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Membrane composition determines the fate of aggregated vesicles†

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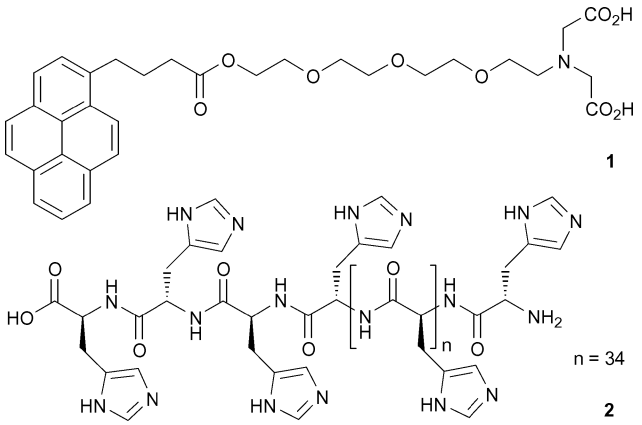
Vesicles incorporating a fluorescent metal-chelating lipid can be linked together by addition of copper(II) and poly-L-histidine, but the stability of adhering vesicles towards fusion depends upon membrane composition.

The structural and functional complexity of tissue depends upon defined adhesion between cells. The information that determines this connectivity is intrinsic to individual cells, as demonstrated by Wilson in 1907, who showed that sycon sponges could reconstitute themselves from dissociated cells.[1] In 1911, Huxley hypothesised the existence of adhesive agents in the cell membrane, agents that we now know are membrane-embedded proteins, cell adhesion molecules (CAMs).[2] We aim to better understand some of the underlying physical principles behind cellular adhesion by constructing vesicle networks linked through multiple weak interactions, such as metal–ligand coordination bonds. Metal ion binding is known to play a role in the adhesion of some CAMs to the extracellular matrix, for example, integrin α2β1 binds to collagen *via* coordination of a collagen glutamate residue to a bound calcium ion.[3] Previous work on vesicle adhesion mediated through metal–ligand complexation has shown that this type of interaction can be strong enough to aggregate vesicles, but the structures formed include intact aggregated vesicles, columnar vesicle fragments and giant vesicles formed by membrane fusion.[4] The reasons for the variety of structures formed, often from very similar systems, is not clear. It is believed to depend on metal ion concentration and identity, but the analysis is further complicated due to the differing phospholipid compositions of the vesicles used by different workers. Herein we describe a system where, as in Nature, multiple weak interactions were used to link vesicles together,[5] allowing the effect of changing the phospholipid composition on the fate of adhering vesicles to be probed.

Our approach uses metal-binding synthetic lipid **1**, embedded in the membranes of phospholipid vesicles, as a CAM mimic (Scheme 1). The copper(II) complex of lipid **1**‡ is designed to coordinate to poly-L-histidine (**2**), a 39-residue multivalent ligand which then acts as an extravesicular "glue" to cross-link the vesicles. This interaction between copper(iminodiacetate) complexes (Cu(IDA)) and histidine, used for affinity chromatography and protein crystallisation experiments, is ideal to mediate vesicle adhesion.[6] It is a relatively weak interaction, particularly when compared to the biotin–avidin and metal chelating interactions often used in other vesicle aggregation studies, and thus closer to the situation *in vivo*, where a series of weak interactions augment each other in a cooperative manner to form a tight bond to a neighbouring cell.[7] Lipid **1** was designed with a tetraethylene glycol spacer to project the binding headgroups away from the vesicle surface and minimise steric repulsion between vesicles. A fluorescent pyrene group



Scheme 1 Metal ion-chelating lipid **1** and poly-L-histidine "glue" **2**.

was included in the design of **1**, allowing vesicles containing lipid **1** to be observed by fluorescence microscopy. This direct method of visualising morphological changes in the vesicles was complemented by monitoring the increase in turbidity caused by vesicle aggregation. Then, to probe the effect of changing bilayer composition on vesicle adhesion, vesicles were formed from phospholipid mixtures with distinct physical properties. Distearoyl phosphatidylcholine (DSPC) and egg yolk phosphatidylcholine (EYPC) are phospholipids which are often used in studies of vesicle adhesion. DSPC bilayers are in a gel-like phase at room temperature, melting into the fluid phase at 54 °C (the transition temperature, T_m), whilst EYPC bilayers are in the fluid phase at 25 °C.[8,9] Furthermore DSPC is a pure compound but EYPC is a naturally occurring mixture of phospholipids, containing a mixture of saturated and unsaturated acyl chains of differing lengths.[9]

