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Reconstitution and Anchoring of Cytoskeleton inside Giant Unilamellar Vesicles

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Among the requirements for all life forms is the ability to self-replicate. In eukaryotic cellular systems, this division is achieved through cytokinesis, and is facilitated by the (re)arrangement and interaction of cytoskeletal proteins with lipids and other proteins localized to the plasma membrane. A fascinating challenge of modern synthetic biology is the bottom-up reconstitution of such processes for the generation of an artificial cell. One crucial step towards this goal is the functional reconstitution of the protein-anchoring machinery to facilitate cytokinesis into lipid vesicles. True to the ideal of a minimal cell-like system, we here describe the formation of an actin-based cytoskeleton within giant unilamellar vesicles (GUVs) made from porcine brain lipid extracts. We demonstrate that the actin filaments are localised and anchored to the interior walls of the GUVs through the spectrin/ankyrin proteins, and produce tightly packed actin bundles. These studies allow for the examination of cytoskeletal rearrangements within a cell-like model membrane system and represent important first steps in reconstituting the minimal machinery required for the division of an artificial cell. In addition, the study of such minimal systems can shed light on protein functions that are commonly unobservable or hidden within the overwhelming complexity of cells.

Introduction

The concept of an artificial cell has fascinated theoretical and evolutionary biologists for several decades. Ideas originating from the theories of self-reproducing machines have been extending into exciting new biological arenas. The creation of a minimal cell would combine molecules into a propagating system that could potentially adapt and evolve.[1–3] Furthermore, the use of "artificial cells" as ultrathin polymer membranes to encapsulate various materials, including cells, enzymes and drugs, was put forward over 40 years ago, and this field continues to create new opportunities in biotechnology and therapeutic applications.[4–6] In 1957, the first polymeric artificial cells were developed; they encapsulated haemoglobin and acted as red blood cell mimics. By the 1970s, artificial cells with encapsulated adsorbents were used clinically for the removal of toxins from the blood. Since then, various artificial cells have been developed, including those that microencapsulate active enzymes and even cells—for example, islet cells for diabetes treatment, or genetically engineered cells or stem cells for regenerative medicine.[4–6] On the other hand, a "bottom-up" reconstitution approach can also be useful to examine minimal systems that are free from the overwhelming cellular complexity. By reducing the components of biochemical systems and expressing them in a "cell-like" environment, one creates a system that has the capacity to examine various biological phenomena from protein expression to protein folding, as well as cellular mechanics, signalling and trafficking.

In one scenario, a minimal cell could possess the ability to process information (that is, translate genetic material into proteins), use nutrients and cofactors as energy, and most importantly, propagate and divide.[1,7] In metazoan native cellular systems, cell division is achieved through the organization and anchoring of the cytoskeleton to the walls of the plasma membrane. Ubiquitously-expressed spectrin proteins play principal roles in anchoring the cytoskeleton to the plasma membrane of eukaryotic cells. Spectrin is coupled to the inner surface of the plasma membrane by a variety of proteins and lipids, but predominantly through its association to ankyrin, which is in turn linked to the cytoplasmic domain of various proteins, including: anion exchange proteins, voltage-gated sodium channels, Na^+/K^+ ATPase, Na^+/Ca^{2+} exchange protein, inositol 1,4,5-triphosphate receptors, the Rh antigen and RhAG ammonium transporter, voltage-regulated K^+ KCNQ2/3 channels, L1CAMs, CD44 and E-cadherin.[8,9] A crucial step in the bottom-up approach towards a self-replicating system is thus, to design a (membrane) compartment simple enough to be easily controllable and reproducible, but also complex enough to accommodate crucial components of the cytoskeleton anchoring machinery.

The plasma membrane of a cell consists of a liquid crystalline lipid/protein centre, an extracellular layer formed by oligosaccharides of glycolipid head groups and the branched polypeptide/oligosaccharide head groups of glycoproteins, and an intracellular side, in which the bilayer is coupled to the membrane-associated cytoskeleton and other proteins.[10] In addi-

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tion to the vast diversity of proteins found in native cellular membranes, the lipid content of various membranes is also incredibly complex and varies greatly amongst different cellular compartments and organelles.[10] When studying model membrane systems, it thus is a daunting task to achieve the complexity found in true native membrane states, especially since each cell and organelle type can contain a variety of lipid and protein compositions. Giant unilamellar vesicles (GUVs), with their cell-like shape and size, are a very promising system if one wishes to leave the mere characterization of model membranes behind and explore their use in synthetic biology.[11,12] The use of complex synthetic mixtures in GUV formation has already shed light on how lipids mix, sort and interact. In addition, the reconstitution of proteins into GUVs has allowed the study of protein-lipid interactions as well as protein functionality in differing membrane environments.[11]

Taking steps towards the reconstitution of metazoan cell division machinery, we sought to polymerise and physiologically anchor an actin cytoskeleton or cortex to the interior walls of GUVs. True to the idea of a minimal cytoskeletal-membrane system, we have reconstituted actin filaments in a cell-like model membrane environment (that is, GUVs made from isolated membrane fractions from porcine brain). The use of membrane fractions maintains the complex lipid composition found in a native brain membrane state, and contains the necessary integral membrane proteins for anchoring the cytoskeleton. In addition, we have also isolated an enriched spectrin/ankyrin pool and utilized this within the GUVs to anchor the actin filaments, for the first time, to the inner walls of the porcine GUVs (Figure 1). While GUVs have been previously made from native membrane fractions,[11] this is the first demonstration of GUVs formed from porcine brain membranes, as well as the first demonstration of a quaternary-protein system reconstituted in and anchored to the interior walls of GUVs. This work not only further validates GUVs as a model "cell-like" compartment, in which multiprotein systems can be reconstituted and examined in the presence of complex lipid mixtures, but also signifies a critical step towards achieving the requirements for division of a minimal cell system.[12]

