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Supplementary information for: Glucose Transport Machinery Reconstituted in Cell Models

Jesper S. Hansen,[†] Karin Elbing,[†] James R. Thompson,[‡] Noah Malmstadt,[‡] Karin Lindkvist-Petersson[*,†]

Department of Experimental Medical Science, Lund University, Lund, Box 117, SE-22100, Sweden, and Mork Family Department of Chemical Engineering, and Materials Science, University of Southern California, 925 Bloom Walk, Los Angeles, CA 90089 USA.

Email: karin.lindkvist@med.lu.se

[*]To whom correspondence should be addressed

[†]Lund University

[‡]University of Southern California

Supporting Methods

Materials

The lipids 1,2–diphytanoyl–*sn*–glycero–3–phosphocholine (DPhPC), 1,2–dipalmitoyl–*sn*–glycero–3 phosphoethanolamine–N–biotinyl (DPPE-Biotin) (Avanti Polar Lipids, USA), ATTO390–DPPE and ATTO488–DPPE (ATTO-TEC GmbH, Germany) were used without further purification. The reagents n–Decyl–β–D–Maltoside (DM) (Affymetrix, USA), agarose type IX-A ultra-low gelling temperature, dimethylformamide (DMF), chloroform, and phosphate buffered saline (PBS) (Sigma-Aldrich, USA) were all of analytical grade and used without further purification. ATTO NHS-ester fluorophores 390 and 565 (ATTO-TEC GmbH, Germany), Amplex Red Glucose/Glucose Oxidase Assay Kit (Life Technologies, USA) and protein desalting micro spin columns (Thermo Scientific, USA) were used as per the manufacturers instructions. AttoFluor® cell chambers (Life Technologies, USA) with standard no. 1 glass coverslips (VWR) were used for giant vesicle formation. Glass-bottom petri dishes with micro-inserts were used for fluorescence microscopy (IBIDI GmbH, Germany). 18.2 MΩ cm Milli-Q water was used in all experiments (EMD Millipore, USA).

Strains and Plasmids

Strain GS115 *aqy1Δ*, described by Fischer *et al.* (2009),[1] expressing *Rattus norvegicus* (r) GLUT1 was made by amplifying the gene from plasmid pYEpH2–rGLUT1 (kindly provided by Professor E. Boles, Goethe University, Germany). GLUT1 was amplified and ligated into the *P. pastoris* pPICZB plasmid (Life Technologies, USA) at the EcoRI and NotI restriction enzyme sites to make plasmid pPICZB–rGLUT1. The vector was linearized using PmeI restriction enzyme before transformation of GS115 *aqy1Δ* according to the manufacturer's instruction (Life Technologies, USA). Correct gene insertion was confirmed by DNA sequencing (Eurofins MWG, Germany).

Screening for Zeocin Resistance to Identify High Expressers of GLUT1

Single transformants were resuspended in 100 µl sterile water in 96–well plates. Five microliters was spotted on YPDS plates containing 0, 1000 or 1500 µg/ml zeocin and incubated at 30°C for 2–3 days. Total membrane was prepared from small scale protein expression tests. Cells were disrupted in breaking buffer (25 mM Sodium phosphate pH 7.4, 5% glycerol, 2 mM DTT and 4 mM EDTA) supplemented with protease inhibitor (Complete EDTA-free, Roche) using FastPrep24 (MP Biomedicals, USA). After removal of cell debris the total membrane was collected using ultracentrifugation 145,000g for 1 h at 4°C. Membrane pellet was resuspended in 20 mM Tris-HCl pH 8.3, 20% glycerol, 10 mM NaCl, 1 mM DTT and 0.2 mM EDTA supplemented with protease inhibitor. The obtained protein samples were run on SDS-PAGE gels followed by Western blotting.

GLUT1 Protein Production and Handling

Cell cultures were precultured in BMGY for 24 hrs (300 ml) and then shifted to BMMY media (1 l) for induction of protein expression according to the manufacturer's instructions (Invitrogen).

