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Engineering multi-compartment vesicle networks

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Yuval Elani,[ab] Antony Gee,[c] Robert V. Law[ab] and Oscar Ces*[ab]

Vesicles serve important functions in the construction of artificial cells. They facilitate biochemical reactions by confining reactants and products in space, and delineate the boundaries of the protocell. They allow concentration gradients to form, and control the passage of molecules *via* embedded proteins. However, to date, manufacturing strategies have focussed on uni-compartmental structures, resulting in vesicles with homogenous internal content. This is in contrast to real cells which have spatial segregation of components and processes. We bridge this divide by fabricating networked multi-compartment vesicles. These were generated by encasing multiple water-in-oil droplets with an external bilayer, using a process of gravity-mediated phase-transfer. We were able to control the content of the compartments, and could define the vesicle architecture by varying the number of encased droplets. We demonstrated the bilayers were biologically functional by inserting protein channels, which facilitated material transfer between the internal compartments themselves, and between the compartments and their external environment. This paves the way for the construction of inter- and intra-vesicle communication networks. Importantly, multi-compartment vesicles allow the spatio-dynamic organisation seen in real cells to be introduced into artificial ones for the first time.

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Introduction

Lipid vesicles are ubiquitous structures in the study of biological systems. Traditionally they are spherical, enclosed bilayer shells, which carry material either in their aqueous interior, or embedded in the hydrophobic bilayer core. Vesicles are used as models of cell membranes, allowing insights to be made into the biophysical laws underpinning membrane and membrane-associated protein behaviour.[1,2] They are also increasingly used as functional tools for a wide range of applications including drug delivery, protein screening, immunoassays, and chemical micro-reactors.[3–6] In synthetic biology, vesicles define the boundary of the proto-cell, allowing concentration gradients to be established, and enabling biochemical reactions and their products to be contained.[7] All these uses require vesicle morphology and chemistry to be engineered according to the purposes of the experiment or application.

Using an assortment of vesicle formation strategies it is now possible to exert influence on parameters including vesicle size, size distribution, curvature and shape, lamellarity, lipid content, bilayer asymmetry, and phase patterning.[8–13] In addition, responsive vesicles—which react to the presence of external stimuli—can be made, intricate biological structures (such as cytoskeleton scaffolding) incorporated, and vesicles can be manipulated to form nanotube-linked networks.[14–16] However, notably lacking from this repertoire of features that can be introduced into vesicles is the spatial segregation of their internal content.

Although there have been several elegant methods of inducing compartmentalisation within vesicles using polymer phase-separation, it is not possible to segregate materials other than these polymers or to define the contents of each compartments, as there is no physical barrier (*i.e.* lipid bilayer) separating the aqueous volumes. This further prohibits protein-mediated communication and material transfer between compartments. Furthermore, these have relied on the presence of large concentration of polymers which may interfere with encapsulated biological process, thus precluding their use as artificial cells.[17,18]

Here, we describe a method where compartmentalisation of vesicle content is achieved by erecting internal bilayer partitions, thus enabling us to construct multi-compartment vesicles with user-defined architectures and internal content.

There are several advantages associated with vesicle compartmentalisation. (i) Spatial separation of distinct chemical reactions or biochemical processes can be achieved. This is important if the constituent reactants or products of the processes in question adversely interfere with one another (*i.e.* they are non-orthogonal). (ii) Compartmentalisation enables the partition of processes to facilitate specialisation of function. The components needed for one process may not be needed for another, therefore physically separating components may improve how resources are utilised. This phenomenon is seen in natural cells in the form of organelles, which are membrane-bound subunits that perform specific

[a]*Department of Chemistry, Imperial College London, Exhibition Road, London, SW7 2AZ, UK. E-mail: o.ces@imperial.ac.uk*

[b]*Institute of Chemical Biology, Imperial College London, Exhibition Road, London, SW7 2AZ, UK*

[c]*Division of Image Sciences and Bioengineering, King's College London, St Thomas' Hospital, Lambeth Palace Road, SE1 7EH, UK*

functions. (iii) It allows reactants to be kept in close proximity to one another, thereby increasing their local concentration and promoting the desired reaction.

