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Biologically-active unilamellar vesicles from red blood cells†

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We demonstrate a method to prepare giant unilamellar vesicles (GUVs) with biologically-active protein activity, by mixing erythrocyte (red blood cell) membrane extract with phospholipids and growing their mixture in a porous hydrogel matrix. This presents a pathway to retain protein biological activity without prior isolation and purification of the protein, though only the activity of the membrane protein GLUT1 is investigated to date. Using the cascade enzymatic reaction glucose oxidase and horseradish peroxidase to assay glucose concentration specifically within the GUV interior, we show that glucose is internalized by GLUT1 whereas adding cytochalasin B, a GLUT1 inhibitor, blocks glucose transport. The method presented here operates at biological ionic strength and is both simple and potentially generalizable.

Introduction

This study considers how to impart biological functionality to giant unilamellar vesicles. The typical cell membrane contains 50% proteins, yet the preponderance of studies involving unilamellar vesicles consist of purified lipids to which previously-purified membrane proteins are added. This limitation presents the challenge to study the complex and dynamic functionality of cell membranes outside the cell's native environment, as if it can be accomplished, to do so would offer interesting potential not only as a platform on the basis of which to study fundamental biophysical and biochemical science, but also from which to explore potential applications in cell-mimicking drug delivery, biological diagnosis, and even soft-robotics.[1,2] Recent advances to wrap native cell membranes around nanoparticles[3,4] eliminate the earlier need for labor-intensive protein identification, purification, and conjugation into synthetic membranes and the resulting biodegradable nanocarriers have been implemented for eukaryotic red blood cell membranes (RBCs),[3] cancer cells,[4] platelets,[5] leukocytes,[6] and bacteria,[7,8] in each case taking advantage of the unique cell membrane properties of these respective cell types, yet with membranes located outside the native cell environment, but giant unilamellar vesicles (GUVs) were not investigated. An earlier pioneering study prepared GUVs from red blood cells by methods based on traditional electroformation, but the method required an environment of low ionic strength.[9]

The question motivating this study was whether protein activity can be retained after forming unilamellar vesicles from cell membrane extract and no prior specific isolation of the membrane protein of interest. Membrane proteins traditionally are difficult to handle and risk losing functionality outside the cell membrane environment. In previous studies that involved exposure to organic solvents, oils and other electric fields in the preparation of synthetic membranes,[9–12] difficulties in incorporating membrane proteins into synthetic phospholipid bilayers often resulted in either a low yield or damage to the membrane protein functionality. Moreover to incorporate transmembrane proteins into such membranes required the expense of labor and time to obtain such proteins, usually by targeted expression and purification.[13]

Experimental

Materials

1,2-Dioleoyl-*sn-glycero*-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn-glycero*-3-phosphoethanol-amine-*N*-biotinyl sodium salt (16:0 Biotinyl PE), 1,2-dipalmitoyl-*sn-glycero*-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) 16:0 Liss Rhod PE were purchased from Avanti Polar Lipids (USA) and used without further purification. Reagents for surface treatments and GUV formation, the agarose type IX-A ultra-low gelling temperature, chloroform, streptavidin, BSA-avidin, BSA and phosphate buffered saline (PBS) (Sigma-Aldrich, USA) were analytical grade and used without further purification. Anti-Glucose Transporter GLUT1 antibody (Alexa

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Fluor® 488) (Abcam, USA), cytochalasin B from Drechslera dematioidea (Sigma-Aldrich, USA) and Amplex™ Red Glucose/Glucose Oxidase Assay kit (Thermo Scientific, USA) were used following the manufacturer's instructions. Glass-bottom Petri dishes were used for GUV formation and fluorescence microscopy (IBIDI GmbH, Germany). Deionized 18.2 MΩ cm Milli-Q water was used in all experiments (EMD Millipore, USA).

For fluorescence imaging labeling, we used the lipid mixture DOPC : Rhod-DMPE : biotin-labeled lipids in the mole ratio 99.7 : 0.2 : 0.1. In case of the glucose internalization study with Amplex™ Red Glucose Oxidase Assay kit, Rhod-DMPE was removed due to the overlapping emission ranges with resorufin after the enzyme interactions.

Bright field microscopy

An optical microscope (Zeiss, A1) was employed to measure GUV formation using a 63× air objective (NA = 0.75, phase control, Zeiss). An EMCCD camera (Andor) was used to acquire the time lapse images at 20 frames per second.