Cells were induced in BMMY for 24 hrs with additional 0.5% v:v methanol supplemented after 18 hrs. Cells were harvested and lysed using an X–Press Disintegrator (AB Biox, Sweden) and resuspended in breaking buffer supplemented with protease inhibitor. After removal of cell debris by centrifugation at 8,000 g for 2×10 minutes the total membrane fraction was isolated by centrifugation at 145,000 g for 90 min at 4°C. Total membrane was washed using 20 mM sodium hydroxide and again collected by centrifugation at 145,000g for 90 min at 4°C. The total membrane fraction was resuspended in a membrane resuspension buffer consisting of 20 mM Tris-HCl pH 8.3, 20% glycerol, 10 mM NaCl, 1 mM DTT and 0.2 mM EDTA supplemented with protease inhibitor. Protein was then solubilized in the membrane resuspension buffer supplemented with 0.9% n-Decyl-β-D-Maltopyranoside (DM) for 1 h at 4°C under slow rotation. The unsolubilized material was removed by centrifugation at 145,000 g for 30 min. Detergent solubilized protein was diluted six times in buffer (20 mM Tris-HCl pH 8.3, 10% Glycerol, 1 mM DTT and 0.2 mM EDTA) to decrease the detergent concentration and then applied on an anion exchange column (Resource Q, GE Healthcare Life Sciences, Sweden). Protein was eluted by a NaCl gradient and concentrated using 50 kDa centrifugal concentrators (Vivaspin, Sartorius AG, Germany) to a final concentration of 10-15 mg/ml. The resulting protein sample was then applied onto a size exclusion column (Superdex200 10/300GL, GE Healthcare, Life Sciences, Sweden) equilibrated with a gel filtration buffer consisting of 20 mM Tris-HCl pH 8.3, 10% glycerol, 150 mM NaCl, 1 mM DTT, 0.2 mM EDTA and 0.11% DM. Purified GLUT1 protein was concentrated using centrifugal concentrator with a 50 kDa cutoff (Thermo Scientific, USA). The protein concentration was monitored by a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) or by using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA). For this study we used a concentration of pure unlabeled GLUT1 of 94.8 μM throughout. Protein samples were flash frozen in liquid nitrogen and kept at -80°C prior to their use.

Fluorescent labeling of GLUT1

Accessible lysine residues of the purified protein were labeled with either ATTO390 or ATTO565 NHS-ester dyes as indicated. To carry out the reaction, 2-fold molar excess of ATTO-NHS dye dissolved in DMF was added to the buffered GLUT1 solution (1/100 volume dilution into aqueous buffer), which was supplemented with sodium carbonate as per the manufacturers instructions to raise the pH to 8.0. The reaction was allowed to proceed for 1 hr at room temperature. Thereafter the reaction mixture was desalted using micro spin gel filtration columns into gel filtration buffer. The resulting fluorescently labeled GLUT1 preparation was placed on ice and used immediately.

Coomassie Blue Staining and Western Blotting of GLUT1 SDS-PAGE Gels

Proteins were separated on SDS-PAGE NuPAGE 4-12% Bis-Tris gels (Thermo Fisher Scientific, USA). Coomassie Blue staining was done using SimplyBlue™ SafeStain (Thermo Fisher Scientific, USA).

For Western blotting, the gels were blotted onto nitrocellulose according to the manufacturer's instructions (Hoefer Inc., USA). The nitrocellulose membrane was blocked for unspecific protein binding in blocking buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 2% fish gelatin, 1% ovalbumin). GLUT1 bands were detected with either primary anti-polyhistidine (anti-His)

antibody of monoclonal origin raised in mouse (Sigma-Aldrich, USA) or goat-anti GLUT1(N-20) (Santa Cruz Biotechnology, USA) using 1:2500 or 1:1000 dilutions, respectively. This was followed by incubation with fluorescently labeled secondary anti-mouse 800 nm or anti-goat 800 nm antibodies from donkey (LI-COR Biosciences, USA) at 1:10,000 dilutions. Signal was detected using Odyssey FC gel imager (LI-COR Biosciences, USA).