Despite the advantages of compartmentalisation, its associated benefits cannot be realised in current vesicle-based systems as they are single compartment structures with homogenous aqueous interiors. The multi-compartment vesicles described here address this shortcoming.

In order for vesicles to be useful in a synthetic biology setting they must fulfil several criteria. They need to be composed of biological materials (lipids), to enable them to be functionalised with further biological components (such as membrane-associated proteins, receptors, and antibodies). Their method of formation should be bio-compatible, and not rely on harmful substances (such as chloroform, toluene, or detergents). The fabrication process should be quick enough (in the order of minutes) so that processes which occur over a short timescale can be incorporated. The content of compartments should be pre-defined and differ from the surrounding solution, and control of the overall structural arrangement must be achieved. Finally, the system must not depend on the presence of a surrounding oil solution, and be fully encased in a bilayer, in order to be truly bio-mimetic. These conditions are not fulfilled with other vesicle-like compartmentalised structures which include: multi-compartment polymersomes;[19] polymersomes-in-polymersomes;[20,21] vesicles-in-vesicles;[22] and multisomes.[23,24]

To construct multi-compartment vesicles we assemble the '*internal*' and '*external*' bilayers in a step-by-step process (Fig. 1A). First, the *internal* bilayers of the final vesicle—which separate compartments from one another—are made, by bringing together monolayer-coated water-in-oil droplets. Oil is excluded from the interface, and droplet-interface bilayers (DIB) form.[25–27] Then, the *external* bilayer—which separates the compartments from the aqueous exterior—is formed by passing the construct through a monolayer at the interface of an oil/water column. As the DIB construct descends, it is enveloped by the interfacial monolayer, thus encasing it in a bilayer and generating a multi-compartment vesicle. This process, referred to in the literature as phase-transfer, was originally developed to manufacture vesicles of a uniform size, defined bilayer asymmetry and high encapsulation efficiency.[10,28] These features are retained when forming multi-compartment vesicles. Using this procedure, we create multi-compartment vesicles, which satisfy the criteria outlined above.

Results and discussion

To form multi-compartment vesicles, water-in-oil droplets were expelled into an oil/water column separated by a lipid monolayer. The oil phase contained 4 mg ml^{-1} of dissolved 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipid, which spontaneously self-assembled both at the water/oil interface of the column and of the individual droplets. The phase-transfer process relied on a density difference between the droplets and the bulk aqueous solution, which was achieved by loading the droplets with 0.5 M sucrose. At lower sucrose concentrations, the gravitational force acting on the droplets was insufficient to overcome the opposing interfacial tension, and droplets remained resting on the water/oil interface. The competition between the two forces also meant that compartments had a minimum size limit of ~500 μm, below which droplets would not penetrate into the aqueous phase. The