Confocal microscopy

Laser scanning confocal microscopy (Leica TCS SP8 STED; Leica Microsystems) employed a 63×/1.4 NA oil-immersion objective lens and involved imaging fluorescent lipids of DMPE-RhB (1,2-dipalmitoyl-*sn-glycero*-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt), at an abundance of 0.2 mol%. Data was processed using Leica LAS X software for 3-D construction after *z*-stack images were acquired. The wavelength of 560 nm was employed to excite the dye and emission at 588 nm was measured.

Extraction of RBC-membrane

The obtained whole blood from mice was washed with ×1 PBS and centrifuged at 800*g* for 5 min at 4 °C in order to remove serum and the buffy coat. The packed RBCs were washed in ice cold 1× PBS and following re-suspended in 0.25× PBS in an ice bath for 20 min for hemolysis under the hypotonic condition. The yield white or pink cell membranes were obtained by the 2–3 times of centrifugation at 800*g* for 5 min. After removed the intracellular contents, the RBC membrane proteins was mostly retained.[3] Finally, the RBC extract was stored at −80 °C. All animal experiments were conducted under protocols approved by the Institutional Animal care and Use Committee of Ulsan National Institute of Science and Technology (UNISTIACUC-15-16).

Formation of RBC-membrane fused GUVs

Coverslips were sonicated in ethanol for 30 min, dried, and subsequently subjected to piranha cleaning for 30 min to make the surface hydrophilic. A solution of low-melting-temperature agarose (Type IX-A) was prepared by heating to 70 °C or alternatively for a few seconds in a microwave. The agarose concentration was 1 to 3 w/v% in deionized water. After cooling agarose solution to 36 °C, the 10–20 v/v% solution of extracted RBC membrane was mixed with the agarose solution at the ratio of 5 to 1. Twenty microliters of the final mixture were pipetted onto a cleaned coverslip, and another coverslip or pipette was used to spread a thin film. The films were allowed to gel at room temperature at least for 2 h. A total of 20 μL of 10 mg mL^{-1} lipid solution in $CHCl_3$ was added and spread evenly over the hybrid agarose. A stream of N_2 gas was blown over the lipid solution to remove the solvent. Followed by, the film was placed in a vacuum chamber (~730 mmHg) for 10 min to remove the residual solvent. Rehydration buffer, PBS (pH 7.4) was then added to the hybrid agarose-lipid film, and the system was allowed to rehydrate for 20 min. The GUVs grown in agarose were harvested by gentle pipetting and were transferred into streptavidin-coated glass Petri dishes. When using the glucose assay involving GO_x, HRP, and Amplex Red, this solution was prepared according to the manufacturer's instructions was employed instead of PBS to rehydrate the gel. For electroformation, small RBC-vesicles were prepared by exposure to a sonication bath for 10–20 min or by extrusion to the desired volume fraction, typically 0.1 to 1 v/v% in deionized water or PBS solution. Dynamic light scattering (DLS) confirmed the vesicle size in the range 50 to 200 nm. Then, these small vesicles in solution were deposited onto ITO slides and exposed to AC fields in the following sequential steps, (i) 2 V, 2 Hz for 30 min (ii) 4 V, 4 Hz for 30 min and finally 10 V, 10 Hz for overnight if required, in a water-saturated chamber. These methods, developed to encourage vesicle fusion to form larger GUVs, broadly follow standard procedures already in the literature.[3,14]

Preparation of the streptavidin-coated surfaces

Petri dishes were pre-incubated with BSA-biotin, washed with BSA, then streptavidin (1 mg ml^{-1} in each case) and subsequently rinsed thoroughly in the same buffer. Biotinylated-GUVs were allowed to anchor to the streptavidin-coated surfaces for 2 h and unbound GUVs were washed away by copious PBS washing. Finally, the specimen was moved to microscopy for imaging.

Labeling the RBC-derived GUVs

Alexa Fluor® 488 labeled antibody (Abcam, USA) and cytochalasin B from Drechslera dematioidea (Sigma-Aldrich, USA) were employed for labeling and blocking GLUT1 in the RBC extracts, respectively. Both were used as purchased to label GLUT1 after diluting from 500 : 1 to 100 : 1 as specified in the manufacturers' instructions. The labeling was completed by mixing these dye solutions with surface-attached GUVs for at least 3 h. Unbound dyes were removed by multiple washing with the same buffer.

