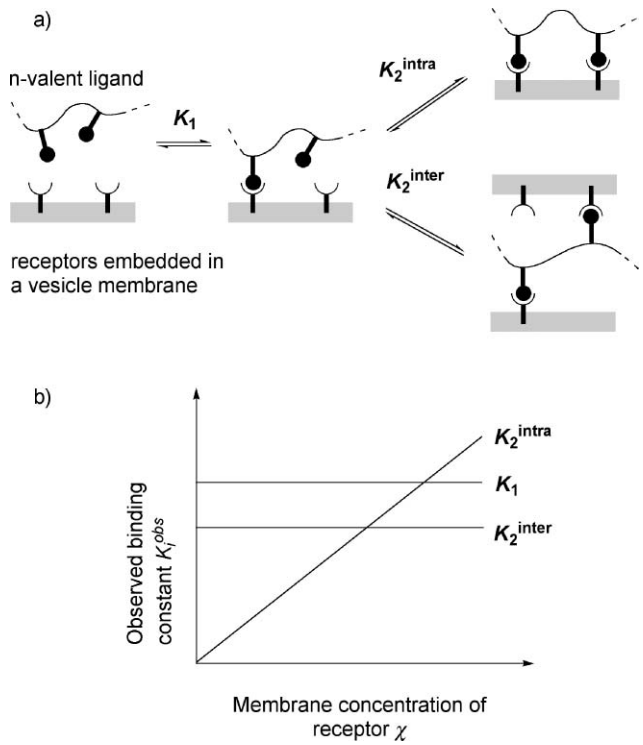


Fig. 4 (a) Schematic representation of the initial binding events between an *n*-valent ligand and vesicle-embedded receptors. (b) Graph depicting the predicted dependence of the intermembrane binding constant (K_2^{inter}) and the intramembrane binding constant (K_2^{intra}) on the membrane concentration of receptor (χ).

In our simple model, following immobilization of the *n*-valent ligand on a vesicle surface, each ensuing i^{th} binding event has these two possible binding modes (K_i^{inter} and K_i^{intra}, $2 \geq i \geq n$). For example, the binding constants K_1 and K_2^{inter} are analogous and independent of the membrane loading of receptor, yet K_2^{inter} should be weaker than K_1 due to the repulsive interactions between vesicles. In contrast, the intramembrane binding constant K_2^{intra} is proportional to the percentage loading, χ, of the receptor in the membrane (eqn (1));[28] experimentally this linear dependence of K_2^{intra} on χ has been borne out by studies of bivalent IgG antibodies binding to membrane-embedded synthetic antigens.[35]

$$K_2^{\text{intra}} = \left[\frac{K_2^{\text{memb}}}{100\ [\text{R}]_{\text{T}} V_{\text{m}}}\right]\chi \qquad (1)$$

where $[\text{R}]_{\text{T}}$ = the total concentration of the receptor in the solution (in mol L^{-1}), and V_{m} = molar volume of the phospholipid (in L mol^{-1}).

A new binding constant, K_2^{memb}, is used in eqn (1); this binding constant is calculated from the concentrations of binding partners within the volume of the membrane only and is independent of membrane loading. Experimentally it can be useful to keep the bulk receptor concentration ($[\text{R}]_{\text{T}}$) constant because the effect of varying χ on ligand binding is immediately apparent from direct comparison of the titration curves.[28]

The observed binding constant for the second binding event is composed of two microscopic binding constants that represent inter- and intramembrane binding:[25]

$$K_2^{\text{obs}} = K_2^{\text{inter}} + K_2^{\text{inter}} \qquad (2)$$

or

$$K_2^{\text{obs}} = K_2^{\text{inter}} + \kappa_2\chi \quad \text{where} \quad \kappa_2 = \frac{K_2^{\text{memb}}}{100\ [\text{R}]_{\text{T}} V_{\text{m}}} \qquad (3)$$