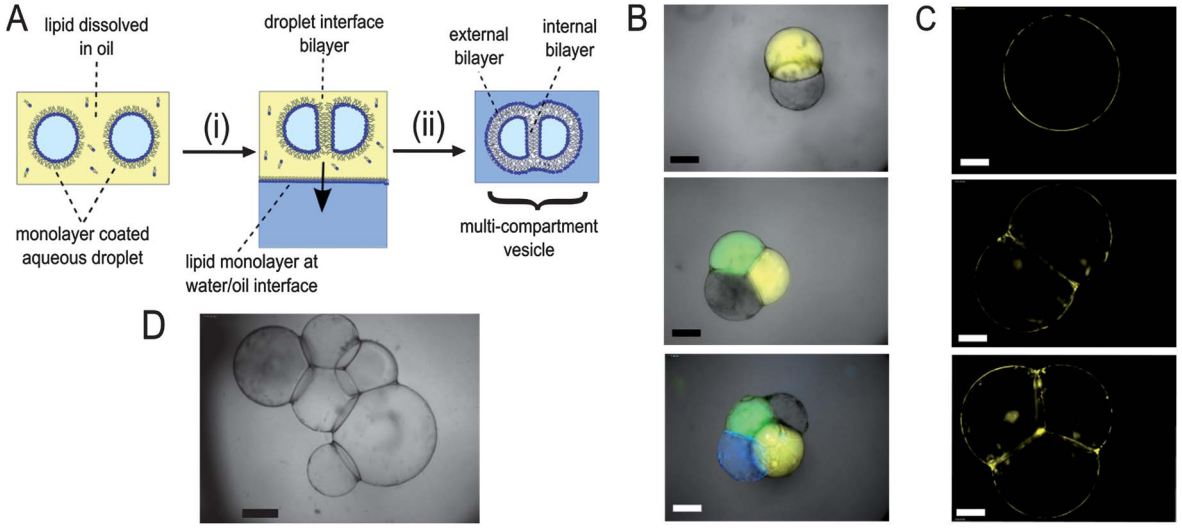

Fig. 1 (A) Schematic of two-compartment vesicle construction. (i) A droplet-interface bilayer (DIB) is formed by bringing together two monolayer-coated water-in-oil droplets. Oil is excluded from the interface and a bilayer forms. (ii) The DIB undergoes phase-transfer by being passed through a lipid monolayer at the interface of a water/oil column. As the second monolayer wraps around the DIB, it becomes encased in a bilayer. (B) Composite brightfield/fluorescent images of two- three- and four-compartment vesicles. The content of each compartment was defined by the initial droplet composition. The compartments could therefore be loaded with distinct cargoes, in this case 100 nm liposomes made of different fluorescently tagged lipids. (C) Fluorescence images of one-, two- and three-compartment vesicles composed of 0.1% fluorescent lipid (Rh-PE). Note the presence of oil pockets, most markedly at the vertices between the compartments. These appear as fluorescent patches due to the presence of dissolved lipids. (D) Six-compartment vesicle. Scale bars = 250 μm. Coloured channels show fluorescence of different lipids: yellow channel for Rh-PE; green channel for 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE); blue channel for Marina Blue 1,2-Dihexadecanoyl-*sn*-glycero-3-Phosphoethanolamine (Marina Blue DHPE).

sizes of the resultant vesicles thus exceed those of natural cells. The increased use and sophistication of microfluidic droplet-based technologies is expected to resolve this issue by offering effective alternative techniques for the production and manipulation of cell-sized droplets. It is important to note that there were occasions where phase-transfer was not successful and droplets burst at the interface (yield of successful two-compartment vesicle formation was found to be 43%, $n = 30$).

As the droplets were slowly expelled over the same location, they fell one above the other on the oil/water interface. It was therefore possible to ensure that a set number of droplets descended through the interface together thus generating vesicles with a defined number of compartments—two droplets produced two compartments, three droplets produced three and so on (Fig. 1B). This could be extended to any number of compartments (Fig. 1D shows a six-compartment vesicle).

Each of the eventual vesicle compartments could contain different pre-defined internal content, achieved by starting with droplets of different compositions. To demonstrate this we constructed two-, three- and four-compartment vesicles carrying different fluorescent cargoes, in this case 5 mg ml^{-1} of 100 nm liposomes containing different fluorescently tagged lipids (Fig. 1B). The fluorescent materials were retained in their individual compartments throughout the course of the experiments (40 minutes). This demonstrated the presence of an effective, continuous barrier between the individual compartments, and between the vesicles and the external aqueous solution. It was found that when the starting droplets contained these liposomes inside, the yield of successful phase-transfer events rose to 83% ($n = 30$). This is because the 'lipid-in' approach increased the probability that droplets were encased with a well packed monolayer (*i.e.* they were effectively stabilised).[26]

Further fluorescent experiments indicated the presence of an internal bilayer partition and of an encasing bilayer. A fraction of fluorescently tagged lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rh-PE), was dissolved in the oil together with DOPC (0.1 wt%), and multi-compartment vesicles were formed in the usual manner. Fluorescent microscopy showed distinct fluorescent boundaries between the compartments themselves, and between the compartments and the bulk aqueous solution (Fig. 1C). These experiments also revealed small regions of trapped oil, which also fluoresced due to the presence of dissolved lipid.