Results and discussion

As the medium in which to grow GUVs, we selected a porous hydrogel. Though aqueous buffer is more commonly employed, the literature shows the promise of the alternative environment of a porous medium.[15] Agarose, Type IX-A

agarose (Sigma Aldrich, USA), a hydrophilic hydrogel of polysaccharides, was selected, though we note that other hydrogels, dextran cross-linked with poly(ethylene glycol) and poly (vinyl alcohol), have also been used.[16,17]

To test biological activity of membrane protein within these membranes, we consider the metabolism of glucose, which is a primary carbon energy source in cellular metabolism for biosynthesis and energy generation, but whose hydrophilic nature (five hydroxyl groups) and relatively large size (molar mass ~180 g mol^{-1}) requires cells to employ integral membrane proteins to transport it across their lipid bilayers. Among more than a dozen GLUT (glucose transporter) proteins, GLUT1 is the first characterized sugar transporter, a hydrophobic integral membrane protein[18] that has been used extensively as a model to investigate metabolic interactions and the functionality and of membrane proteins.

Extraction of red blood cell (RBC) cell membrane was conducted after isolation from mouse blood (Imprinting Control Region (ICR) 6–8 weeks mice), hemolysis by hypotonic treatment conditions and cell membrane purification using procedures in the literature.[3] After RBC membrane extractions, SDS-PAGE analysis shows minor perturbation of the composition of membrane proteins.[3]

First, a dissolved 1% (w/w) agarose (Type IX-A) solution in deionized water is mixed with 20% (v/v) of the extracted RBC membranes at room temperature at the ratio of 5 to 1. Approximately 20–30 μL of this ungelled solution is deposited onto cleaned glass Petri dishes and spread evenly with a glass rod to generate a hybrid film of mixed RBC membranes and agarose. These crosslinked, water-swellable polymers act as hosts for GUVs as they grow and appear to lessen membrane protein denaturation during this process. After spreading these films, 20 μL of 10 mg mL^{-1} neutral zwitterionic lipids of DOPC (1,2-dioleoyl-*sn-glycero*-3-phosphocholine) in chloroform is spread evenly over them, and the residual solvent is removed first by passing N_2 gas and then placing the films in a mild vacuum (~730 mmHg) for 10 min. Lastly, an aqueous solution in either deionized water or PBS buffer is poured into the Petri dish to rehydrate this DOPC-coated, partially-dried hydrogel, containing agarose and RBC-membranes. During this rehydration, GUVs are observed to form, swell with water, and then fuse into larger vesicles as small vesicles became crowded in this porous environment. Without added DOPC, GUVs did result but in lower yield and smaller size. The GUVs were removed from agarose by gentle suction.

Fig. 1A shows illustrative fluorescence images of GUVs taken at 4 consecutive times during vesicle formation; the largest vesicle size stabilized at ~10 μm within 30 min [Fig. S1 (inside the gels), S2 (different spots in gels), and S3† (floating GUVs in solution)] including agarose gel on the surface and aqueous solution, albeit with a heterogeneous size distribution ranging from a few μm to tens of μm in size depending on *xyz* locations in gels and swelling time, perhaps owing to irregular pore size in the hydrogel, as shown in Fig. 1B and Fig. S4.† The effect of agarose fraction on forming micron-sized GUVs was insignificant, probably owing to heterogeneous pore size. In order to compare the size growth of GUVs, the combined protocols of electroformation[14] and spontaneous swelling[19] were attempted; in these experiments, we applied electric fields to small RBC-derived vesicles roughly 100 nm in size, in the traditional way for electroformation typically under 10 Hz, with findings illustrated in Fig. S5.† However, the size of vesicles prepared this way reach only a few μm. In the literature, larger GUVs were achieved by electroformation at the relatively high frequency of 500 Hz, this protocol required low ionic strength, <10 mM (ref. 9) and risk of protein denaturation and

