SUPPLEMENTARY INFORMATION

Gap junction-mediated delivery of polymeric macromolecules

AUTHORS

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MATERIALS & METHODS

Chemical reagents.

NaCl, $CaCl_2$, ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 kDa, 20 kDa, 40 kDa, 70 kDa fluorescein isothiocyanate dextran, N-Ethylmaleimide (NEM), carbenoxolone disodium salt (CBX), and polybrene were purchased from Sigma-Aldrich. Fetal Bovine serum (FBS), trypsin, penicillin, streptomycin, L-glutamine (PSLG), phosphate buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), and F-12 medium were purchased from GE Healthcare.

Cell culture.

Human retinal pigmented epithelial (RPE) cells (ARPE-19) and HeLa cells were from American Type Culture Collections (Manassas, VA). RPE cells were cultured in 1:1 F12/DMEM supplemented with 10% FBS, 20 mM HEPES, and 1% penicillin, 1% streptomycin, 1% L-glutamine. HeLa cells were cultured in DMEM supplemented with 10% FBS, 20 mM HEPES, and 1% penicillin, 1% streptomycin, 1% L-glutamine.

Cloning of Cx43-delCT-mRFP plasmid construct.

The plasmid coding for connexin 43 with a deleted C-terminus (Cx43-delCT-mRFP) was constructed from the original Cx43-mRFP plasmid, a generous gift from Dr. Dale Laird. Cx43-mRFP was previously subcloned into pLJM1 backbone, a gift from Dr. David Sabatini (Addgene #19319). The entire construct, excluding the C-terminus region of Cx43, was then amplified using PCR primers containing the Xma1 restriction site. Once amplified, a restriction enzyme digest using Xma1 enzyme was done to generate compatible ends for ligation. A ligation reaction was then performed to circularize the vector containing the Cx43-delCT-mRFP construct. This construct was then confirmed by Sanger sequencing.

Development of connectosome donor cell line.

To produce the connectosome donor cell line, HeLa cells were stably transfected with the Cx43-delCT-mRFP plasmid described above via lentiviral transfection. Lentiviruses were generated by co-transfecting the transfer plasmid (Cx43-delCT-RFP), the packaging plasmid pCMV-dR8.91, and the envelope plasmid (VSVG) into 293T packaging cells with FuGENE. 48 hours after the transfection, virus-containing supernatant was collected, filtered, and added to HeLa cells with 8 μg/mL of polybrene for transduction. Transduced cells were then sorted for mRFP fluorescence using a FACS Aria cell sorter (BD). After selection, these cells were cultured in DMEM media

supplemented with 10% FBS, 20 mM HEPES, and 1% penicillin, 1% streptomycin, 1% L-glutamine at 37 C with 5% CO2.

Scratch loading assay and analysis.

Cx43-delCT-mRFP HeLa cells were grown on acid-cleaned 22 x 22 mm glass coverslips in 6-well plates (Corning) until they reached 100% confluency. The cells were rinsed three times with DMEM media supplemented with 10% FBS, 20 mM HEPES, and 1% penicillin, 1% streptomycin, 1% L-glutamine. The media was then removed, and 500 µL of 1 mg/mL FITC dextran (either 10 kDa, 20 kDa, 40 kDa, or 70 kDa molecular weight) was added to the cells. Next, a pipette tip was used to scrape a longitudinal line across the monolayer of cells. The cells were then incubated for 30 min at 37 ºC. Following incubation, the cells were washed three times again with media and imaged using confocal fluorescence microscopy. FITC Dextran was visualized under the spinning disk confocal microscope with a 488-nm laser. A control using the gap junction inhibitor carbenoxolone (CBX)[2] was performed in which cells were treated with 100 µM of CBX prior to addition of FITC dextran. 100 µM of CBX was prepared in media and cells were incubated in 100 µM CBX for 30 minutes prior to sample preparation and scratching. All subsequent steps involved 100 µM CBX media.

Connectosome formation and purification.

Connectosomes were formed by following established protocols for generating giant plasma membrane vesicles (GPMVs).[3, 4] Connectosomes were formed by rinsing donor Cx43-delCT-RFP HeLa cells twice with GPMV buffer (10 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 150 mM NaCl) and once with active buffer (10 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 150 mM NaCl, 2 mM NEM (N-ethylmaleimide)). The cells were then incubated overnight at 37 ºC in active buffer. Following incubation, the active buffer containing Connectosomes was collected from the cells and subsequently centrifuged at 300 x g for 5 minutes to pellet any cell debris. The supernatant containing the Connectosome vesicles was collected and spun again at 17,000 x g for 20 min at 4 ºC to concentrate the vesicles. Finally, the Connectosomes pellet was resuspended in fresh GPMV buffer.