Unilamellar vesicles (0.8 μm diameter, 20 mM lipid in 20 mM MOPS buffer, pH 7.4) composed of DSPC doped with **1** (5 mol%) or EYPC doped with **1** (5 mol%) were prepared by extrusion of a phospholipid suspension through polycarbonate membranes at a temperature above the phase transition temperature (25 °C for EYPC and 60 °C for DSPC). The fluorescence spectra of these two vesicle suspensions showed only pyrene monomer emission at 377 nm and no excimer emission, indicating no phase separation of lipid **1** in the membrane.[10] Titration of copper(II) (10 μM) into suspensions of **1**/DSPC or **1**/EYPC vesicles (2 μM lipid) progressively diminished the intensity of the monomer emission through fluorescence quenching by copper(II).[11] Analysis of the fluorescence titration data for **1**/DSPC vesicles showed strong binding of **1** to copper(II), and the formation of complexes Cu(**1**)$_n$ with $n = 1$ and 2. The average binding constant obtained was 4×10^7 M^{-1}, consistent with the data of Arnold *et al.*[12] Titration of copper(II) into vesicles lysed with excess Triton X-100 gave comparable titration curves, indicating that the full complement of **1** in both leaflets of the bilayer is available for complexation.

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As expected, the titration of copper(II) into a suspension of **1**/DSPC vesicles (now at 2 mM lipid) resulted in only a minor increase in turbidity (Fig. 1), showing that vesicle morphology was not significantly altered by complexation of **1** to one equivalent of copper. However, titration of poly-L-histidine **2** (0.5 mM) into the Cu(**1**)/DSPC vesicles resulted in a strong increase in turbidity (Fig. 1). The increase in turbidity slowed and started to plateau after the addition of 0.125 equivalents of poly-L-histidine, or five histidine residues per Cu(**1**) complex. The concentration of poly-L-histidine to give a 50% turbidity response was 4 μM (0.16 mM histidine residues).[13] Fluorescence microscopy showed this increase in turbidity was due to the formation of vesicle aggregates (Fig. 1, Figs. 2a and 2b). Prior to addition of poly-L-histidine, fluorescence microscopy showed vesicles with diameters 1.1 ± 0.3 μm. After addition of poly-L-histidine, a typical fluorescence micrograph showed 15 aggregates in the field of view with an average cross-sectional area of (5 ± 3) × 10^6 nm^2 (8 ± 5 vesicles per aggregate). The changes in turbidity observed after addition of copper and poly-L-histidine were found to be stable for at least 30 minutes and the aggregated Cu(**1**)/DSPC vesicles were stable towards fusion for at least 24 hours. The stoichiometry of the complex leading to this increase in optical density was determined using a Job Plot, which showed that the maximum increase in turbidity was achieved when the ratio of Cu(**1**) lipid to histidine residues was 1 : 2, suggesting a $(His)_2Cu(IDA)$ complex has optimal cross-linking ability (Fig. 2c). Equilibrium constants for histidine complexation to Cu(IDA) complexes are 4.5 × 10^3 M^{-1} for single histidine ligation and up to 10^6 M^{-1} for bidentate coordination of a $His(X)_3His$ unit to Cu(IDA) (where X is any amino acid), so the results of the Job Plot suggest that complexation is largely *via* the stronger bidentate coordination of $His(X)_3His$ units in the poly-L-histidine to Cu(**1**).[14]

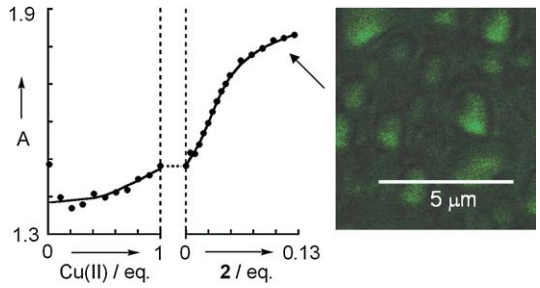

Fig. 1 Changes in the absorbance at 700 nm (●) observed during the addition of copper(II) followed by poly-L-histidine (**2**) to unilamellar vesicles (0.8 μm diameter) composed of 5 mol% lipid **1** in DSPC. Inset: Confocal fluorescence micrograph showing the result of the addition of copper(II) (1 eq.) followed by poly-L-histidine (**2**) (0.125 eq.) to vesicles composed of 5 mol% lipid **1** in DSPC.