UV-Visible Absorbance Spectroscopy of the Glucose Assay Reaction

Absorbance measurements of the glucose assay reaction mixture were carried out in 1 ml cuvettes using a Beckman DU-640 spectrophotometer (Beckman Coulter Inc., USA). The assay was carried out as per the manufactures instructions.

Circular Dichroism (CD) Spectroscopy of Purified GLUT1

Prior to CD spectroscopy the protein buffer was changed from the gel filtration buffer to 0.13% DM in PBS using small desalting spin columns (Thermo Scientific, USA). Spectra were recorded using a protein concentration of 0.2 mg/ml.

CD spectra were acquired with a Jasco J-810 spectrometer (Jasco Inc., Italy). The sample temperature was controlled to 25°C by a built-in Peltier device. The spectra were collected with 20 nm/min scan speed between 250 and 195 nm with a response time of 0.25 s and a data pitch of 0.1 nm. Baselines were collected in the same manner and spectra were baseline corrected. Cuvettes used were quartz with a 0.1 cm path length. Results were obtained in millidegrees (mdeg) and subsequently converted to mean residue ellipticity (MRE) ($[\theta]\times$mdeg cm^2 dmol^{-1}) based on 491 amino acid residues (not including the start codon methionine) and a molecular weight of 54083.7 g/mol for the GLUT1.

GLUT1 Reconstitution During Giant Vesicle Formation

Formation of protein reconstituted giant vesicles was carried out in AttoFluor cell chambers using 25 mm diameter standard no. 1 glass coverslips. Prior to use, coverslips were first sonicated in a 1 M NaOH solution for 30 min, followed by thorough rinsing in Milli-Q water. To make a clean hydrophilic surface, coverslips were plasma treated for 1 min with a handheld model BD-20 laboratory corona treater (Electro Technic Products Inc., USA).

Following our previously published protocol[2], briefly we diluted fluorescently labeled GLUT1 in gel filtration buffer at a 1:5 ratio into molten agarose dissolved in Milli-Q water (1% (w:v), 25°C). Ten microliters of the still molten protein in agarose solution was deposited onto a cleaned glass coverslip and spread across the surface evenly by swiping another coverslip across the surface. Following gelation of the hydrogel, a layer of DPhPC lipids (10 µl, 10 mg/ml in $CHCl_3$) supplemented with 1 mol% DPPE-Biotin was pipetted onto the partially dried agarose film in a N_2 gas stream to immediately evaporate the solvent. The resultant lipid-hydrogel film was rehydrated in aqueous buffer (typically 400 µl).

For imaging of pure GLUT1-swelled giant vesicles 200 mM sucrose in PBS, pH 7.4 was used as the rehydration buffer. As previously noted, a period of 30 min was found to be sufficient to form a dense population of giant vesicles on the surface in all cases.

For microscopy giant vesicles were harvested by aspiring the rehydration medium and diluting 5 fold into 200 mM glucose in PBS. This suspension was then transferred to coverslip petri dishes pre-incubated with BSA-Biotin, washed with BSA, then avidin (1 mg/ml in each case) and subsequently rinsed thoroughly in the same buffer. The sugar density gradient causes the giant vesicles to settle and attach on the biotinylated coverslip surface for imaging.