Process visualisation

Real-time observation of a two-compartment vesicle formation process revealed that the system passed through several distinct stages (Fig. 2). First, after the droplets were expelled into the column, they descended through the oil and eventually settled

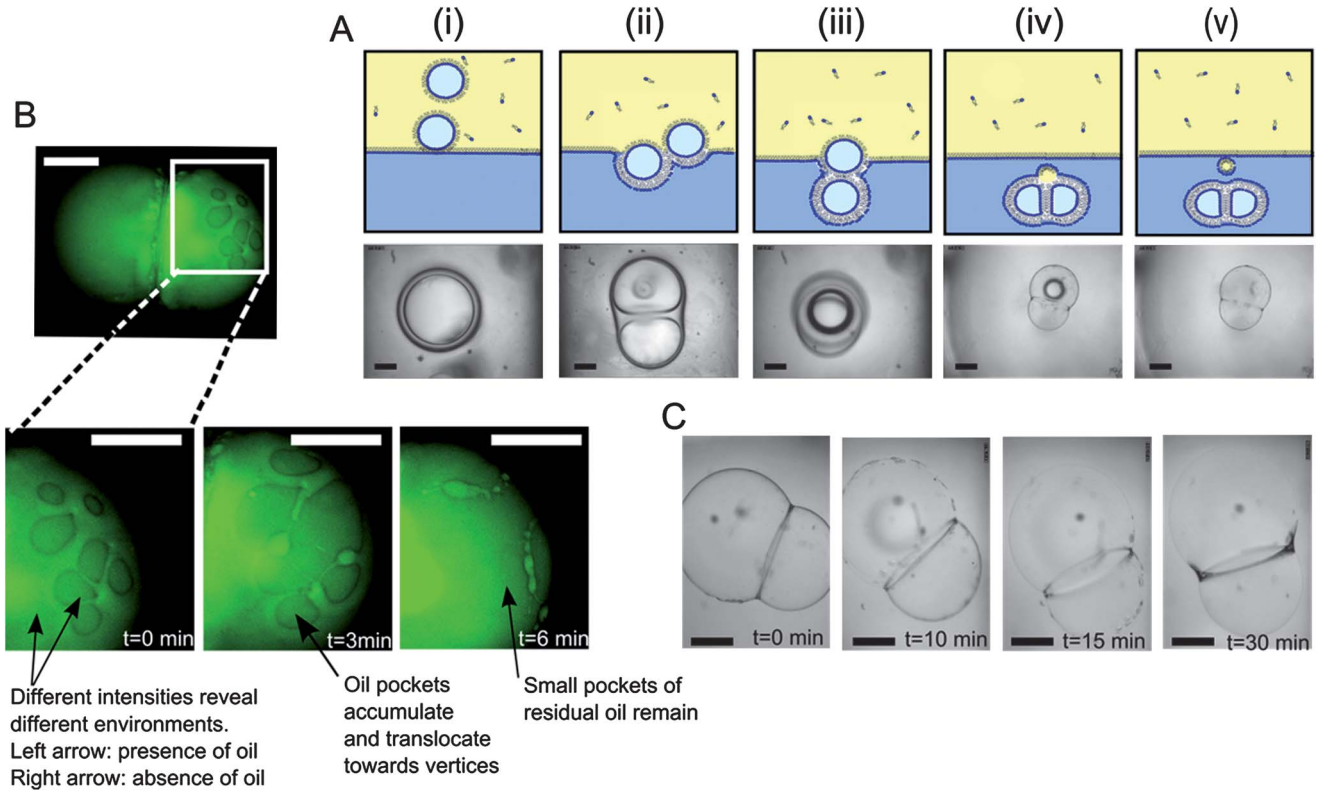

Fig. 2 (A) Schematic of the stages involved in two-compartment vesicle formation and corresponding experimental images. Note the presence of lipid monolayers at all water/oil interfaces. (i) Seconds after the water-in-oil droplets were introduced into the column, the first droplet settled on the interface. (ii) A second droplet reached the interface, which started to deform as both droplets descended into the aqueous phase. (iii) The two droplets continued to descend, one above the other, until they were submerged. (iv) Residual oil from the phase-transfer process accumulated as an oil lens. (v) The oil lens was ejected upon reaching a critical size. (B) Tracking of trapped oil using Fluo-4 dye revealed the movement of oil patches within the vesicle (areas of higher fluorescence reveal the regions of oil). (C) Over a period of 10 minutes, oil accumulated at the compartment vertices. Scale bar = 250 μm.

at the oil/water interface. The droplets made contact with one another and a DIB was formed (a process deduced by the characteristic optical patterns between the droplets).[25] Then, after *ca.* 5–60 s, the droplets descended into the lower aqueous volume by gradually deforming the oil/water interface around them. The monolayer-coated droplet pair was encased by a second monolayer as it passed into the aqueous phase, resulting in the formation of a stable external bilayer. At this point there was a quantity of trapped oil present, which accumulated at the top of the structure as a small oil lens (a phenomenon which has been observed and discussed elsewhere).