Thus by extension each i^{th} binding event of a multivalent ligand can be represented by:

$$K_i^{\text{obs}} = K_i^{\text{inter}} + \kappa_i\chi \qquad (4)$$

We can use this model to analyse the behaviour of our system. At $\chi = 5$, the geometric mean of K_1, K_i^{inter} and K_i^{intra} ($2 \geq i \geq 39$) for **2** (K_{av}(**2**)) is only slightly greater than the initial binding event K_1 determined using ligand **4**. This suggests that intramembrane binding of Cu(**1**) is intrinsically unfavourable, given that at a membrane loading of 5% mol/mol the effective local concentration of Cu(**1**) available for intramembrane binding is 0.06 M. Furthermore, the valence-corrected binding constant does not increase significantly when the valency of the poly-L-histidine is increased, *i.e.* K_{av}(**2**) ~ K_{av}(**3**), though more extensive vesicle aggregation is observed for **3** compared to **2**. This suggests each of K_2^{obs} to K_n^{obs} could be relatively similar; indeed, if we assume all K_i^{obs} ($2 \geq i \geq n$) have a similar value (*i.e.* ~ K_2^{obs}) then the averaging effect of valence-correcting binding constants for highly multivalent ligands means that K_{av} will approximate K_2^{obs} for large values of *n*, and increasing ligand valency further will not change K_{av} significantly.[25] However, due to the concomitant six-fold increase in ligand length decreasing the unfavourable effect of vesicle-vesicle repulsion, the extent of crosslinking increases (though this increase in K_i^{inter} for ligand **3** seems not to be sufficient to increase K_{av}(**3**) significantly). Our results and binding model (Fig. 4b) show the crucial role played by membrane loading of receptor; in effect it can be more important than ligand valency in determining the net binding strength K_{av}.

Conversely, researchers sometimes desire strong multivalent binding to a single vesicle surface without vesicle aggregation. Coating vesicles with PEG-capped lipids has often been used to prevent unwanted vesicle aggregation, as it increases steric hindrance between vesicles and diminishes K_i^{inter}.[21,36] Our model suggests an alternative method to decrease crosslinking; building structural features into the receptor that give high intrinsic membrane binding constants (K_i^{memb}), thus leading to high values of K_i^{intra} at low membrane loadings and promoting intramembrane over intermembrane binding. On this point, it is interesting to note that multivalent ligand-mediated vesicle aggregation has been observed in many synthetic systems, often despite high

receptor membrane concentrations (up to 9% mol/mol).[9–11,34] This suggests that in synthetic systems intramembrane binding is often intrinsically weak, and specific features to encourage intramembrane binding over crosslinking need to be incorporated into synthetic receptors, for example perfluoroalkyl groups to facilitate intramembrane clustering of receptors.[37]

Conclusion

We have used vesicle aggregation by poly-L-histidine to explore how increasing ligand valency affects the balance between intra- and intermembrane binding of multivalent ligands. In our model system, poly-L-histidine ligands of different valencies were added to DSPC vesicles containing the synthetic copper(II) iminodiacetate receptor Cu(**1**) embedded in their membranes. Turbidity and ITC studies revealed that at a 5% mol/mol membrane concentration of Cu(**1**), increasing the valency of the poly-L-histidine increased vesicle aggregation but did not lead to significantly stronger binding when analysed on a per binding unit basis.

To explain our observations we have proposed a binding model that accommodates intra- and intermembrane binding. This model suggests the ability of poly-L-histidine to form multiple links to receptors on the same vesicle surface is intrinsically poor but should improve at high membrane loadings of receptor Cu(**1**). Indeed, the difficulty of forming multiple bonds to receptors embedded within the same vesicle surface must be a common effect in synthetic systems, as many other researchers also observe extensive vesicle crosslinking by multivalent ligands. Our binding model also suggests that at low membrane loadings of receptor the multivalent ligand will bind more weakly than the separated monovalent binding units.[25] In this case vesicle crosslinking will predominate over intramembrane chelation.

We are now endeavouring to create synthetic receptors and multivalent ligands with structural features that will favour either intramembrane binding or intermembrane crosslinking. We hope this will give some insight into how cells control the balance between receptor clustering and agglutination, both of which involve cells binding to multivalent ligands.