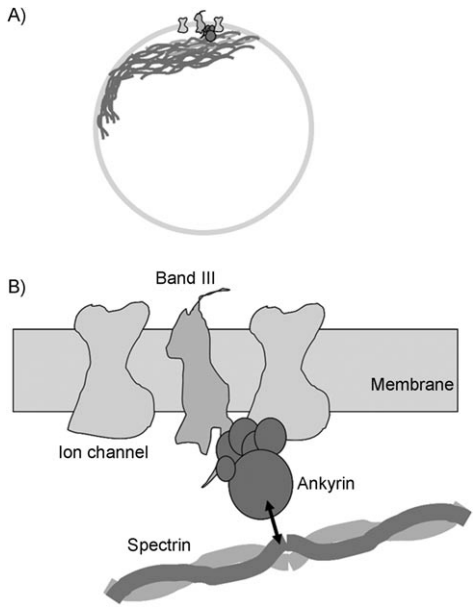

Figure 1. A) Cartoon of a giant unilamellar vesicle containing membrane-associated proteins (for example, ion channels and band III protein), which bind ankyrin and, hence, spectrin and the bundles of actin filaments to the lipid bilayer. B) Close-up of (A), with the protein components (ion channels, band III, ankyrin, spectrin) needed to anchor actin filaments to the membrane. This Figure serves as a simple schematic of how we envisage the actin is anchored to the walls of the GUVs. For a comprehensive review of how ankyrin, spectrin and actin can interact with the plasma membrane in eukaryotic cells see references 8 and 9.[8,9]

Results

GUVs from porcine brain containing integral membrane proteins

Important studies on cytoskeletal organization have been realised using erythrocytes and brain tissue. In addition, ankyrins and spectrins are expressed at high levels in the vertebrate brain, and there has been much work characterizing and purifying these cytoskeletal components from bovine and porcine brain.[13–16] With the goal of mimicking a physiologically relevant environment for the anchoring of cytoskeleton inside GUVs (Figure 1), and as the spectrin-based cytoskeleton in brain has already been thoroughly characterized, we decided to isolate membrane fractions from porcine brain to form GUVs consisting of a native lipid composition and containing integral membrane proteins capable of binding spectrin/ankyrin.

Porcine brain was brought to the laboratory for homogenization and membrane/protein extraction within an hour of harvest. Following protocols by Davis and Bennett,[13] the brains were homogenized, membrane fractions were separated through centrifugation, the brain membranes were demyelinated through a sucrose bed and the membrane associated proteins were separated from the membrane fractions containing integral proteins with a NaOH (0.1 N) extraction. The membrane fractions were washed extensively in buffer to restore a neutral pH and were finally centrifuged by ultracentrifugation. These membrane pellets were then diluted to an approximate final protein concentration of 5–6 mg mL^{-1} and stored in trehalose (20 mM), sodium phosphate (10 mM), sodium azide (5 mM) and EGTA (0.2 mM). Due to high proteolytic activity in these brain extracts, all buffers and membrane/protein fractions used contained a cocktail of protease inhibitors (see the Experimental Section).

Approximately 10 μL of this extracted membrane fraction was spotted onto ITO coverslips and GUVs were grown through electroswelling as previously described.[17] GUVs grown from these preparations resulted in various vesicles (both multi- and unilamellar) ranging in size between five and 100 μm (Figure 2). GUVs were visualized either with the lipid dye DiD-C18 or by immunostaining with specific antibodies targeting proteins known to be integral to brain membranes. Figure 2 illustrates a typical GUV from porcine brain stained

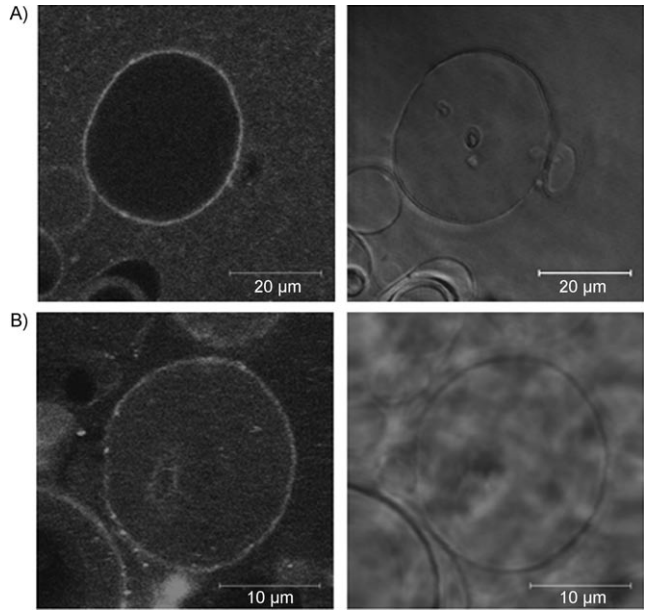

Figure 2. Giant unilamellar vesicles prepared from porcine brain extracts. Porcine brain membranes were spotted onto ITO cover slips and GUVs were formed by electroswelling as described in the see the Experimental Section. GUVs were visualized by immunostaining against A) Na/K ATPase and B) Ca^{2+} ATPase with mouse monoclonal antibodies and a secondary goat anti-mouse antibody conjugated to Cy5 (left). Right panels are the corresponding phase contrast images. Scale bars in (A) and (B) are 20 and 10 μm, respectively.