Facilitative Glucose Transport in Giant Vesicles

Encapsulation of the glucose assay into giant vesicles was carried out by adding all of the glucose assay components to the vesicle rehydration buffer. Preparation of stock solutions and handling of HRP, GOx and Amplex Red were carried out as per the manufacturers instruction (Life Technologies, USA). Formation of GLUT1-swelled and glucose assay encapsulated giant vesicles was carried out essentially as described in the previous section. However, the rehydration buffer and the sinking buffer are different, as both glucose and to some extent sucrose (the latter presumably due to glucose impurity) activate the reaction scheme. We found that raffinose works well in the rehydration buffer, since this sugar did not cause any notable fluorescence over time with the reaction mixture. The rehydration buffer consisted of 100 mM raffinose in PBS, pH 7.4. Following giant vesicle formation, the vesicle suspension was diluted 5 fold into a sinking buffer consisting of 67.8 mM KCl in PBS, pH 7.4. The osmotic balancing between sinking buffer and rehydration buffer was carried out by using an Osmomat-30 freeze fracture osmometer (Gonotec, Germany).

The giant vesicles were allowed to sink for 1.5 hrs in a 15 ml Falcon tube placed on ice. Seventy microliters from the bottom of the Falcon tube were transferred to the micro-inserts of a glass-bottom petri dish (IBIDI GmbH, Germany). The giant vesicles were then allowed to settle for another 30 minutes. The vesicles attached to the bottom of the petri dish were then washed with 2.5 chamber volumes of the sinking buffer (67.8 mM KCl in PBS, pH 7.4). Prior to addition of glucose, a background micrograph was taken. Only vesicles displaying faint fluorescent background and a clear phase contrast were visualized for GLUT activity measurements. Glucose was then added from a 10 mM stock solution of glucose in sinking buffer to a final concentration of 1 mM in the reaction chamber, unless otherwise stated. For blocking the glucose transport activity the giant vesicles were pre-incubated with 10 μM cytochalasin B for 5 min before adding the glucose. Time-lapse experiments were carried out by acquiring micrographs of the same vesicle for 10 min with a 30 sec delay cycle.

Microscopy

Microscopy was performed on a confocal microscope (model LSM 510 META; Carl Zeiss MicroImaging, Jena, Germany). All micrographs were acquired using Plan-Neuofluar objectives (40× NA 1.3 oil-immersion for individual vesicles and 20× for mixed vesicle populations). Illumination was provided at excitation 488 nm by an Argon ion laser and excitation 561 nm and 405 nm by diode-pumped solid-state lasers.

Fluorescence was imaged through a bandpass filter centered at around 525 nm for the ATTO488 dye, at 595 nm for the ATTO565 dye and resorufin, and with a bandpass filter centered at 435 nm for ATTO390. Acquisition was performed using LSM 510 software (Carl Zeiss MicroImaging). Z-stacks were separated by 1.0 μm in all cases. Micrographs of resorufin fluorescence emission

were acquired with a 3.0 μm depth in all cases. This yielded the most reproducible output for the entire giant vesicle population, balancing between having a good signal-to-noise and without saturating the PMT.

Image Processing

All images were analyzed with ImageJ software.[3] All fluorescence micrographs are background normalized using the in-built ImageJ rolling-ball filter. Z-stack images for standard deviation projections and 3D rotations were created using standard ImageJ stack tools. All images are presented without any other adjustments. Quantification of fluorescence intensity was performed by measuring a 10.55 μm diameter circle (giant vesicles are per definition ≥10 μm in diameter) in the center of each giant vesicle using the built-in ImageJ measuring tool.

Supporting Figures

Large Scale Protein Production of GLUT1 in Yeast *P. pastoris*

GLUT1 was overexpressed in an *aqy1*-deleted strain of *P. pastoris*. To generate a strain with high expression levels of GLUT1, a high-throughput zeocin assay was established to screen hundreds of transformants (Figure S1A). The transformants that survived at the highest zeocin concentrations showed to have the highest protein expression levels (Figure S1B).