Experimental

11-Hydroxy-3,6,9-trioxaundecyl *para*-toluenesulfonate (6)

To a stirred solution of tetra(ethylene glycol) **5** (344 mg, 1.7 mmol, 1 eq.) in dichloromethane (20 mL) was added fresh Ag_2O (720 mg, 3.1 mmol, 1.8 eq.), *para*-toluenesulfonyl chloride (398 mg, 2.1 mmol, 1.2 eq.), and KI (66 mg, 0.4 mmol, 0.23 eq.). The reaction mixture was stirred at 0 °C for 15 minutes, then filtered through a small pad of silica gel and washed with ethyl acetate. The solvent was removed from the filtrate under reduced pressure and the residue purified by column chromatography (ethyl acetate, silica gel) to give the desired monotosylated product (465 mg, 1.3 mmol, 79%) as a colourless oil. $R_f = 0.17$ (ethyl acetate, silica gel); ν_{max}(Nujol mull)/cm^{-1} 664, 768, 815, 920, 1009, 1095, 1126, 1177, 1596, 1724, 3421; δ_H(300 MHz, $CDCl_3$, 25 °C) 2.48 (s, 3H, tosyl CH_3), 3.6–3.8 (m, 14H, 7 × glycol chain CH_2), 4.19 (t, $J = 4.8$ Hz, 2H, CH_2OTs), 7.37 (d, $J = 8.5$ Hz, 2H, 2 × *meta* aromatic C*H*), 7.83 (d, $J = 8.5$ Hz, 2H, 2 × *ortho* aromatic C*H*); δ_C(100 MHz, $CDCl_3$, 25 °C) 22.1 (tosyl CH_3), 62.2, 69.1, 69.7, 70.7, 70.9, 71.1, 72.9, 83.7 (all glycol CH_2), 128.4 (tosyl CH), 130.3 (tosyl CH), 131.9 (tosyl CSO_3), 143.6 (tosyl *C*CH_3); *m*/*z* (ES-HRMS) 371.1135 (M + H$^+$. $C_{15}H_{24}NaO_7S^+$ requires 371.1140). Elemental analysis: Found: C, 50.27; H, 7.17; S, 8.71; Calc. for $C_{15}H_{25}O_{7.5}S$ ($C_{15}H_{24}O_7S{\cdot}0.5H_2O$) C, 50.41; H, 7.05; S, 8.97%.

11-Bis(*tert*-butoxycarbonylmethyl)amino-3,6,9-trioxaundecanol (7)

11-Hydroxy-3,6,9-trioxaundecyl *para*-toluenesulfonate **6** (174 mg, 0.5 mmol, 1 eq.) was dissolved in a stirred solution of di(*tert*-butyl) iminodiacetate (173 mg, 0.5 mmol, 1 eq.) and sodium carbonate (106 mg, 1 mmol, 2 eq.) in acetonitrile (10 mL). The resulting solution was allowed to stir at reflux for 5 days under dry air. The solvent was removed under reduced pressure and the crude product purified by column chromatography (98 : 2 chloroform–methanol, silica gel) to give the product (187 mg, 0.42 mmol, 84%) as a colourless oil. $R_f = 0.15$ (98 : 2 chloroform–methanol, silica gel). ν_{max}(Nujol mull)/cm^{-1} 1149, 1223, 1735, 3419; δ_H(300 MHz, $CDCl_3$, 25 °C) 1.48 (18H, s, 2 × C(CH_3)$_3$), 2.97 (t, $J = 6.0$ Hz, 2H, CH_2N), 3.52 (s, 4H, 2 × CH_2CO$_2$^{t}Bu), 3.63–3.70 (m, 12H, 6 × glycol CH_2), 3.75 (br t, $J \sim 5$ Hz, 2H, CH_2OH); δ_C(75 MHz, $CDCl_3$, 25 °C) 28.6 (C(*C*H$_3$)$_3$), 53.8 (CH_2N), 57.0 (*C*H$_2$CO$_2$^{t}Bu), 62.1, 70.6, 70.7, 70.9, 71.0, 72.9, 81.2 (all glycol CH_2), 171.1 (*C*O$_2$^{t}Bu); *m*/*z* (ES-HRMS) 422.2748 (M + H$^+$. $C_{20}H_{40}NO_8^+$ requires 422.2748).