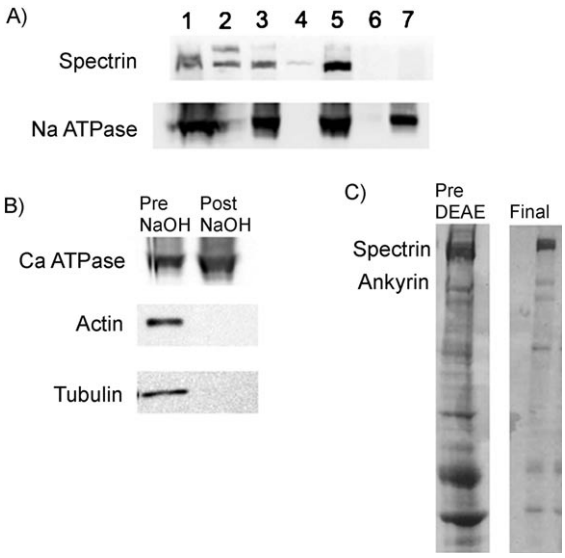

Figure 3. SDS-PAGE and immunoblot analysis of various porcine membrane and protein fractions. A) Immunoblots against porcine spectrin (260 kDa) and Na/K ATPase (110 kDa) at various steps of the preparations (see the Experimental Section). Lane 1: Whole brain homogenate. Lane 2: Supernatant from whole brain homogenate centrifugation. Lane 3: Membrane fractions after centrifugation through a 1 M sucrose bed (demyelinated). Lane 4: Starting membrane supernatant. Lane 5: Starting membrane fraction. Lane 6: Rapidly sedimenting membrane fraction. Lane 7: Slow sedimenting membrane fraction. B) Immunoblots against porcine Ca^{2+} ATPase (140 kDa), actin (42 kDa) and tubulin (57 kDa) pre and post NaOH extraction. C) Coomasie blue stained SDS-PAGE of highly enriched spectrin/ankyrin protein fraction. The left panel shows the pre DEAE protein fraction while the right panel shows the final purified fraction used in the current study.

with a mouse monoclonal antibody against (A) Na/K ATPase and (B) Ca^{2+} ATPase. The primary antibodies were detected through a secondary goat antibody against mouse IgG conjugated to a Cy5 fluorescent dye. GUVs are also depicted as phase contrast images (Figure 2, right panels). It can be seen that the antibody localizes along the surface of the GUV. Since there are multiple washes after primary and secondary antibody incubation, we believe that the immunostaining of the membrane is indicative of the presence of both Na/K ATPase and Ca^{2+} ATPase in the GUV membrane. Controls performed on the GUVs with an unspecific primary antibody against human spectrin, which does not recognize porcine brain spectrin, or in the presence of secondary antibody alone did not result in staining of the GUVs (data not shown). In addition, mouse monoclonal antibody against Na/K ATPase and Ca^{2+} ATPase did not bind to GUVs prepared from porcine total brain lipid extract (TBLE) containing no proteins (not shown). Immunoblots of the various membrane fractions against the Na/K ATPase as well as spectrin are displayed in Figure 3 A. Spectrin/ankyrin is very tightly associated to the membrane fractions. However, after membrane extraction with NaOH, all membrane associated proteins that are not integral are separated from the membrane. Immunoblots against spectrin, Ca^{2+} ATPase, actin and tubulin demonstrated the absence of cytoskeletal components in the membrane preparations after NaOH extraction, while the integral Ca^{2+} ATPase remained present (Figure 3 B).

GUVs from porcine brain retain functional ion channels

The presence of integral membrane proteins in porcine GUVs does not imply that these proteins retain proper structure and/or activity. To ensure that the NaOH extraction of the membranes, which is necessary to remove actin, ankyrin and spectrin, did not inactivate the integral membrane proteins, we set up a functional assay for Ca^{2+} ATPase. We prepared the GUVs in the presence of ATP and Calcium Green, a sensitive calcium probe that becomes brightly fluorescent in the presence of Ca^{2+}. By growing GUVs in the presence of these components, we ensured that the interior of the GUVs contained both ATP and Calcium Green. We then washed the GUVs extensively with buffer containing ATP (1 mM) but no Calcium Green (10–20 chamber volumes or ~2–4 mL of buffer) in order to remove any excess Calcium Green from the media surrounding the GUVs. These GUVs showed virtually no fluorescence signal (Figure 4 A). Upon injection of $CaCl_2$ (5 mM) and ATP (1 mM) into the GUV chamber the interior of the GUVs became bright fluorescent green (Figure 4 B), while GUVs grown and washed in the absence of ATP remained at a nearly undetectable fluorescent level (Figure 4 C). These results demonstrate that the integral membrane ion transporters reconstituted in our GUVs from porcine brain remained active. Next we examined the spectrin/ankyrin binding capabilities of the porcine brain GUVs.

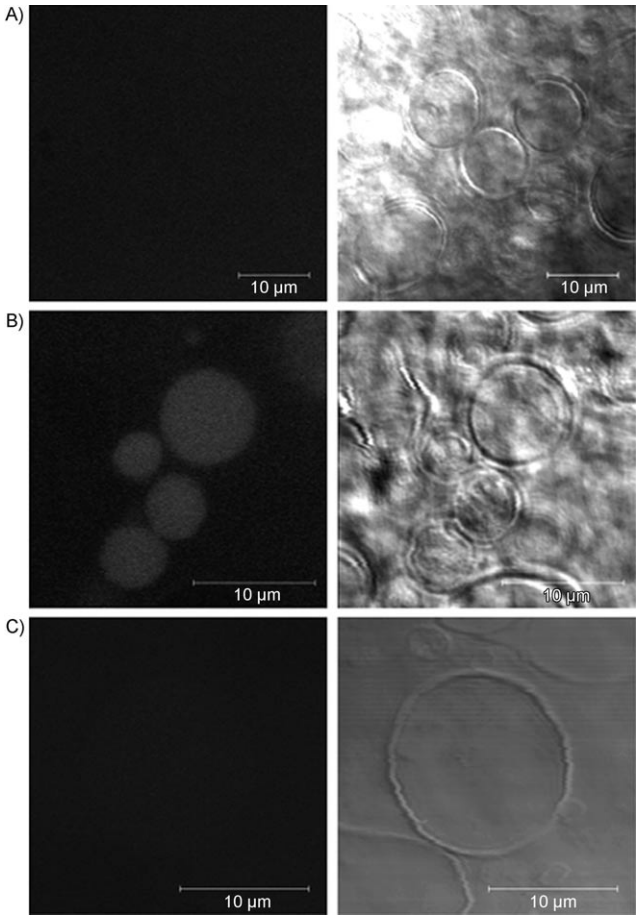

Figure 4. GUVs from porcine brain retain active ion channels. A) GUVs grown in the presence of ATP (1 mM) and calcium green indicator (10 μM). In the absence of Ca^{2+}, the indicator is not fluorescent. B) GUVs prepared as in (A); however, after formation, the GUVs were treated with Ca^{2+} (5 mM). C) GUVs were grown in the presence of 10 μM calcium green indicator, but in the absence of ATP. Following formation, GUVs were treated with Ca^{2+} (5 mM) as in (B). Left panels indicate fluorescence images while right panels indicate phase contrast images; scale bars are 10 μm.