Dextran loading into connectosomes.

A stock solution of either 10 kDa or 70 kDa FITC dextran was prepared at a concentration of 1 mM in PBS buffer, pH 7.4. Connectosomes were incubated with 10 kDa or 70 kDa dextran at a final concentration of 200 µM, and chelating agents EDTA and EGTA at a final concentration of 5 mM for 1 hr at room temperature. After 1 hr, connectosomes were centrifuged at 17,000 x g for

20 min, the supernatant containing unencapsulated dextran was aspirated, and the pellet containing connectosomes was resuspended in fresh GPMV buffer containing 2 mM calcium.

To estimate the amount of FITC dextran encapsulated within the connectosomes, free dextran was serial diluted to generate a fluorescence-based calibration curve, supplementary figures S2 and S3. The FITC fluorescence of the calibration curve along with the loaded connectosome samples was measured using a Varioskan Flash plate reader (Thermo Scientific) with excitation at 480 nm and emission at 520 nm. Samples containing 70 kDa FITC dextran were diluted prior to measurement to ensure the fluorescence detected fell within the calibration curve limits, as concentrations higher than 10 μM were above the saturation limit of the plate reader detector. Using the equation for the best-fit line from the calibration curves, and the fluorescence of the connectosome samples, we calculated the molar concentration of dextran within each dextran loaded sample for 10 kDa and 70 kDa dextran.

Calculate percent of connectosomes loaded.

To estimate the percent of connectosomes loaded with dextran, we imaged connectosomes during the loading process using confocal microscopy. Connectosomes were imaged for FITC fluorescence to quantify dextran content using a 488-nm laser and 525 nm filter with a 50 nm width, as well as mRFP fluorescence to visualize the connectosomes themselves using a 561-nm laser and 617 nm filter with a 73 nm width. Connectosomes were imaged upon addition of 200 μM dextran, for both 10 kDa and 70 kDa molecular weights, and prior to the addition of chelating agents so that calcium was present and the hemichannels remained closed to exclude dextran, supplementary figure S1. FITC fluorescence intensity within the lumens of connectosomes was then quantified along a line through the center of each vesicle, Figures 3A – C, and the average of the minimum intensity values for all vesicles evaluated was used to determine the unloaded baseline condition. Connectosomes were then imaged 1 hr after the addition of chelating agents so that calcium was removed and the hemichannels were opened to allow dextran to diffuse into the vesicles, supplementary figure S1. Again, a line was drawn through the center of connectosomes and luminal FITC fluorescence intensity was quantified for these vesicles that were in the absence of calcium. The average of the maximum FITC intensity values of the background near all vesicles was determined, and this value was used to define 100% loading of dextran. Saturation of the vesicle lumen intensity with this background value would indicate complete loading of dextran, Figures 3A – C and supplement figure S1. However, not all vesicles reached saturation with the background, so partial loading of connectosomes was also determined. Partial loading was defined by luminal FITC fluorescence intensities that were

above 50% of the background intensity, Figures 3A – C and supplement figure S1. To quantify percent of loaded connectosomes, we combined the number of completely loaded vesicles and partially loaded vesicles, based on our described criteria, and divided that number by the total number of vesicles in the image. We repeated this calculation for 10 total images (n = 10) for each condition (+calcium and -calcium) for both 10 kDa and 70 kDa molecular weight dextran samples, Figure 3D.

Connectosome delivery to cells.

Recipient RPE cells were plated on a 6-well plate at a density of 100,000 cells per well and a total media volume of 2 mL per well. Dextran loaded connectosomes were prepared as described above, and from the molar concentration of dextran in connectosome samples, we determined the effective dextran concentration in the connectosome solution. One day after plating RPE cells, 1 mM of dextran within 10 kDa dextran loaded connectosomes and within 70 kDa dextran loaded connectosomes were each delivered to separate sets of three wells containing RPE cells. Additionally, 1 mM of 10 kDa and 70 kDa FITC dextran dissolved in PBS buffer, were each delivered to a third and fourth set of 3 wells of cells, respectively. Cells were incubated in the dark at 37ºC with 5% CO_2 for 24 hr. After 24 hr, media was aspirated from the cells and replaced with 500 µL of trypsin. Cells were incubated for about 1 min again at 37ºC, and then the trypsin was removed and replaced with fresh trypsin. This process was repeated 3x to wash away any connectosomes stuck to the cells, supplement figure S4. After the third 1 min trypsin wash, cells were fully detached from the wells by incubation with 500 µL of trypsin for 5 min at 37ºC, 5% CO_2. The trypsin was then quenched with 1 mL of media, and cells from each well were transferred to Eppendorf tubes and centrifuged at 300 x g for 5 min. The resulting cell pellet was resuspended in 300 µL of PBS in preparation for flow cytometry analysis.