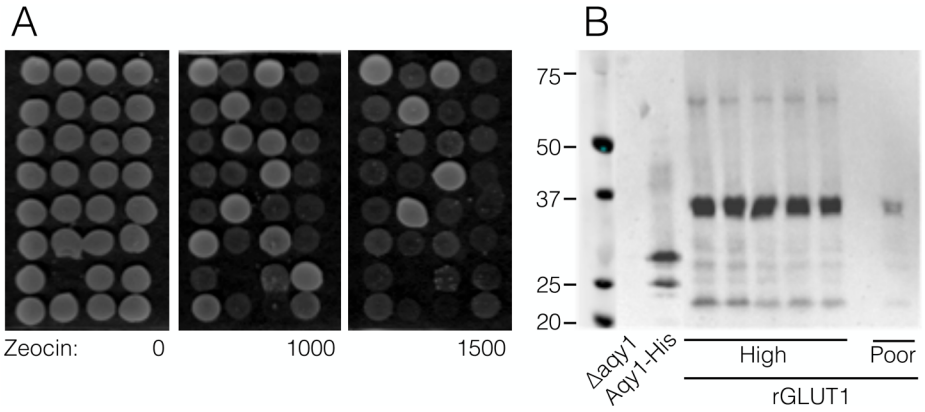

Figure S1. High zeocin resistance correlates with high protein production. (A) GLUT1 transformed colonies were spotted on plates with increasing zeocin (μg/ml) concentrations to select for high zeocin resistance. (B) Western blot of total membrane from GLUT1-His strains growing on high zeocin concentrations. The *aqy1*-deleted strain is included as a negative control and pure Aqy1-His as a positive control. Protein is detected by a His-antibody.

Optimization of purification protocols and membrane washing procedures are often necessary to obtain large quantities of integral transmembrane proteins. For GLUT1, we found that sodium hydroxide washing of total membrane prior to protein solubilization resulted in extensive removal of adhering proteins and other contaminants but with no loss of GLUT1 protein (Figure S2A, Table S1). Minimal protein loss was observed in the solubilization step, where the

membrane protein is removed from the membrane and kept soluble in aqeous solution by detergent (Table S1).

Table S1. GLUT1 recovery per purification step.

Purification steps	GLUT1 recovery (%) compared to total membrane
NaOH washed membrane	100.0 ± 2.6
Solubilized protein	96.0 ± 3.9
Ion-exchange chromatography	88.0 ± 10.4
Size exclusion chromatography	52.0 ± 7.1

Finally, the solubilized protein was purified by ion exchange and size exclusion chromatography, resulting in a homogeneous and pure fraction of GLUT1 proteins (Figure S2). The total yield after protein purification was 2-3 mg of pure GLUT1 per 1 l of shaker flask culture (10 g wet cells). This is about 20 times more protein per gram wet cells than previously reported.[4]

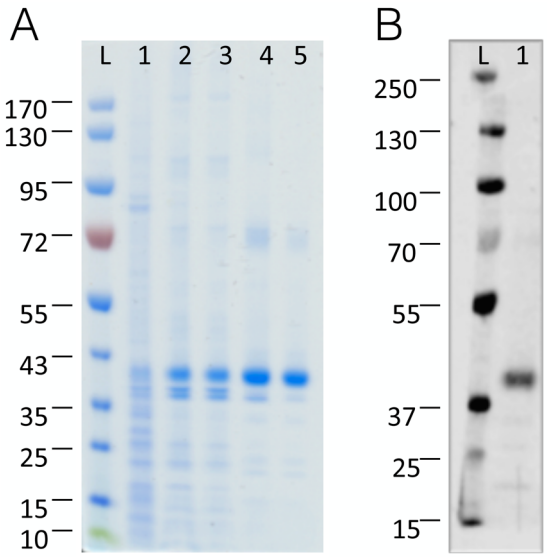

Figure S2. GLUT1 purity was validated by Coomassie Blue staining of SDS-PAGE gels and by Western blotting. (A) The purity of GLUT1 during purification is shown by loading 2 µg total protein in each lane. Samples were loaded as follows: L. is ladder; 1. Total protein; 2. Sodium hydroxide washed membrane; 3. Solubilized membrane protein; 4. Anion exchange purified GLUT1; 5. Size exclusion purified GLUT1. (B) Purified GLUT1 after size exclusion chromatography. Protein was detected by GLUT1 antibody. Lanes: L is ladder; 1. Purified GLUT1 protein.