Anchoring of the cytoskeleton inside porcine GUVs

GUVs were grown as described above in the presence of purified actin (5 μg) treated with phalloidine–Alexa 488 conjugate (1 unit). After electroswelling, the GUVs were washed extensively with buffer in order to remove the majority of the actin filaments on the outside of the GUVs. The resulting GUVs contained actin filaments dispersed throughout their interior (Figures 5 A and B). Resolving the single filaments was difficult due to large undulations of the GUV membrane as well as the free diffusion of actin filaments within the GUV. We did observe some free floating filaments in the surrounding buffer medium, but the majority of the filaments were contained within the GUVs (Figure 5 A).

Highly enriched protein fractions containing spectrin and ankyrin were isolated using a protocol developed by Davis and Bennett with slight modifications (see Experimental Section).[13,14,16] GUVs were prepared from these fractions in the presence of actin and spectrin/ankyrin. The filaments in such GUVs were no longer dispersed throughout the GUV interior.

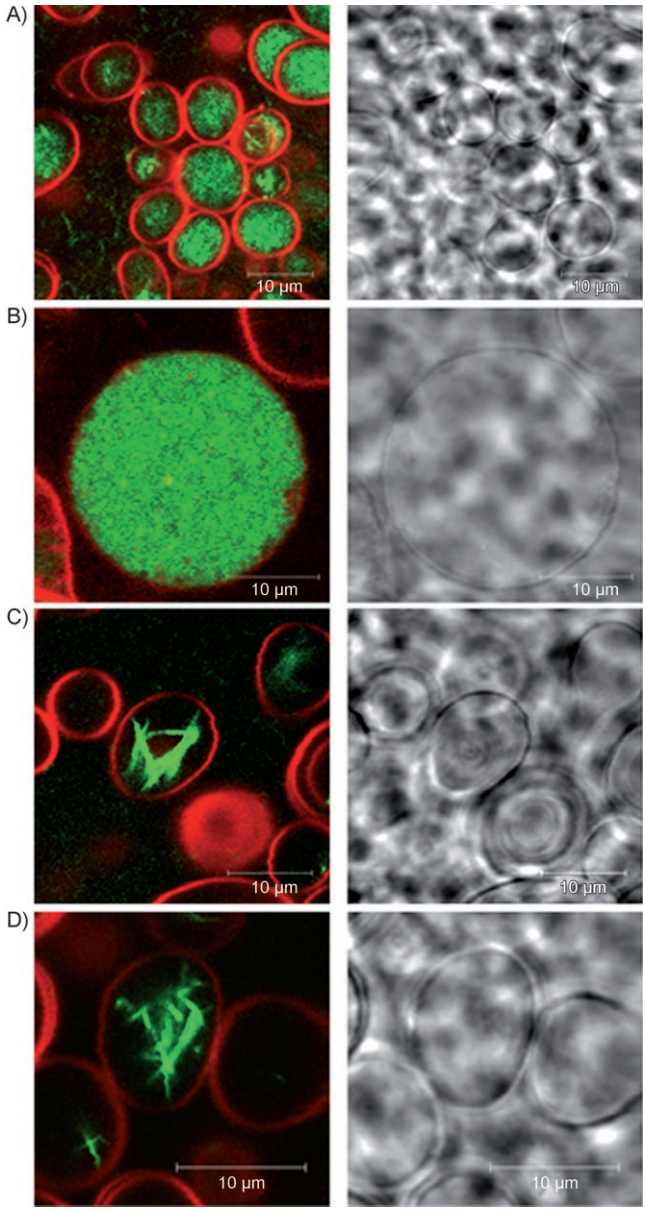

Figure 5. Spectrin/ankyrin proteins can anchor actin filaments to the interior walls of GUVs and result in dense actin bundle formation. A) and B) GUVs prepared from porcine brain membrane extracts (visualized with the lipid dye DiD C18) in the presence of actin (visualized with phalloidin–Alexa 488 conjugate). C) and D) GUVs formed as described in (A) and (B) with the addition of a highly enriched fraction of spectrin/ankyrin proteins; scale bars are 10 μm.

Instead, the actin filaments displayed dense packing near the walls of the GUVs (Figures 5 C and 5 D), as according to the sketches in Figure 1. In the majority of cases, the filaments appeared anchored to the interior wall of the GUV, as can be seen by the stack of cross-sectional images displayed in Figures 5 A and 5 B. The series begins on the outer wall of the GUV (Figures 6 A and B panel 1) and progressively moves towards the GUV equator (each image represents a 1 μm shift towards the equator of the vesicle in the *z*-direction). Figure 6 A demonstrates actin filaments closely adhering to the wall and following the curvature of the GUV (purple arrow). In Fig-