Flow cytometry.

A Guava easyCyte Flow Cytometer (Millipore Sigma) with 488 nm and 532 nm excitation lasers was used to analyze fluorescence of recipient cells after dextran delivery. All data were collected at 35 µL/min and flow cytometry data were analyzed using FlowJo (Treestar). A rectangular gate was drawn in forward scattering versus side scattering plots to exclude debris and contain the majority of cells. Within this gate, cell populations were further analyzed by plotting histograms of FITC fluorescence intensity to determine shifts in dextran uptake within cells from each treatment group; 10 kDa and 70 kDa dextran loaded connectosomes, and 10 kDa and 70 kDa free dextran.

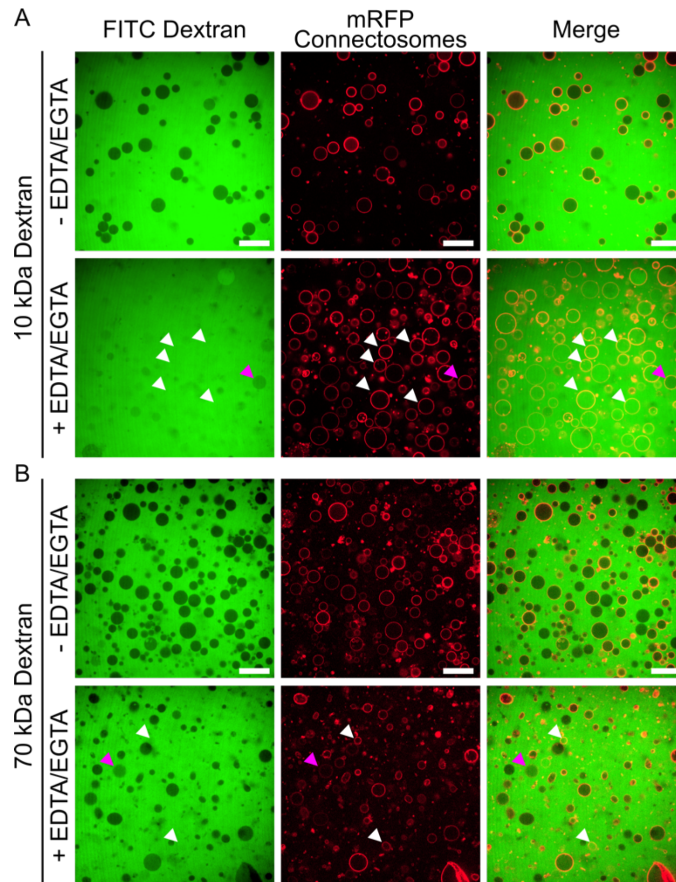

Figure S1. Example images of FITC dextran loading into connectosomes by chelation of calcium. Fluorescence confocal images of connectosomes in a solution containing 10 kDa FITC dextran with and without 5 mM chelators present (A). When chelating agents EDTA/EGTA are added, calcium in the solution is removed, and connectosome vesicles are load with 10 kDa FITC dextran as shown by the white arrows. Some connectosome vesicles remain only partially loaded as shown by the magenta arrows, where they do not reach complete saturation of fluorescence with the background. Images of connectosomes in a solution of 70 kDa FITC dextran with and without 5 mM chelators present (B). The white arrows point to completely loaded connectosomes and the magenta arrows point to partially loaded connectosomes. Scale bars indicate 20 μm.

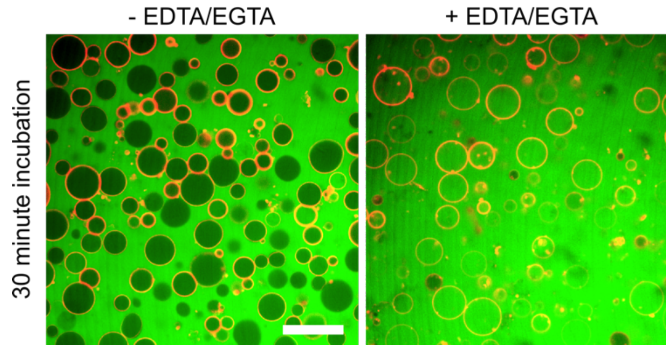

Figure S2. Evaluate passive permeation across connectosome membranes. Fluorescence confocal images of connectosomes labeled with mRFP in a 200 μM solution of 10 kDa FITC dextran. The left image displays connectosomes without the addition of chelating agents at a 30 minute incubation time, and the right image displays connectosomes in the presence of chelating agents at a 30 minute incubation time. Scale bars indicate 20 μm.