[29–31] When enough oil accumulated, the lens was ejected and floated upwards. This process of oil accumulation and ejection occasionally occurred twice, depending on the amount of trapped oil from the phase-transfer process. Any residual oil left in the system continued to accumulate, and eventually resided in the vertices between the compartments, which are the lowest energy points in the structure.[32] The accumulation of oil in these regions played a role in stabilising the vesicle, as it reduced the packing frustration of the bilayers at the junctions.[32] The accumulation and movements of the oil was revealed by manufacturing multi-compartment vesicles containing Fluo-4 dye, then observing them with fluorescent microscopy. This allowed the trapped oil to be tracked through the different emission intensity of the dye in the presence of oil (Fig. 2B).

The process of sequential oil-lens formation, ejection, and residual oil accumulation took approximately 10 minutes, and the formation of vesicles with more than two compartments also went through all the stages described above. The multi-compartment vesicles had a lifetime of *ca.* 60 minutes after which time the external bilayer eventually ruptured (a process likely due to the presence of a large osmotic gradient between the vesicle interior and exterior),[33] and the internal vesicle content was released into the surrounding aqueous solution.

Protein insertion experiments

In order to show that the internal and external boundaries of the vesicles were indeed bilayers, we conducted a series of experiments on two-compartment vesicles which demonstrated the successful insertion of the transmembrane protein, alpha-Hemolysin (αHL). αHL is a heptameric protein whose constituent components self-assemble on unilamellar membranes to form a pore of 1.4 nm diameter,[34] and has therefore been used to demonstrate successful bilayer formation in many model membrane systems.[35–37] The protein enables the influx of Ca^{2+} ions, which are otherwise impermeable to the membrane. We were therefore able to determine successful protein insertion using Fluo-4, a Ca^{2+} sensitive fluorescent dye.