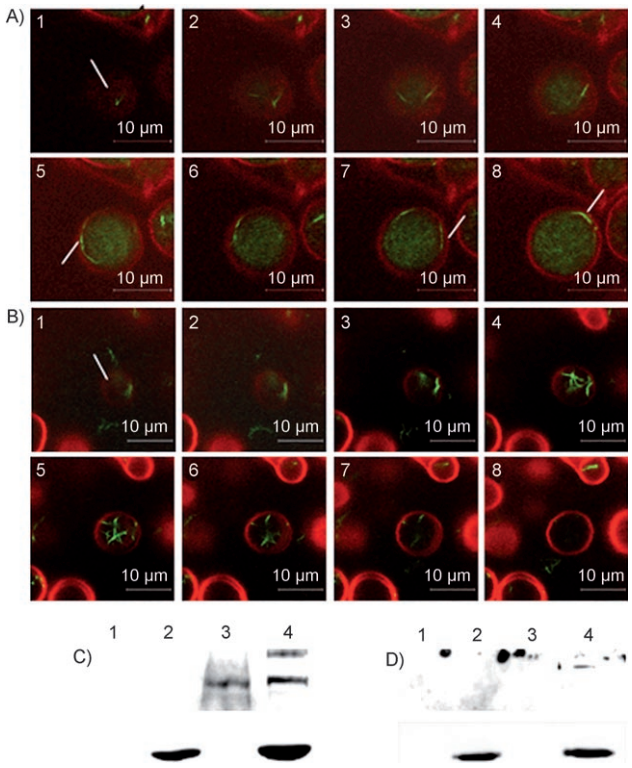

Figure 6. In the presence of spectrin/ankyrin, actin associates directly with integral proteins in the lipid bilayer of the GUV. A) and B) *z*-Stack fluorescence images visualized with DiD C18 (red) and actin–phalloidin Alexa 488 conjugates (green). Images begin at the outer wall of the GUV (panel 1) and move towards the equator of the vesicle (panel 8) in 1 μm increments per panel. A) The white line in panel 1 indicates the outer-membrane wall, while the white lines in panels 5, 7 and 8 indicate an actin filament following the curvature of the GUV membrane wall. B) The white line in panel 1 indicates the outer-membrane wall of the GUV; scale bars are 10 μm. C) Membrane-precipitation assay showing interactions between porcine brain membrane fractions, spectrin/ankyrin and actin. D) Membrane-precipitation assay showing interactions between porcine total brain lipid extract (contains no proteins), spectrin/ankyrin and actin. In (C) and (D) immunoblots on the top panel were probed for spectrin and immunoblots on the bottom panel were probed for actin. Lane 1: porcine membrane (C)/TBLE(D) alone. Lane 2: porcine membrane/TBLE plus actin. Lane 3: porcine membrane/TBLE plus spectrin/ankyrin. Lane 4: porcine membrane/TBLE plus spectrin/ankyrin and actin.

ure 6B, it can be seen that the filaments are located directly below the GUV wall and are no longer visible when the focus reaches the equator of the vesicle (panel 8).

We then examined the nature of protein–GUV lipid interactions. We developed a membrane precipitation assay, in which the membrane fractions were incubated with actin and/or spectrin/ankyrin for 10–15 min and then pelleted by centrifugation. The pellet was then resuspended and washed with excess buffer containing 0.05% Tween 20 to remove any unspecific protein interactions. In essence, the membrane containing the integral proteins was pelleted and washed rather than beads conjugated to antibodies as in a traditional IP assay. The washing/centrifugation procedure was repeated fivefold prior to addition of SDS loading dye and resolution by SDS-PAGE (see Experimental Section). Immunoblots showed that in the absence of ankyrin and spectrin, the actin filaments could still interact with the lipids of the GUV (Figure 6C). In addition, the membrane precipitation assay demonstrated that spectrin alone could also associate with the porcine membrane fractions as could actin in the presence of spectrin (Figure 6C). In the presence of spectrin/ankyrin, ~30% more actin was retained by the membrane fractions, as approximated by PAGE band quantitation (compare lanes 2 and 4 in Figure 6C, lower panel). It has been previously demonstrated that both spectrin and actin can bind membranes through their electrostatic interactions with the exposed, charged head groups of the lipids.[18–20] To better examine spectrin/actin–lipid interactions, we repeated the membrane precipitation assay with a total brain lipid extract (TBLE) containing no proteins. The membrane precipitation assay demonstrated the ability of actin to associate with the TBLE GUVs (Figure 6D). However, the spectrin/ankyrin pool did not associate with the membranes under these conditions, unless in the presence of actin (Figure 6D), although very weakly. This may indicate that the spectrin/ankyrin can associate with the actin filaments, but not with the TBLE of the GUV wall. This idea is also supported by band intensity analysis of Figure 6D, lower panel lanes 2 and 4, in which there is an approximate 1.3-fold (or about 20%) increase in actin retention of the TBLE in the presence of spectrin/ankyrin. Comparison of the intensity of the bands in the gels indicates that the porcine membrane retained about 1.8-fold more actin than the TBLE (compare lower panels of Figures 6C and D, lanes 2), while in the presence of ankyrin and spectrin, the porcine membrane retained approximately 2.5-fold more actin than the TBLE and 1.9-fold more actin than the TBLE with spectrin/ankyrin (compare lower panels in Figures 6C lane 4, and 6D lanes 2 and 4). Taken together, these results indicate the first reconstitution of natively anchored cytoskeleton to the interior walls of GUVs.

Discussion

Minimal systems allow for the study of biochemical pathways in a cell-like environment that is free from the overwhelming complexity found in living cells. Working with systems like giant unilamellar vesicles (GUVs) is a fascinating task that allows the exploration of whether the key phenomena of living matter, such as the capacity to divide and self-replicate, can be reconstituted in such artificial environments with dramatically reduced complexity.[1] In the present work, we have reconstituted the anchoring of actin filaments to the interior of native lipid–protein composition GUVs through the spectrin/ankyrin proteins, which bind to integral membrane proteins.

There has been significant previous work involving the incorporation of membrane proteins into GUVs.[11] Previous studies have demonstrated that the lipid composition of the membrane bilayer can directly influence the function (and hence the proper folding) of the incorporated proteins.[17] Therefore, since the anchoring of actin to the eukaryotic plasma membrane requires integral membrane proteins, we opted to extract both the integral membrane proteins as well as the native lipids from porcine brain. We did this since it is not trivial to recombinantly express these large, multi-subunit proteins

and then ensure that they incorporate into the GUVs properly with respect to one another. In addition, the already thorough characterization of the spectrin-, ankyrin- and actin-based cytoskeleton in porcine brain made this tissue ideal for the extraction of actin-based cytoskeletal components for reconstitution.[13–16] We confirmed the functional state of the membrane proteins in the porcine GUVs, in particular, the integral protein Ca^{2+} ATPase was shown here to transport free Ca^{2+} into the interior of the GUVs. While recombinant Ca^{2+} ATPase has been previously reconstituted in vesicles made of synthetic lipids,[21] this is the first demonstration of GUVs formed from native porcine brain membranes that contain functional transmembrane proteins. This also highlights the efficacy of electroswelling for forming complex component containing unilamellar vesicles with functional proteins within.