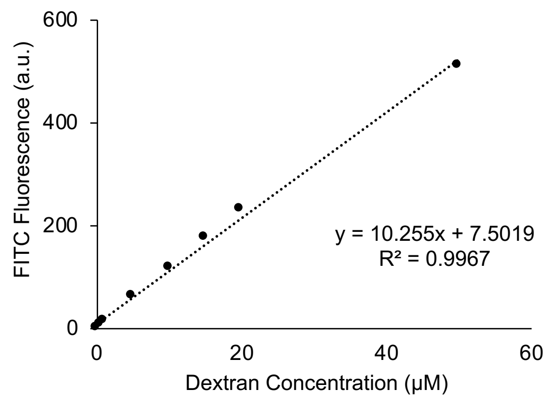

Figure S3. Calibration curve of 10 kDa FITC dextran in GPMV buffer. This calibration curve was generated by measuring the fluorescence at 520 nm with an excitation wavelength of 480 nm of FITC labeled 10 kDa dextran dissolved in GPMV buffer (10 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 150 mM NaCl). A line was fit to this curve and the equation along with the coefficient of determination are shown. This plot was used to calculate the concentration of 10 kDa dextran in connectosome samples.

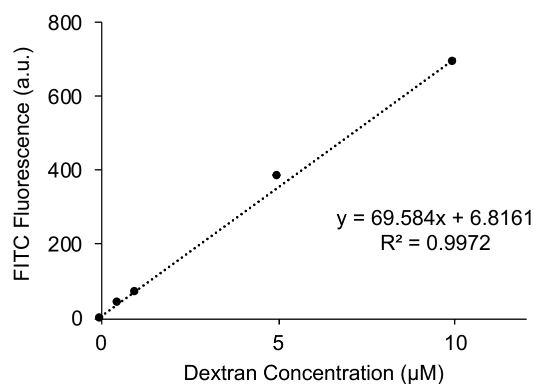

Figure S4. Calibration curve of 70 kDa FITC dextran in GPMV buffer. This calibration curve was generated by measuring the fluorescence at 520 nm with an excitation wavelength of 480 nm of FITC labeled 70 kDa dextran dissolved in GPMV buffer (10 mM HEPES, pH 7.4, 2 mM $CaCl_2$, 150 mM NaCl). A line was fit to this curve and the equation along with the coefficient of determination are shown. This plot was used to calculate the concentration of 70 kDa dextran in connectosome samples.

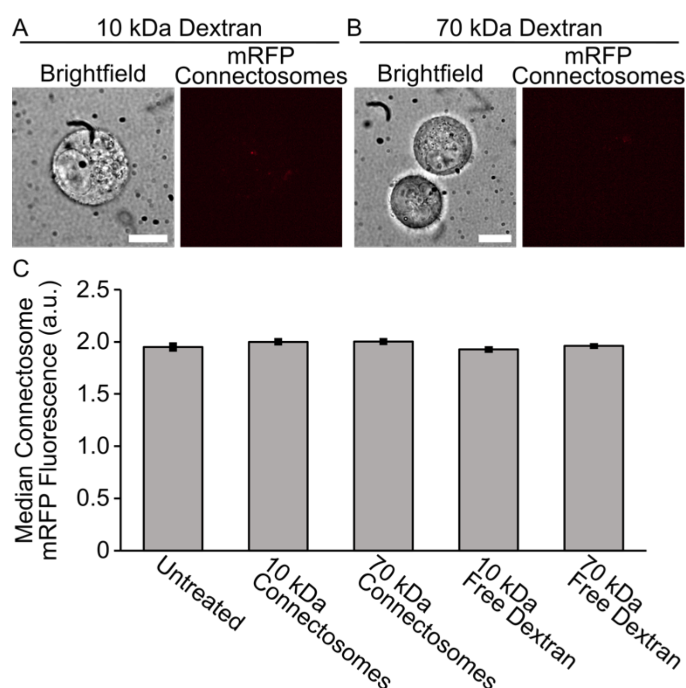

Figure S5. Evaluate connectosome mRFP fluorescence on recipient cells post trypsanization. Example confocal images of recipient cells (rounded) after 3x washes with trypsin to remove connectosomes stuck to the cell surface for 10 kDa dextran loaded connectosome delivery (A) and 70 kDa dextran loaded connectosome delivery (B). Scale bars indicate 10 μm. A bar chart quantifies the median mRFP fluorescence intensity of recipient cells for each treatment condition to evaluate connectosomes remaining on the cell surface (C). All conditions exhibit relatively similar median mRFP fluorescence, indicating no significant amount of connectosomes remained on the surface of recipient cells during analysis. Error bars indicate standard deviation, n = 3.

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