To demonstrate protein insertion in the *internal* partitioning bilayer, one of the compartments contained Fluo-4 and αHL and the other contained $CaCl_2$. Initially, the dye-containing compartment showed only a low level of fluorescence due to the lack of Ca^{2+} ions. Over a period of ~40 minutes, the fluorescence intensity increased as protein inserted into the bilayers and Ca^{2+} ions flowed through the pores (Fig. 3A). The initial lag before fluorescence increase is observed is likely due to the time necessary for sufficient protein monomers to insert and

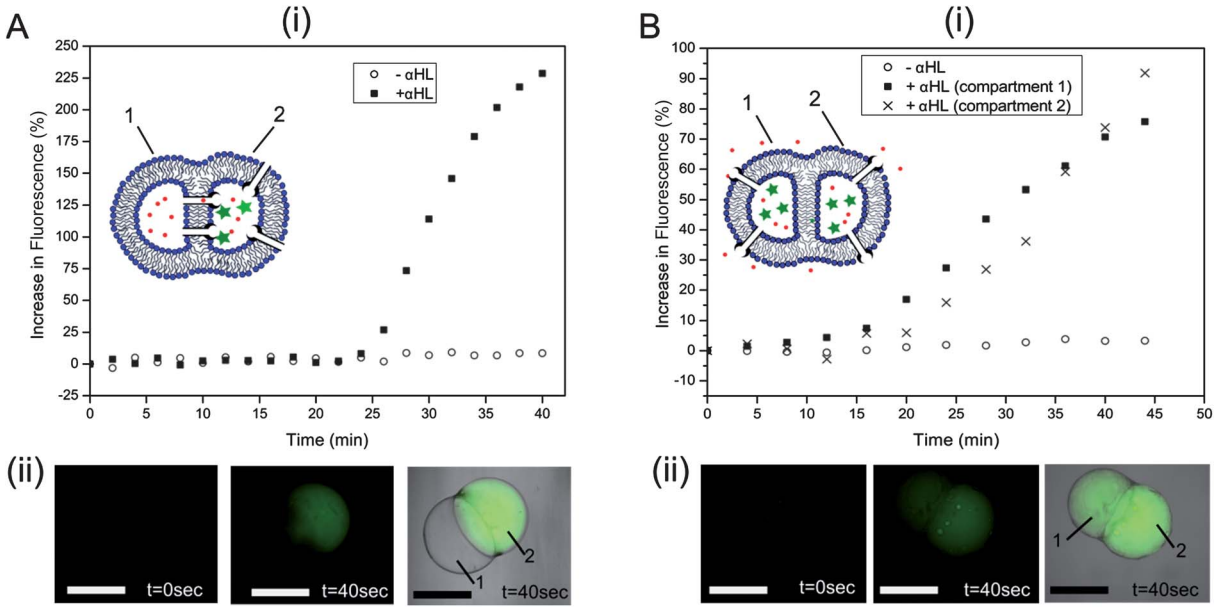

Fig. 3 (A) Experiment demonstrating alpha-Hemolysin (αHL) insertion into the internal bilayer. Inset shows schematic of experimental outline. Compartment 1 contained Ca^{2+} (red circles). Compartment 2 contained αHL monomers and Fluo-4 (a Ca^{2+} sensitive dye, green stars). External solution contained DI water only. As the monomers inserted into the membrane and assembled to form a pore (black cylinder), Ca^{2+} influx occurred. (i) Graph showing increase in fluorescence of compartment 2 in the presence of αHL and in its absence (ii) Fluorescence images at $t = 0$ and $t = 40$ min, and a composite brightfield/fluorescence image after 40 min. (B) Experiment demonstrating αHL insertion into the external bilayer. Compartments 1 and 2 both contained Fluo-4. The external solution contained Ca^{2+} and αHL monomers. As the protein assembled on the external bilayer, Ca^{2+} influx occurred in both compartment simultaneously, leading to an increase in fluorescence. (i) Graph showing fluorescence increase of compartments 1 and 2 in the presence of αHL and in its absence. (ii) Fluorescence images at $t = 0$ and $t = 40$ min, and a composite brightfield/fluorescence image after 40 min. Scale bar = 500 μm.