11-Bis(*tert*-butoxycarbonylmethyl)amino-3,6,9-trioxaundecyl 4-(pyren-1-yl)butanoate (8)

To a solution of compound **7** (170 mg, 0.4 mmol, 1 eq.) in dry dichloromethane (2 mL) was added 1,3-dicyclohexylcarbodiimide (90 mg, 0.44 mmol, 1.1 eq.), 1-pyrenebutyric acid (126 mg, 0.44 mmol, 1.1 eq.) and *N*,*N*-dimethylaminopyridine (4.80 mg, 0.04 mmol, 0.1 eq.). The reaction mixture was stirred under argon at room temperature for 2 days. After evaporation of the solvent under reduced pressure, the residue was dissolved in ethyl acetate (5 mL), and stirred at 0 °C for 1 hour, then filtered and the precipitate washed with cold ethyl acetate. After evaporation of the filtrate, the residue was purified by column chromatography (1 : 1 : 1 : 0.03 ethyl acetate–cyclohexane–dichloromethane–triethylamine, silica gel) to give the desired ester (121 mg, 0.175 mmol, 44%) as a yellow oil. $R_f = 0.24$ (1 : 1 : 1 : 0.03 ethyl acetate–cyclohexane–dichloromethane–triethylamine, silica gel). ν_{max}(Nujol mull)/cm^{-1} 1149, 1218, 1249, 1605, 1732; δ_H(300 MHz, $CDCl_3$, 25 °C) $\delta = 1.45$ (s, 18H, 2 × C(CH_3)$_3$), 2.21 (tt, $J_1 = 7.7$ Hz, $J_2 = 7.2$ Hz, 2H, ArCH$_2$CH_2CH$_2$), 2.51 (t, $J = 7.2$ Hz, 2H, ArCH$_2$CH$_2$CH_2), 2.94 (t, $J = 5.8$ Hz, 2H, CH_2N), 3.41 (t, $J = 7.7$ Hz, 2H, ArCH_2CH$_2$CH$_2$), 3.50 (s, 4H, 2 × CH_2CO$_2$^{t}Bu), 3.58–3.64 (m, 10H, 5 × glycol CH_2), 3.70 (t, $J = 4.9$ Hz, 2H, glycol CH_2), 4.26 (t, $J = 4.7$, 2H, CO$_2$CH_2), 7.87 (d, $J = 7.5$ Hz, 1H, Ar CH), 7.97–8.20 (m, 7H, 7 × Ar CH), 8.32 (d, $J = 9.4$ Hz, 1H, Ar CH); δ_C(75 MHz, $CDCl_3$, 25 °C) 27.2, (ArCH$_2$*C*H$_2$CH$_2$), 28.6 (C(*C*H$_3$)$_3$), 33.1, Ar*C*H$_2$CH$_2$CH$_2$), 34.2, ArCH$_2$CH$_2$*C*H$_2$), 53.8 (*C*H$_2$N), 57.0 (*C*H$_2$CO$_2$^{t}Bu), 63.9, 69.5, 70.7, 70.8, 70.9 (all glycol *C*H$_2$), 81.2 (*C*(CH$_3$)), 123.7, 125.1, 125.2, 125.3, 126.2, 127.1, 127.8, 127.9 (all Ar *C*H), 129.1, 130.3, 131.4, 131.8, 136.1 (all Ar *C*), 171.1 (CO_2R), 173.8 (CO_2R); *m*/*z* (ES-HRMS) 692.3793 (M + H$^+$. $C_{40}H_{54}NO_9^+$ requires 692.3793).

Elemental analysis: Found: C, 67.75; H, 7.53; N, 1.90 Calc. for $C_{40}H_{55}NO_{10}$ ($C_{40}H_{53}NO_9 \cdot H_2O$): C, 67.68; H, 7.81; N, 1.97%.

11-Bis(carboxymethyl)amino-3,6,9-trioxaundecyl 4-(pyren-1-yl)butanoate ($H_2$1)