Folding and Stability of GLUT1

Integral membrane proteins are challenging to produce in large amounts and it is even more difficult to retrieve stable and properly folded proteins in solution. Hence, the produced GLUT1 was examined concerning its folding properties and stability. Circular Dichroism (CD) spectroscopy was performed to confirm that the produced GLUT1 protein has a correct secondary structure. A typical CD spectrum of an α-helical protein has two minima, one at 208 nm and the other at 222 nm.[5] GLUT1 adopts this characteristic spectrum, clearly confirming that GLUT1 has an α-helical structure in solution (Figure S3A). SDS-PAGE confirmed that the resulting purified GLUT1 proteins were pure and essentially free of contaminants (Figure S2A, lane 5).

The stability of GLUT1 was tested at different temperature conditions, i.e. at 21ºC, 4ºC and -80ºC. The protein stability was evaluated by the elution profile following size exclusion chromatography (Figure S3B). The samples could withstand all the different conditions, however, higher order oligomers started to appear after overnight storage at 21ºC, suggesting that the protein requires ≤4ºC temperatures for long-term stability (Figure S3B).

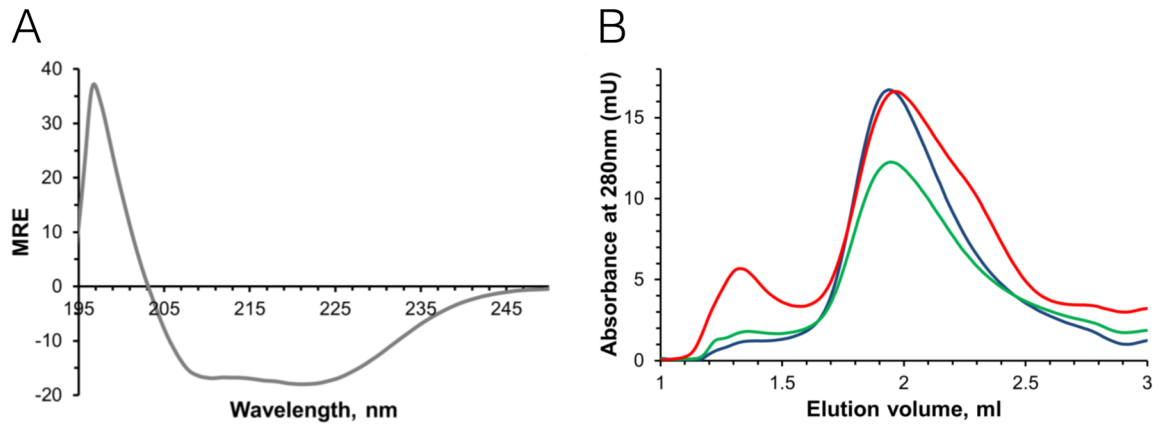

Figure S3. Secondary structure and stability of purified GLUT1 in the detergent solubilized state. (A) Circular dichroism spectroscopy of purified GLUT1 (0.2 mg/ml). MRE is mean residue ellipticity ([θ]×mdeg cm^2 $dmol^{-1}$). (B) GLUT1 was kept at -80°C for a week (blue line), at 4°C for three days (green line) or at 21°C overnight (red line) before subjected to size exclusion chromatography. At 21°C overnight storage higher oligomers started to appear (red line).

Protein Incorporation and Encapsulation During Giant Vesicle Formation

To verify GLUT1 incorporation into the membrane of giant vesicles during formation, purified GLUT1 was nonspecifically labeled with ATTO565-NHS dye. Unreacted dye was separated from the protein by desalting. Labeled protein was diluted into molten agarose and 10 µl of agarose-GLUT1 solution was spread onto a clean cover glass surface. The agarose solution was allowed to gel, followed by rehydration in sucrose containing aqueous buffer solution. As previously reported for SoPIP2;1 aquaporins,[2] this resulted in a densely packed film of giant vesicles formed from the hydrogel surface (Figure S4).