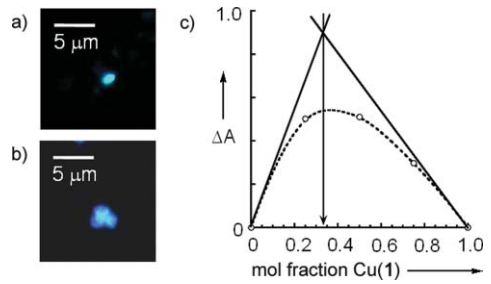

Fig. 2 Fluorescence micrographs of unilamellar vesicles (0.8 μm diameter) composed of 5 mol% lipid Cu(**1**) in DSPC: a) before the addition of poly-L-histidine, and b) after the addition of poly-L-histidine (5 eq. of histidine residues). c) Job Plot of DSPC vesicles containing Cu(**1**) (0.1 mM Cu(**1**), 2 mM DSPC, 800 nm) mixed with poly-L-histidine solution (0.1 mM in histidine residues).

In comparison, the titration of copper(II) (10 mM) into a suspension of **1**/EYPC vesicles (2 mM lipid) resulted in a large increase in turbidity after addition of 0.4 equivalents of copper (Fig. 3). Visualisation of this mixture by confocal fluorescence microscopy showed that very large vesicles had been formed, with diameters between 20 and 100 μm. The sigmoidal nature of the increase in turbidity is suggestive of a cooperative process, where the complexation of **1** to copper(II) leads to vesicle fusion and the formation of giant vesicles. Addition of the poly-L-histidine "glue" (0.5 mM, 20 mM histidine residues) to the fused Cu(**1**)/EYPC vesicles resulted in no further increase in turbidity, and no increase in size was observed in the fluorescence micrographs. Presumably no aggregation of these giant EYPC vesicles was observed because there was a lower concentration of vesicles after fusion.

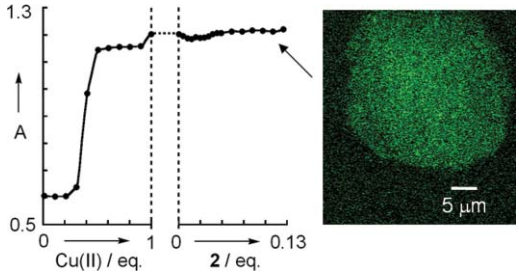

Fig. 3 Changes in the absorbance at 700 nm (●) observed during the addition of copper(II) followed by poly-L-histidine (**2**) to unilamellar vesicles (0.8 μm diameter) composed of 5 mol% lipid **1** in EYPC. Inset: Confocal fluorescence micrograph showing the result of the addition of copper(II) (1 eq.) followed by poly-L-histidine (**2**) (0.125 eq.) to vesicles composed of 5 mol% lipid **1** in EYPC.

To ascertain membrane integrity during fusion and aggregation of vesicles, 5/6-carboxyfluorescein (5/6 CF, 0.1 M) was encapsulated within both **1**/DSPC and **1**/EYPC vesicles, and the effect of adding copper(II) ions (1 eq.) or poly-L-histidine (5 eq. of histidine residues) on the release of 5/6 CF ascertained. Vesicle fusion was initiated by addition of copper(II) to vesicles composed of 5 mol% **1** in EYPC, but there was no significant 5/6 CF release (only 5% after 20 minutes). The addition of poly-L-histidine then resulted in significant release of 5/6 CF (85% after 20 minutes). Poly-L-histidine initiated no such release of 5/6 CF from DSPC vesicles containing 5 mol% Cu(**1**); 28% release after three days compared to 17% in the absence of copper(II). These observations suggest that Cu(**1**)/DSPC vesicles remain intact upon aggregation, and fusion of Cu(**1**)/EYPC vesicles proceeds *via* a non-leaky concerted membrane fusion event between adjacent vesicles, as observed by Lehn *et al.*[4a]

Initially we ascribed the fusion of **1**/EYPC vesicles to the fluidity of EYPC bilayers. At 25 °C, EYPC bilayers are in the fluid phase, unlike gel-phase DSPC bilayers, so molecules of lipid **1** are free to roam across the surface of EYPC vesicles.[15] They are able to migrate to the interface between adhering vesicles and maximise adhesion, perhaps in doing so weakening the membrane and causing membrane fusion.[16] Since the phase transition temperature of DSPC bilayers is 54 °C, above this temperature lipid **1** will also be free to travel to the inter-vesicle interface and possibly initiate membrane fusion. Therefore we carried out the same titrations with copper(II) and poly-L-histidine on DSPC vesicles doped with 5% lipid **1** at 60 °C. However, even at this elevated temperature we observed behaviour similar to that at 25 °C, with a small increase in turbidity upon addition of copper(II) and a stronger increase upon addition of poly-L-histidine (Fig. 4a). These observations suggest the higher fluidity of EYPC at 25 °C is not the determining factor for vesicle fusion. EYPC is fluid because it contains large amounts of unsaturated phospholipids. Such unsaturated phospholipids, including palmitoyl oleoyl phosphatidylcholine