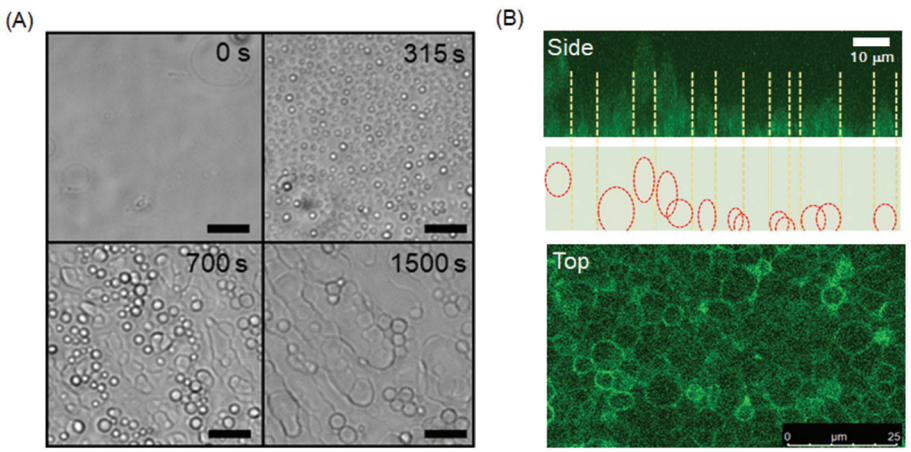

Fig. 1 Stages of growing hybrid GUVs from extracted red blood cell membrane mixed with DOPC lipid in phosphate buffer solution and growth in agarose gel. (a) Representative top-down bright field images at indicated elapsed times from zero to 1000 s showing progressive GUV formation. GUVs are visible after 5 min, and their size saturates within 30 min. All scale bars are 5 μm. (b) In the top panel, edge-on confocal images produced by z-slice; dotted vertical yellow lines call attention to the individual GUVs whose contours are outlined schematically in the middle panel as guides to the eye. In the bottom panel, top-down confocal images. In both panels, the GUVs are with rhodamine-labeled DMPE. Scale bar is 10 μm (upper panel) and 25 μm (down panel).

possible oxidation of unsaturated lipids owing to dehydration steps, with uncertain consequences. Using the methods of our present study, the final GUVs contained 25–30% lipids relative to the amount initially deposited onto the agarose films, as determined from our fluorescence quenching experiments with the resulting GUVs [Fig. S6†].

Fig. 2A and B show representative images of these biotin–avidin coated surface-attached RBC-derived GUVs. Antibodies for the protein GLUT1 were introduced, labeled with the fluorescent dye Alexa 488 (Abcam, USA), after GUV attachment onto a glass slide previously modified to anchor them to the surface by biotin–streptavidin linkage. For this purpose, biotinylated lipid (Avanti, USA) and streptavidin linked to bovine serum albumin (BSA) (Sigma-Aldrich, USA), were employed. Fig. 2C and Fig. S7,† an overlay and individual images of fluorescence and bright field microscopy from the antibody labeled GUVs, show that the antibody attached to GLUT1 protein at irregular spots, possibly in part due to obstruction by agarose debris attached to the GUV membrane[15] and low fraction of GLUT1 (~6%) in the RBC membranes[20,21] Indeed, direct reconstitution from agarose gel is known to introduce inhomogeneous protein distribution while residing in the agarose gel, possible reflecting variable mechanical stress on the membranes and inhomogeneous orientation of the membrane proteins, sometimes inside-pointing and sometimes outside-pointing.[22] Despite uncertainty about these issues, the biological activity of GLUT1, whose function is to shuttle only glucose across cell membranes by the process known as facilitative diffusion[23–25] from higher to lower concentration, could be assessed.

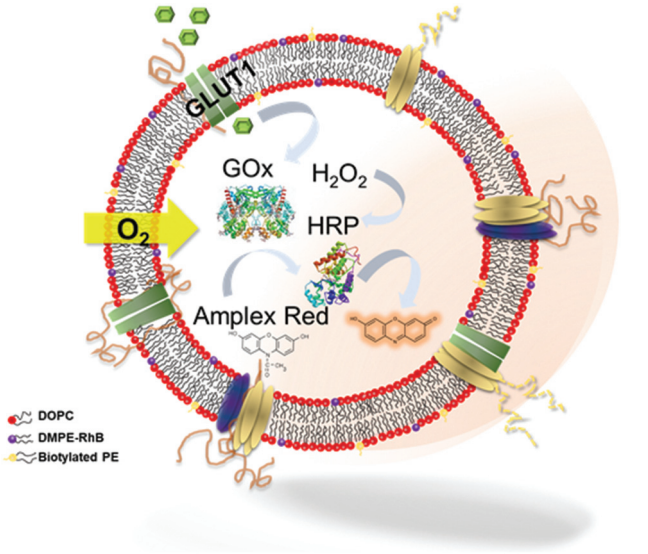

Fig. 3 Scheme of method to check for glucose internalization from outside to the GUV interior. Glucose enters the GUV mediated by the action of GLUT1, then reacts with the enzyme cascade, glucose oxidase and horseradish peroxidase.