Compound **8** (120 mg, 0.2 mmol, 1 eq.) was dissolved in trifluoroacetic acid (1.65 mL). The solution became brown in colour after a few minutes stirring. After stirring for 5 hours at room temperature, further trifluoroacetic acid (0.75 mL) was added, and the mixture stirred for another 5 hours. The solvent was removed under reduced pressure, then the resultant sticky grey-green oil triturated with dry ether (3 mL). The residue was redissolved in dichloromethane and the solution evaporated under reduced pressure, which gave the product as a yellow sticky tar. The crude product was then purified by reversed-phase column chromatography (60 : 40 acetone–water, octadecyl-functionalised silica gel) to give the desired product as a colourless oil (59 mg, 0.10 mmol, 51%). ν_{max}(Nujol mull)/cm^{-1} 714, 850, 1138, 1192, 1676, 1735; δ_H(300 MHz, $CDCl_3$, 25 °C) 2.13–2.20 (m, 2H, $ArCH_2CH_2CH_2$), 2.46 (t, $J = 5.8$ Hz, 2H, $ArCH_2CH_2CH_2$), 3.35 (t, $J = 7.4$ Hz, 2H, $ArCH_2CH_2CH_2$), 3.44–3.66 (m, 16H, 8 × glycol CH_2), 4.23 (s, 4H, 2 × CH_2CO_2H), 7.83 (d, $J = 7.5$ Hz, 1H, Ar C*H*), 7.94–8.15 (m, 7H, 7 × Ar C*H*), 8.27 (d, $J = 9.0$ Hz, 1H, Ar C*H*); δ_C(75 MHz, $CDCl_3$, 25 °C) 27.1 ($ArCH_2CH_2CH_2$), 33.1 ($ArCH_2CH_2CH_2$), 34.1 ($ArCH_2CH_2CH_2$), 56.0 (CH_2N), 56.4 (CH_2CO_2H), 63.7, 65.6, 69.4, 70.4, 70.7 (all glycol CH_2), 123.7, 125.2, 125.3, 126.3, 127.1, 127.8, 127.9 (all Ar CH), 129.0, 130.2, 131.2, 131.7, 136.0 (all Ar C), 168.2 (CO_2CH_2), 174.2 (CO_2H); *m/z* (ES^+) 580.4 ($[M + H]^+$), 602.4 ($[M + Na]^+$), 618.4 ($[M + K]^+$); *m/z* (ES-HRMS) 580.2524 ($M + H^+$. $C_{32}H_{38}NO_9^+$ requires 580.2541). Elemental analysis prior to reverse phase chromatography: Found: C, 52.80; H, 5.08; N, 1.75; Calc. for $C_{35}H_{42}Cl_2F_3NO_{12}$ ($C_{34}H_{38}F_3NO_{11}.CH_2Cl_2.H_2O$): C, 52.77; H, 5.31; N, 1.76%. Elemental analysis after reverse phase chromatography: Found: C, 60.80; H, 6.01; N, 2.23; Calc. for $C_{32}H_{39}NNaO_{10.5}$ ($C_{32}H_{36}NNaO_9 \cdot 1.5H_2O$) C, 61.14; H, 6.25; N, 2.23%.

Synthesis of vesicles

Unilamellar vesicles were prepared by dissolving the appropriate phosphatidylcholine (16 mg, 20 μmol) and the required amount (1 μmol, 5 mol% of synthetic lipid relative to phospholipid) of synthetic lipid in spectroscopic-grade ethanol-free chloroform (5 mL), followed by removal of the solvent to give a thin film of phospholipid on the interior of a round-bottomed flask. The appropriate buffer (20 mM MOPS, 100 mM NaCl, pH 7.4 at 25 °C, 1 mL) was added to the flask, and the thin film detached by vortex mixing to give a suspension of multilamellar vesicles. These were extruded through a single 800 nm polycarbonate membrane at ~60 °C, a temperature above the T_m of DSPC (54 °C) using an Avestin Liposofast extrusion apparatus to give unilamellar vesicles. The final concentration of phospholipid in these parent vesicle suspensions was 20 mM, and that of the synthetic lipid 1 mM.

Turbidimetric measurements

The parent vesicle suspensions were diluted 1 in 10 to give suspensions 2 mM in lipid, which were added to a cuvette and left to equilibrate for 5 minutes at 25 °C before addition of external reagents. After each addition of an aliquot of ligand solution (containing ligand **2**, **3** or **4**, each solution 2 mM in histidine residues in 20 mM MOPS, 100 mM NaCl, pH 7.4) the absorbance spectra of the suspensions were measured from 200 to 900 nm. All turbidity measurements were repeated several times.

Fluorescence microscopy

Vesicle aggregates were observed on glass slides after dilution of the parent vesicle solutions 1 in 10 with buffer solution to give solutions that were 2 mM in lipid.

ITC measurements

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 ligand solution (containing ligand **2**, **3** or **4**, each solution 2 mM in histidine residues in 20 mM MOPS, 100 mM NaCl, pH 7.4). All calorimetric measurements were repeated several times.

Fluorescence

The parent vesicle solutions were diluted 1 in 1000 with buffer solution, then added to a cuvette and left to equilibrate for 5 minutes at 25 °C. Pyrene fluorescence was measured by scanning the emission spectrum from 360 to 560 nm (excitation 346 nm).

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