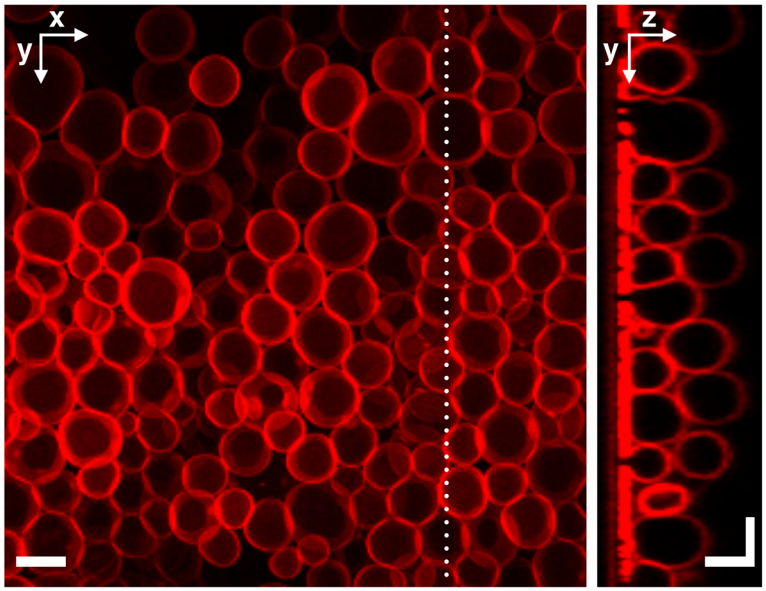

Figure S4. Rehydrating a DPhPC-coated partially dried hydrogel containing pure ATTO565-labeled GLUT1. Fluorescence micrographs showing a top-down standard deviation projection of a hydrated lipid gel from a z-stack (left) and a z-reslice through the white dotted line (right). All scale bars equal 10 µm.

Harvested giant vesicles were allowed to settle on the bottom of a petri dish by diluting the vesicle suspension into a glucose containing buffer solution. As seen in Figure S5 the giant vesicle membrane is clearly fluorescent, showing that GLUT1 is incorporated into the lipid bilayer.

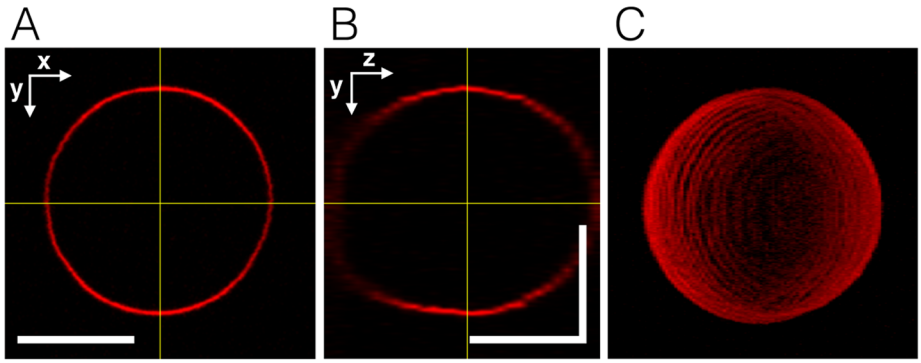

Figure S5. GLUT1 incorporation during giant vesicle formation. GLUT1-swelled giant vesicle shows that the protein is incorporated into the lipid bilayer and that it is homogenously distributed throughput the membrane (A) Confocal slice from z-stack of a giant vesicle with incorporated ATTO565 fluorescently labeled GLUT1. (B) Orthogonal yz-view of the same vesicle through the vertical yellow line of image (A). (C) Standard deviation projection from 3D z-stack of the vesicle. Scale bars equal 10 µm.