Since the main goal of this study was to anchor actin filaments to the GUVs, our primary concern was to ensure that these transmembrane proteins reconstituted in the GUVs were functional with respect to protein-binding (that is, spectrin/ankyrin and actin). Actin has been previously polymerized inside GUVs consisting of simple lipid compositions, and there is also work describing the anchoring actin to the outer walls of GUVs.[18,22–25] However, the interior anchoring of actin filaments to GUVs has only been achieved through electrostatic interactions or through formation of biotin-streptavidin complexes.[18,22,23] Here we demonstrate actin filament binding and anchoring inside complex lipid–protein GUVs, mediated by native spectrin/ankyrin. In general, the GUVs from porcine brain were highly undulatory in nature making imaging a challenge. Nonetheless, the undulations of the interior actin filaments were greatly reduced in the presence of spectrin/ankyrin, reinforcing the idea that they were immobilized and anchored to the walls of the GUVs. In the vast majority of cases, *z*-stack analysis indeed revealed that these actin bundles were located in discrete regions within the GUV, often near the membrane wall (Figure 6A and B). Repeated imaging over the course of several hours also revealed that these filaments were not free floating, but were fixed to specific locations within the GUV (data not shown).

Previous studies have indicated gross vesicle deformation in GUVs encapsulating polymerized actin filaments.[23] Although we did observe a few deformed vesicles, as well as some vesicles with protruding filaments, in the vast majority of GUVs formed in the presence of spectrin and ankyrin, the actin filaments only formed the dense packed bundles described above. Even in the absence of spectrin and ankyrin, the vast majority of the GUVs remained somewhat spheroid (Figures 5C and D and 6A and B). The exact cause of these observations is difficult to pinpoint for they are likely dependent upon actin amounts, the mode of polymerisation, and the lipid (and protein) composition of the GUVs. (When working with model systems, one inherently also encounters artefacts of that model system.) Furthermore, our actin filaments were polymerized and stabilized with phalloidin, salt and spectrin/ankyrin whereas, in previous studies, polymerization of actin inside GUVs was induced by electrostatic means and/or by temperature changes.[18,22,23] A study by Wagner and colleagues[26] examined how the structure of cross-linking molecules influences the structural and mechanical properties of actin networks. While this study did not specifically examine the effect of spectrin/ankyrin, they showed similar actin bundle formation in the presence of specific cross-linking molecules.[26] The study also demonstrated that the spacing structure of the cross-linker molecules predominantly determines their mechanical effectiveness, and not the affinity of the actin-binding domains. Here, the spectrin/ankyrin interaction with actin resulted in similar bundle formation, which may have influenced filament length and hence vesicle deformation (or lack thereof). It has also been shown that spectrin/ankyrin can greatly strengthen the plasma membrane of a cell (facilitating bleb formation and mass cell deformation without cell rupture), and spectrin rod domain repeats have been demonstrated to induce a large increase in the surface shear viscosity of lipid monolayers. This "stiffening" of the membrane could have contributed to the lack of vesicle deformations. According to our results, the interaction of actin with spectrin/ankyrin stabilized the actin filaments into dense bundles. It is likely that these dense actin networks anchored to the GUV walls induced local membrane tension. This idea is supported by our observation of undulations of the GUV wall during imaging. In the case of GUVs with spectrin/ankyrin anchored actin, the bundles are rather fixed and static. (Nonetheless, the membrane undulations did persist, however the degree/amplitude of membrane undulations in the presence and absence of actin/spectrin/ankyrin was not investigated in detail.) In addition, the spectrin/ankyrin used here was not highly pure, but only a highly enriched fraction (Figure 3C). We cannot exclude the possibility that some contaminant proteins had an effect upon the actin organization. It should also be noted that electroswelling is not 100% efficient in GUV formation. That is, the surface of the ITO sample slide still contained much of the originally spotted membrane fractions and actin filaments. Hence, many of the ruptured GUVs (if any) may have sedimented onto this layer, while those GUVs that electroswelled up from this layer contained densely packed actin bundles that did not deform the membranes. It is also worth mentioning that this large lipid pool below the electroswelled GUVs also made epi-fluorescence illumination difficult (due to very high background fluorescence), and hence we opted to use confocal imaging, despite the large undulatory nature of GUVs. The goal of the present study was to reassemble the spectrin/ankyrin and actin interactions on the interior walls of GUVs, and we are currently examining these spectrin–actin–GUV interactions, and their effect on vesicle shape, in more detail.

We further examined the actin filament interactions with porcine lipids in the presence and absence spectrin/ankyrin using a membrane precipitation assay (Figures 6C and D). As a control, we examined porcine total brain lipid extracts, since both spectrin and actin have been shown to bind lipids through electrostatic interactions.[18,19] In the absence of spectrin and ankyrin, both our membrane-protein containing porcine extracts and the TBLE were capable of binding actin, although the porcine membrane extracts did exhibit greater retention of actin. Upon addition of spectrin/ankyrin, both the

amount of actin associated with our porcine brain membrane extracts as well as the amount of actin associated with the TBLE lipids increased, indicating spectrin/ankyrin induced cross-linking actin filaments (Figures 6C and D). Furthermore, spectrin/ankyrin could only interact with our porcine brain membranes and not significantly with TBLE lipids. Spectrin–lipid binding is due in part to the ankyrin-binding domain, and can be inhibited through the addition of ankyrin to spectrin, and likely explains why we do not see spectrin binding to the total porcine brain lipid extract (and is indicative that we had intact spectrin/ankyrin complexes). The association between spectrin and ankyrin is very strong, and traditional purification protocols require harsh treatment of the spectrin/ankyrin complexes for proper dissociation (KBr).[16] Furthermore, our assay utilized extensive washing in the presence of detergent, which could disrupt the electrostatic interactions between the lipids and the spectrin. While we could not find a commercially available ankyrin antibody that would detect porcine ankyrin, the previously well-characterized spectrin/ankyrin purifications also produced similar banding patterns in Coomasie stained SDS-PAGE as observed in Figure 3C. Taken together, these data suggest that we indeed anchored actin filaments to the interior walls of GUVs through the specific interaction between spectrin/ankyrin and integral membrane proteins. To our knowledge, this is the first demonstration of a four-component protein interaction reconstituted in a close-to-native state inside GUVs.