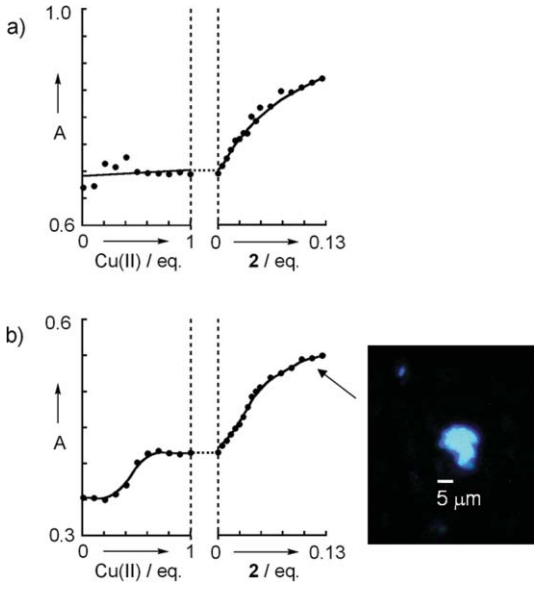

Fig. 4 a) Changes in the absorbance at 700 nm during the addition of copper(II) followed by poly-L-histidine (**2**) to unilamellar vesicles (0.8 μm diameter) composed of 5 mol% lipid **1** in DSPC at 60 °C (●). b) Changes in the absorbance at 700 nm during the addition of copper(II) followed by poly-L-histidine (**2**) to unilamellar vesicles (0.8 μm diameter) composed of 5 mol% lipid **1** in 1 : 1 DSPC : POPC at 25 °C (●). Inset: fluorescence micrographs showing the result of the addition of copper(II) (1 eq.) followed by poly-L-histidine (**2**) (0.125 eq.) to vesicles composed of 5 mol% lipid **1** in 1 : 1 DSPC : POPC.

(POPC), are known to promote fusion events between vesicles due to their narrow headgroups and wide tails.[17] They stabilise the highly negatively curved surfaces found at the junction between fusing vesicles (the "stalk") and also decrease the order and packing within the membrane, making the membranes more prone to fusion.[18] To test this hypothesis, we carried out the same titrations with copper(II) and poly-L-histidine on DSPC vesicles containing 48% of the unsaturated phospholipid POPC and 5% lipid **1** at 25 °C. Unsaturated phospholipids and DSPC form mixed vesicles at room temperature that contain islands of gel DSPC in a fluid matrix.[19] The changes in turbidity observed were combinations of the changes observed with pure EYPC and DSPC vesicles (Fig. 4b). The reproducible sigmoidal increase in turbidity at 0.4 equivalents of copper(II), as found for EYPC, implies that POPC does promote fusion, though the further increase after the addition of poly-L-histidine suggests the larger vesicles resulting from fusion could be aggregated by the addition of polyhistidine. Visualisation of the mixture by fluorescence microscopy showed giant conglomerates of large vesicles (>5 μm diameter), small vesicles and fragments of vesicles, suggesting that the presence of POPC causes fusion, aggregation and membrane disruption all to occur. Based upon these observations, we propose the following model to explain why contacting EYPC vesicles fuse, yet DSPC vesicles give stable aggregates at 25 °C. The high fluidity of EYPC bilayers allows lipid **1** to migrate to the vesicle adhesion interface to form intervesicular complexes of the form $Cu(IDA)_2$. When the strength of this multivalent binding is close to its peak, at a 1 : 2 ratio of copper(II) to lipid **1**, unsaturated phospholipids at the interface promote the formation of a "stalk" between membranes, initiating irreversible membrane fusion. In comparison, although DSPC bilayers are fluid at 60 °C, the lack of fusogenic unsaturated lipids in the bilayers allows vesicle adhesion to be reversible.

The elucidation of the underlying factors that control the balance between stable vesicle adhesion and membrane fusion is a key step towards understanding cell adhesion. We have found that the copper(iminodiacetate)–histidine interaction can mediate vesicle adhesion and that the balance between membrane adhesion and fusion seems to be determined by the composition of the membrane, rather than membrane fluidity. Thus controlling the composition of the membrane should allow stable vesicle networks to be constructed and lead to improvements in vesicle-based drug delivery systems. We now wish to gain a better understanding of how receptor preorganisation can maximize the strength of inter-vesicular binding, as part of our studies towards constructing vesicle-based nanostructures as tissue mimics.

Acknowledgements

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Notes and references

‡ Lipid **1** was made using standard synthetic procedures and gave satisfactory spectroscopic data (see Supporting Information†). Poly-L-histidine hydrochloride (DP 39, MW 6 700 mol^{-1} g) was purchased from Sigma-Aldrich Co. Ltd and used as received.

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