Encapsulation of active enzymes, GOx and HRP, and Amplex Red were successfully accomplished by growing the giant vesicles in rehydration buffer containing all of the glucose assay components. Following formation, the giant vesicles were first diluted 5 fold into sinking buffer and then transferred to visualization chambers where settled giant vesicles were washed with sinking buffer to further remove excess exterior glucose assay components. Although, Amplex Red is generally considered a cell-impermeable compound,[6] its ability to permeate artificially made membranes, at least to some extent, has previously been reported.[7] However, lipid bilayers of DPhPC, used in this study, exhibit lower permeability of small ionic and neutral molecules compared to other phospholipids,[8] which may explain the no (or little) leakage in our cell model.

Kinetics of the Glucose Assay Reaction in a Cuvette

Figure S6 shows the rate of conversion of Amplex Red to resorufin by GOx and HRP in the presence of glucose measured by UV-Vis spectroscopy. Also shown is the reaction with 10 μM of CB added. The presence of CB in the reaction did not cause noticeable effects on amplitude or kinetics of the reaction.

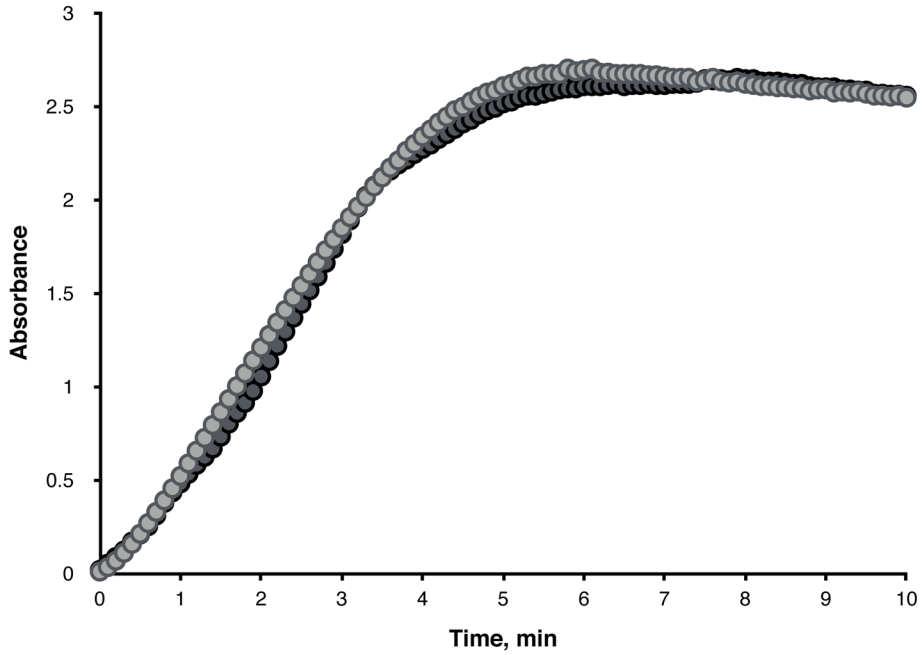

Figure S6. UV-Vis spectroscopy of the enzymatic glucose assay measured in a cuvette without and with CB. Glucose was added at 50 μM to the reaction mixture consisting of 0.2 U/ml HRP, 2 U/ml GOx and 50 μM Amplex Red in a buffer of 100 mM raffinose in PBS, pH 7.4 (grey, filled circles). The reactants were mixed rapidly before starting the measurements. Conversion of Amplex Red to resorufin was measured with absorbance at 560 nm. To an otherwise identical reaction mixture 10 μM of CB dissolved in DMSO was added and equilibrated with the reaction components for 5 min before addition of glucose (black, filled circles). As seen the presence of CB in the reaction mixture did not affect the assay.

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