assemble on the membrane, and in addition, to EDTA chelating the ions before they can bind to the dye. Indeed, a similar time-dependent diffusion of ions through such pores has been observed and commented upon previously.[23] To show successful protein insertion into the *external* bilayers, a vesicle was constructed where both compartments contained Fluo-4 and the bulk external solution contained $CaCl_2$. αHL was then injected into the external solution in the vicinity of the vesicle. The fluorescence intensity of both compartments increased simultaneously over a period of ~40 minutes (Fig. 3B). In both experiments, the control cases where no protein was present showed no observable increase in fluorescence. This confirmed that the constructs were not simply vesicle aggregates with two individual bilayers separating the compartments, but were instead vesicles with a single, internal bilayer partition. The experiments also demonstrated that the bilayers were functional with respect to their biological activity, and showed that it would be possible to modify the membrane for specific purposes.

It is possible that there may have been regions of oil residing in the bilayer. Indeed, Kirchner *et al.* used Raman spectroscopy to show that vesicles generated using a jetting technique have regions of micron thickness oil inclusions, with other regions existing as pure lipid bilayers with no oil present.[38] If this is the case with the vesicles described here then it is likely that proteins inserted into the regions of the vesicle with no residual oil, thus explaining the observation of successful protein insertion. If there is oil present in the bilayers, it is evidently at low enough levels to prevent full reconstitution of membrane proteins.

It is noteworthy that that Noireaux and Libchaber have observed what appeared to be multi-compartment vesicles in their work on vesicle bioreactors.[4] However, the assemblies were not formed by design; the number of compartments and their individual internal contents could not be user-defined, experiments on the performance of vesicles were not conducted, and it was not demonstrated if the vesicles were formed *via* the phase-transfer, or were simply as a result of vesicle fusion. Similarly, Carrara and Luisi generated extended vesicle aggregates (referred to as colonies) by modifying the lipid chemistries of the constituent vesicles.[39] In both these cases however, it was not demonstrated that a single bilayer separated the vesicle compartments, and indeed it is likely that vesicles were still distinct entities.

Materials and methods

All lipids were purchased from Avanti Polar Lipids, except Marina Blue DHPE which was purchased from Life Technologies. All chemicals were purchased from Sigma Aldrich, unless otherwise stated.

Multi-compartment vesicle formation

All vesicles were composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) unless otherwise stated. To generate the multi-compartment vesicles lipids were first dissolved in an a 75 : 25 (v/v) mixture of hexadecane and mineral oil by sonication at 50 °C for 30 minutes, to give a 4 mg ml^{-1} solution. The lipid/oil mixture (1 ml) was then deposited above deionised (DI) water (1 ml) in a LabTek Chamber Coverglass (Thermo Scientific). The resulting water/oil column was left for 90 minutes so that a well packed monolayer could form at the interface. Before the droplets were inserted, a sucrose solution (0.1 ml, 1.5 M) was injected at the bottom of the column. This later acted as a cushion for the eventual vesicles (due to their lower density) to ensure they would not make contact with the glass surface which would have led to their rupture.

To form the water-in-oil droplets, 1 mm inner diameter tubing was filled with the oil/lipid solution, and droplets of 0.5 M sucrose in DI water were pipetted in. After the appropriate number of droplets were made, they were expelled onto the column using a syringe pump, at a flow rate of 1 μl min^{-1}, and multi-compartment vesicles were generated.

In experiments where droplets had 5 mg ml^{-1} of lipids within them, lipids were present as 100 nm liposomes formed by extruding a water/lipid suspension 11 times through a polycarbonate membrane (Avanti Polar Lipids). Bilayers doped with 0.1 wt% fluorescent lipids were formed from a lipid mixture that was dissolved in chloroform, dried under a stream of nitrogen, and placed under a lyopholiser overnight before use.

Process visualisation and fluorescence experiments

All experiments were visualised with a Nikon Eclipse TE2000-E inverted microscope. Fluorescent experiments used an illuminating mercury arc lamp, and were imaged with the appropriate filter sets. Images were taken with a QICAM camera (QImaging) and were analysed using ImageJ software. In the oil tracking experiments, vesicles contained 10 μM Fluo-4 and 0.5 M sucrose. The camera exposure time for fluorescent lipid experiments was 100 ms.