Previous studies have shown actin anchored to the exterior walls of GUVs.[18,22–25] A recent and interesting study by Liu and colleagues demonstrated that actin polymerization can induce changes in the membrane domain organization of GUVs and stabilize existing domains.[25] The GUVs used in that study were of a relatively simple composition (four lipids) and the actin filaments were anchored through N-WASP interactions with PIP2 and Arp2/3. We examined our porcine GUVs for the existence of membrane domains but did not observe any domain formation or rearrangement on a large spatial scale (within the optical resolution), both in the presence and absence of the actin networks (Figure 5 and data not shown). Nonetheless, our actin networks were often localized to specific regions on the GUV wall. It could be that domains exist, however they may be too small to resolve optically in our current system. Also, we cannot rule out domain rearrangements (below optical resolution) upon actin polymerization and anchoring to the GUV walls.

Also important to the discussion is the lipid composition of the vesicles used. Here we utilized a demyelinated total brain porcine extract. Commercially available total brain lipid extracts (Avanti) contain low levels (i.e., $<17\%$) of phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, phosphatidic acid, and phosphatidylinositol, along with a major component consisting of sphingomyelin, gangliosides, and ceramides, which are mainly removed during the demyelination through the sucrose gradient (see Experimental Section). While it was beyond the scope of the current work to perform a detailed analysis on the effects of individual lipids in the vesicles, such studies are of importance and are under investigation.

In conclusion, the current study is an important step towards the far goal of creating an artificial cell. In order to achieve a minimal system for cell division, there is a requirement for organizing and anchoring the cytoskeleton within such vesicles. The next steps will be to control the organization of the actin networks in the GUVs (that is, specifically localize the anchoring of the filaments) and potentially introduce motor proteins to induce constriction of the vesicles. Such studies are currently underway.

Experimental Section

Materials: All reagents, salts, protease inhibitors and buffers were purchased from Sigma. Antibodies against porcine brain spectrin (ab11182), human erythrocyte spectrin (ab2808), Na/K ATPase (ab2871), Ca^{2+} ATPase (ab2825), actin (ab11004), tubulin (ab7291), as well as goat anti-mouse and goat anti-rabbit IgG HRP conjugates (ab6789 and ab6721, respectively) were purchased from Abcam (Cambridge, UK). Secondary goat anti-mouse Cy5 conjugates were purchased from Jackson ImmunoResearch Laboratories (Suffolk, UK). The lipid probe DiD-C18, purified actin from rabbit skeletal muscle, calcium green and phalloidin conjugated to Alexafluor 488 were purchased from Molecular Probes (Invitrogen). Total porcine brain lipid extract (TBLE) was purchased from Avanti Polar Lipids (Alabaster, USA). All confocal images were taken on a Zeiss Meta LSM 500, and all immunoblots were visualized using a Fuji LAS 3000 chemiluminescence detection system.

Isolation of porcine membrane fractions: Porcine brains were obtained fresh from a slaughterhouse and used within an hour of harvest. About 90–100 g of brain was used for one preparation, following a protocol developed by Davis and Bennett.[13] Note that all buffers used in all methods described below contained a cocktail of protease inhibitors including: PMSF (200 nM), pepstatin A (1 $\mu g\,mL^{-1}$), benzamidine (2 mM), leupeptin (100 μM), aprotonin (800 nM), chymostatin (100 μM), and antipain (100 μM). Briefly, the brain tissue was washed in fresh PBS buffer to remove menengis and then was homogenized in homogenization buffer (250 mL) containing sodium phosphate (10 mM), sodium azide (5 mM), EDTA (0.2 mM) at pH 7.5. The homogenate was centrifuged at 900 *g* for 15 min and the resulting supernatant was centrifuged at 20000 *g* for 45 min. The resulting membrane pellet was resuspended in homogenization buffer (100 mL) with sucrose (700 mM) and centrifuged at 30000 *g* for 45 min in order to demyelinate the membrane fraction. The resulting myelin layer was removed along with the supernatant and the pellet was washed three times with wash buffer. This produced the "starting membrane" fraction. At this point, the starting membrane pellet was split into two equal portions, one for the isolation of the spectrin/ankyrin pool (see below), and one for the production of porcine membrane fractions free of nonintegral membrane associated proteins.

One half of the above pellet was then resuspended in NaOH (100 mL, 0.1 N) and extracted at 4° C for 30 min. The extracted mixture was then centrifuged at 30000 *g* for 45 min through wash buffer containing sodium phosphate (10 mM), sodium azide (5 mM), EGTA (0.2 mM), DTT (0.5 mM), 0.05% (*v/v*) Tween 20 and 10% (*w/v*) sucrose and the resulting pellet was washed with wash buffer until the pH was returned to 7.5 (ca. 5×). This membrane fraction was the "rapidly sedimenting" membrane fraction. The supernatant from the 10% sucrose spin was also centrifuged for 60 min at 40000 rpm to produce a slowly sedimenting membrane fraction. This membrane fraction was washed with wash buffer

until the pH returned to 7.5. After the final washes, the two membrane fractions (rapid and slow sedimenting) were again centrifuged and then resuspended in GUV buffer, which consisted of wash buffer containing trehalose (20 mM) and KCl (10 mM), distributed into aliquots, snap frozen in liquid nitrogen and stored at −80 °C. Aliquots were used within several days of preparation and were only thawed once for use. Upon thawing, no membrane fractions were refrozen or reused. Aliquots were 10 μL at an approximate lipid concentration of 30–40 mg mL^{-1} and a protein concentration of approx. 5 mg mL^{-1}. Quantitation was achieved by using a nanodrop spectraphotometer. As can be seen in Figure 2A (lanes 6 and 7), the rapidly sedimenting membrane fractions contained no Na/K ATPase and hence all experiments were performed with the "slowly sedimenting" membrane fractions.