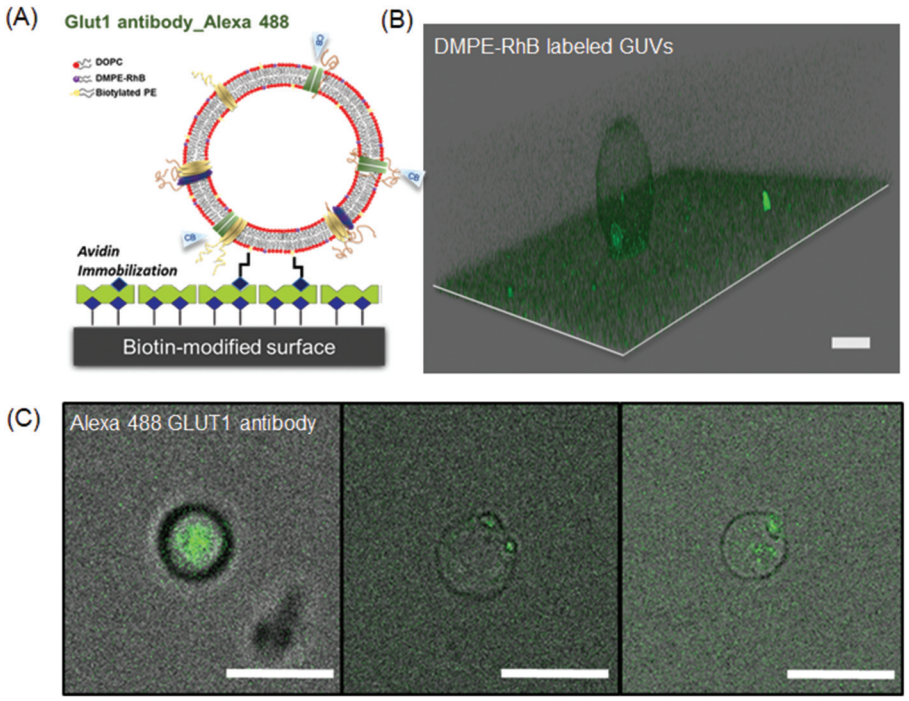

Fig. 2 Identifying the glucose-transporter membrane protein GLUT1 by exposure to fluorescently-labeled antibody. (a) GUVs are attached to a biotin-coated glass slide by avidin linkage. (b) A 3D reconstructed fluorescence confocal image (green) of a surface-attached vesicle labeled only with DMPE-RhB. (c) A top-down overlay images of Alexa 488 labeled GLUT1 antibodies (green) on the RBC-derived GUVs (bright field). Inhomogeneous distributions of antibodies on the GUVs possibly attributed to the low fractions (~6%) of GLUT1 in the RBC extracts and debris of aggregates between agarose and RBC membranes. All scale bars are 10 μm.

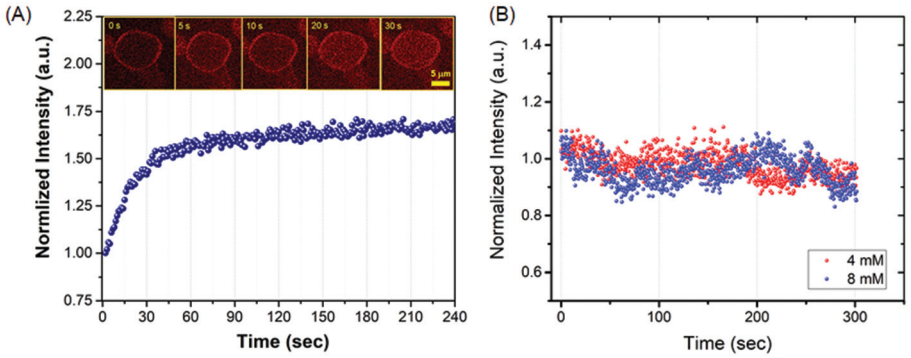

Fig. 4 D-Glucose added to the GUVs without (a) and with (b) accompanying presence of cytochalasin B, which blocks GLUT1 activity. These examples are for the single GUV depicted in the left panel. In both panels, resorufin fluorescence intensity inside the GUVs, normalized by its original value, is plotted against time after adding 4 mm glucose. Inset figures show the change of the fluorescence signal in the GUV interior due to the internalization of glucose. With blocking agent cytochalasin B present (b), the fluorescence intensity is nearly time-independent. The fluorescence intensities are normalized by the initial averaged mean intensity inside the vesicle at t = 0 in order to quantify intensity increase due to the newly produced resorufin.