Protein insertion experiments

All aqueous solutions were made of 25 mM Tris–HCl, 500 mM KCl, pH 8.0 buffer. All droplets contained 0.5 M sucrose in buffer. αHL from *Staphylococcus aureus* was purchased as a lyopholised powder (Sigma Aldrich) and dissolved in DI water to a concentration of 0.5 mg ml^{-1}, before being diluted with the sucrose/buffer solution to give a final concentration of 60 ng μl^{-1}. In the experiments where proteins were inserted in the *internal* bilayer, one compartment contained 20 μM Fluo-4 and 200 μM ethylenediaminetetraacetic acid (EDTA) to chelate residual Ca^{2+} ions that may have been present. The second compartment contained 0.5 mM $CaCl_2$, and the external solution contained only buffer. In the experiment where protein was inserted in the external bilayer, both compartments contained 20 μM Fluo-4 and 200 μM EDTA. 50 μl αHL in buffer was injected in the surrounding solution, which consisted of 100 mM $CaCl_2$. The camera exposure time was 800 ms.

Conclusions

We have constructed vesicles with distinct internal compartments separated by internal bilayer partitions. This was achieved by encasing multiple droplets joined by interface bilayers

with an external bilayer, using a process of gravity-mediated phase-transfer. The vesicle architecture was determined by the number of droplets encased by the bilayer, and the internal contents and volumes of each compartment could be rigorously defined by starting with droplets of different compositions and sizes respectively. Both the internal and external bilayers were fully functional, as demonstrated by the successful reconstitution of transmembrane proteins. This paves the way for generating intra-vesicle communication networks mediated by membrane proteins, and demonstrates the potential of vesicle communication with the external environment.[40]

Spatial segregation of vesicle internal content has implications which span several disciplines. Most notably, in *in vitro* synthetic biology, the spatio-dynamic organisation seen in real cells can start to be introduced into artificial ones.

Compartments can be dedicated to specific functions, and distinct regions of the vesicle membrane patterned with different membrane-associated proteins. This latter feature—seen in real cells, where membranous structures have specific proteins associated with them—can be introduced as the internal and external membranes are formed separately. The sequential assembly of compartments also allows multipart systems to be built out of simpler components in a modular manner: individual compartments performing distinct operations (*e.g.* stimuli sensing and subsequent response), can be coupled together to form a complex functioning unit. This could facilitate a shift in synthetic biology away from monofunctional elements and towards more integrated systems.[41] Finally, because the method uses DIBs as templates around which a bilayer is encased, many of the promising capabilities of DIB networks—such as energy harvesting, light sensing, and bio-batteries—could be transformed into a vesicle format, so they could exist as true cell-like bio-devices.[42]

The presence of multiple compartments also increases the scope and sophistication of possible 'smart' drug delivery systems, a concept explored by Villar *et al.* in their work on multisomes.[23] The incorporation of intra-vesicle communication networks, and of biological ligands such as antibodies, could lead to delivery systems where reactive drugs are formed *in situ* at the target site.

This work should be seen in the context of the emerging trend of using droplet-based technologies as models for artificial cells and minimal tissues,[43] primarily due to the great degree of control that can be exerted.[27,44] These systems can often be easily translated to a microfluidic format, which carries the advantages of low sample volumes, automation and high throughput. Indeed, efforts are on-going to fabricate multi-compartment vesicles in a microfluidic chip using non-gravitational methods of formation. This would allow smaller vesicles to be generated, as well as increase their lifetime which is currently limited. It would also pave the way to the formation of more extended networked multi-compartment vesicles, with defined 2D and 3D architectures and connectivities, such as have previously been attained with DIBs.[27] We are also exploring the use of more soluble oils, which will allow us to fine tune the quantities of oil trapped in the vesicle.

Acknowledgements

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