Isolation of highly enriched spectrin/ankyrin pool: Following the protocol above, the porcine membranes were prepared up to the "starting membrane" fraction stage. At that point, we followed a protocol described by Davis and Bennett with some modifications.[14–16] One half of the starting membrane pellet was extracted with wash buffer containing KCl (500 mM) for 30 min. The extract was centrifuged for 45 min at 30000 *g* and the resulting pellet was further extracted with wash buffer containing KI (800 mM) for 60 min. Spectrin and ankyrin remained in the supernatant upon centrifugation at 30000 *g* for 45 min. Ammonium sulphate was added to the isolated supernatant at 4 °C to 60% saturation, and the proteins were precipitated at −20 °C followed by centrifugation at 1000 *g* for 15 min. The resulting pellet was resuspended in wash buffer dialysed against wash buffer containing KCl (500 mM). This suspension was centrifuged at 100000 *g* for 60 min to remove small vesicles, and the supernatant was concentrated through a 300 kDa MWCO Vivaspin 50 concentrator in order to remove smaller unwanted proteins. Since the associated spectrin/ankyrin complex is far bigger than 300 kDa, it did not pass through the filter. The concentrate, which was enriched in spectrin/ankyrin, was then dialysed to KCl (150 mM) in ankyrin buffer and loaded onto a 10 mL DEAE sepharose fast-flow column (Amersham; Figure 3C, left). The column was washed with 10 bed volumes of wash buffer containing KCl (150 mM) and the column was eluted in steps with wash buffer containing 300, 500 and 750 mM KCl. The spectrin/ankyrin eluted at 300 mM KCl, as determined by SDS-PAGE and Coomasie blue staining. The spectrin/ankyrin containing fractions were concentrated through a 300 kDa MWCO Vivaspin 50 concentrator as described above (Figure 3C, right). The preparation was distributed into aliquots, snap frozen in liquid nitrogen and stored at −80 °C. Aliquots of 50 μL were prepared at a protein concentration of approximately 5 mg mL^{-1}. The presence of spectrin was confirmed through immunoblotting (Figure 6). Unfortunately a commercially available porcine-brain-ankyrin antibody was not available, although ankyrin is clearly visible through Coomasie blue staining (Figure 3C).

GUV preparation from porcine brain fractions: GUVs were prepared as previously described.[17] Aliquots of porcine membrane fractions were spotted evenly over ITO coverslips. The chambers were then sealed and filled with GUV buffer. The GUVs were grown using electroswelling for 2 h at 1.2 V. Where indicated, actin (5 μg) and/or spectrin/ankyrin (5 μg) was added to the membrane aliquote and thoroughly mixed prior to spotting onto the ITO coverslip. Where indicated, DiD-C18 (0.2 μL, 2 μM) was added to the mixture in order to stain the lipid bilayer. Samples containing actin were also treated with one unit of phalloidin–Alexa 488 conjugate (following the manufacturer's recommended protocol). The GUVs were visualized using a Zeiss Meta LSM 500 confocal microscope.

Immunostaining of GUVs: GUVs prepared from porcine brain were incubated with a solution of 1:500 primary antibody (mouse anti-Na/K ATPase, or Ca^{2+} ATPase as indicated) in GUV buffer and left at room temperature for 60 min. The chamber was then washed with 10 chamber volumes (10×200 μL) of wash buffer. Next, a 300 μL solution of 1:500 secondary goat-anti-mouse antibody conjugated to the Cy5 fluorescent dye in GUV buffer was injected into the GUV chamber and incubated at room temperature for 60 min. After this incubation, the chamber was washed with approximately 20 chamber volumes of GUV buffer containing Tween 20 (0.05%). The GUVs were then visualized by using a Zeiss Meta LSM 500.

Assay for Ca^{2+} ATPase activity in porcine GUVs: GUVs were prepared as described above in the presence of calcium green indicator (10 μM) and, where indicated, ATP (1 mM), following the manufacturer's (Invitrogen) recommended protocol. After electroswelling, the sample chamber was washed with excess wash GUV buffer (containing 1 mM ATP, where indicated). The chamber was then filled with GUV buffer containing $CaCl_2$ (5 mM), and ATP (1 mM) where indicated, and imaged as described above.

"IP-like" assay for spectrin/ankyrin and actin binding to porcine membrane fractions and immunoblots: "slowly sedimenting" membrane fractions (5 μL) were incubated with actin (5 μg) and/or highly enriched spectrin/ankyrin (5 μg) on ice for 15 min. The mixtures were then diluted to 500 μL with wash buffer containing Tween 20 (0.05%) and centrifuged at 14000 rpm for 15 min. The resulting membrane pellet was resuspended and washed in 500 μL wash buffer containing Tween 20 (0.05%, 5×) before the final pellet was resuspended in 50 μL wash buffer, SDS loading dye was added and the samples were boiled and resolved with 8% SDS-PAGE. The resolved SDS gels were transferred onto nitrocellulose and blocked for 60 min with a milk solution (5%) in Tween-Tris buffered saline (TTBS) buffer containing Tween 20 (0.05%), Tris-HCl pH 7.5 (20 mM), and NaCl (0.5 M). Primary antibodies were incubated for at least 2 h followed by 3×X 5 min washes with TTBS and an incubation of secondary antibody for 20 min. Blots were washed excessively with TTBS and treated with ECL solution prior to imaging chemiluminescence with a Fuji LAS 3000.

Abbreviations: GUV: giant unilamellar vesicle; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBLE: total brain lipid extract; PMSF: phenylmethylsulfonyl fluoride; DTT: dithiothreitol; EGTA: ethylene glycol tetraacetic acid; IP: immunoprecipitation; TTBS: Tween–Tris buffered saline; PBS: phosphate buffered saline; ITO: indium titanium oxide; ATP: adenosine triphosphate; Na/K ATPase: sodium/potassium ATPase; Ca^{2+} ATPase: calcium ATPase; KI: potassium iodide; KCl: potassium chloride; KBr: potassium bromide.

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