To assess this activity, we employed cascade enzymatic reactions within the GUV interiors. The Amplex Red glucose assay kit (Thermo Fisher, USA) was used as received. Briefly, active enzymes of horseradish peroxidase (HRP), glucose oxide (GOx), Amplex Red and buffers were mixed according to the manufacturers' instructions and used as the hydration solution for the GUV-formation step. Fig. 3 spells out the enzyme reaction schemes. First, internalized D-glucose transported by GLUT1 is converted by GOx into D-glucono-1,5-lactone and hydrogen peroxide, H_2O_2. Next, this newly-produced H_2O_2 triggers the HRP enzyme and a substrate of non-fluorescent Amplex Red to react, and after it binds to HRP, it converts to resorufin, which is fluorescent.[26]

The fluorescent product was detected inside GUVs, while they resided within the agarose medium, after adding 4 mM glucose solution outside the GUVs [Fig. 4A and Fig. S8A†]. The formation of resorufin is accompanied by oxidation of Amplex Red, catalyzed by horseradish peroxidase (HRP). As the minimum 6 h were needed to prepare the surface-attached GUVs containing the Amplex Red, HRP, and GOx owing to copious steps of anchoring, washing and antibody labeling, during this time there transpired partial oxidation with resulting production of resorufin in the fluorescence background. Nevertheless, H_2O_2- and HRP-independent oxidation of Amplex Red to resorufin have lower yield than HRP/H_2O_2-mediated oxidation, and neither Amplex Red nor HRP can cross biological membranes.[27,28] Hence, this background is a minor issue when it comes to determining the activity of GLUT1 in the reconstituted RBC-derived GUVs from the production of resorufin upon glucose addition. In Fig. 4A, the insets show the time-dependent fluorescence signal within the GUVs: increase by 65% compared to the intensity at t = 0 followed by a plateau. Despite small deformation of the GUV shape from osmotic pressure mismatch across the membrane by the glucose addition, no significant disruptions of GUV morphology were observed. Glucose transport from exterior to interior persisted up to 10 min and could be repeated when additional glucose was added to the exterior (Fig. S8B†). Upon repetitive addition of glucose into GUVs, the kinetics of the glucose transport was slower when the GUVs were removed from the agarose medium and attached instead to avidin-coated glass surfaces, as shown in Fig. 4B and Fig. S8B.† While the fluorescence intensity showed 1.5 folds increase during the first 60 s upon the addition of glucose when GUVs surrounded by agarose medium (Fig. 4A), the surface-attached GUV situation required longer time (>200) to reach the same increase (Fig. S8C†), possibly due to enhanced surface contact *via* agarose, but a detailed explanation of kinetics is beyond the scope of this study.

Finally, we characterized the response after blocking the activity of this membrane protein [Fig. 4B]. The inhibitor, cytochalasin B (CB), which inhibits glucose transport by blocking with sulfhydryl groups[29] the GLUT1 substrate efflux site, was added as sketched in Fig. 4B, thus confirming the absence of glucose internalization with no specific fluorescence increase upon the 4 mM addition of glucose. This fungal toxin[30] is known to accomplish near-complete blockage of GLUT1 activity. When CB was added to surface-attached GUVs and glucose was added afterward (8 mM), no glucose transport was observed. Testing with negative control, we confirmed that DOPC GUVs showed almost no intensity change when glucose was added outside them.

Conclusions

Going beyond the known method to produce GUVs from erythrocytes using electroformation under conditions of low ionic strength,[9] this study shows the biological activity, of the membrane protein considered here, even at biological ionic strength and without the additional protein purifications. The efficiency of GUV formation and its functionality were signifi-

cantly assisted by allowing the GUV self-assembly to occur within the pores of agarose hydrogel. The method may likewise assist the insertion of foreign proteins, but as our purpose in this study was to explore use of the native erythrocyte cell membrane, this direction of inquiry was not pursued.

The experimental methods introduced here appear to be general, although their generality has not yet been tested. If so, they may also be useful in reconstituting other types of biological membranes, among them cancer cells,[4] platelets,[5] leukocytes,[6] and bacteria,[7,8] in each case taking advantage of the unique cell membrane properties of these respective cell types. This offers an experimental platform to explore other protein function mechanisms within this cell-like environment, and also in the future to visualize protein dynamics within lipid bilayers using methods based on confocal microscopy to probe small regions on the vesicle surfaces. Another potential use of this experimental platform can be to study questions of metabolism in cases of more subtle chemical reactions than the glucose enzymatic cascades considered here.

Conflicts of interest

There are no conflicts to declare.

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