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Display of Single-Domain Antibodies on the Surfaces of Connectosomes Enables Gap Junction Mediated Drug Delivery to Specific Cell Populations

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Abstract

Gap junctions, transmembrane protein channels that directly connect the cytoplasm of neighboring cells and enable the exchange of molecules between cells, are a promising new frontier for therapeutic delivery. Specifically, cell-derived lipid vesicles that contain functional gap junction channels, termed Connectosomes, have recently been demonstrated to substantially increase the effectiveness of small molecule chemotherapeutics. However, since gap junctions are present in nearly all tissues, Connectosomes have no intrinsic ability to target specific cell types, which potentially limits their therapeutic effectiveness. To address this challenge, here we display targeting ligands consisting of single domain antibodies, on the surfaces of Connectosomes. We demonstrate that these targeted Connectosomes selectively interact with cells that express a model receptor, promoting selective delivery of the chemotherapeutic doxorubicin to this target cell population. More generally, our approach has the potential to boost cytoplasmic delivery of diverse therapeutic molecules to specific cell populations while protecting off-target cells, a critical step toward realizing the therapeutic potential of gap junctions.

Graphical Abstract

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Supporting Information

Supporting figures as referenced in the text.

The authors declare no competing financial interest.

Introduction

Gap junctions, transmembrane protein channels that connect the cytoplasm of adjacent cells, are providing a promising new route for therapeutic drug delivery^{1–4}. Formed from hexameric connexin proteins, gap junctions are known to facilitate exchange of metabolites, ions, second messengers and other signaling molecules between cells⁵. Additionally, gap junctions enable drug permeation through tissues by allowing drugs to move from the cytoplasm of one cell to that of its neighbors⁶. Through this phenomenon, known as the bystander effect, gap junctions have been shown to enhance the efficacy of many therapeutics, including paclitaxel⁷, doxorubicin⁷, gemcitabine⁸, etoposide⁷, and others⁹. Further, gap junctions are known to transport small interfering RNAs (siRNAs) between cells¹⁰, and it has recently been proposed that gap junction channels underlie the ability of exosomes to deliver siRNA to cells².

Towards mimicking the natural ability of cells to exchange chemotherapeutics through gap junctions, we recently developed Connectosomes, cell-derived lipid vesicles that contain functional gap junction channels in their membrane surfaces¹. By forming gap junction interfaces with cells, Connectosomes access the cytoplasm directly, decreasing the minimum effective concentration of the chemotherapeutic doxorubicin by more than 100-fold in comparison to traditional liposomal formulations of the drug. However, since connexin proteins are found ubiquitously in cells throughout most tissues¹¹, nonspecific interactions between Connectosomes and healthy tissues could limit their translational relevance. Therefore, developing a means of targeting Connectosomes to specific cell populations is an important step toward realizing their potential as therapeutic delivery vehicles.

To target tumor cells, biochemical moieties that recognize tumor-specific cell surface receptors are frequently displayed on the surfaces of therapeutic delivery particles¹². By promoting preferential interaction of the particles with tumor cells that overexpress specific receptors, these targeting ligands improve the specificity of drug delivery. Specifically, the advantages of targeting have been well-documented for synthetic nanoparticles, where ligands including antibodies^{13–15}, organic compounds^{16, 17}, and peptides¹⁸ have been used to direct therapeutics to a diverse range of specific cell populations¹⁹. Recently, targeting approaches have also been extended to cell-derived materials, and several groups have

harnessed the cell's own machinery to incorporate targeting peptides into cell-derived vesicles²⁰.

Towards designing a targeted drug delivery system which can deliver therapeutic molecules directly into the cytoplasm of a specific population of target cells, here we utilize a recently developed system²¹ to display targeting proteins consisting of single domain antibodies on the surfaces of Connectosomes. Specifically, we have engineered a mammalian cell line (HeLa) which co-expresses connexin 43 and a targeting protein on the cell surface. From these cells we extract targeted Connectosomes. Our results show that display of targeting proteins selectively enhances Connectosome binding by 25-fold to HeLa cells that express a model target receptor. Further, using targeted Connectosomes to deliver doxorubicin reduces the minimum effective dose of doxorubicin by six-fold for cells expressing the target receptor in comparison to off-target cells cultured in the same dish. Taken together, these data illustrate the potential of targeted Connectosomes as efficient and specific vehicles for delivery of drugs to the cell cytoplasm.

Materials and Methods

Chemical reagents

CellTrace Calcein Red-Orange AM and trypan blue were purchased from Life Technologies. Sodium phosphate, DTT (dithiothreitol), PFA (paraformaldehyde), doxycycline, glycine, NaCl, imidazole, CaCl₂, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), DMSO (dimethyl sulfoxide) and doxorubicin were purchased from Sigma-Aldrich. Leupeptin and pepstatin were purchased from Roche. PMSF (phenylmethanesulfonyl fluoride), and β -ME (β -mercaptoethanol) were purchased from Fisher Scientific. Fetal bovine serum (FBS), trypsin, penicillin, streptomycin, L-glutamine, PBS (phosphate buffered saline), and DMEM (Dulbecco's modified Eagle medium) were purchased from GE Healthcare. Puromycin was purchased from Clontech. Geneticin (G418) was purchased from Corning. 7-AAD (7-amino-actinomycin D) was purchased from Affymetrix eBioscience. Extrusion membranes were purchased from VWR. All chemical reagents were used without further purification.

Plasmid constructs

The plasmid coding for the targeting protein (GFPnb-mRFP) was constructed by first excising eGFP from the Tf-R Ecto-eGFP AP180 CTD plasmid developed in our previous work²¹ with BamH1 and SalI digestion and inserting the PCR amplified mRFP (Addgene plasmid #13032, pcDNA3 backbone), a gift from Dr. Douglas Golenbock (University of Massachusetts Medical School). The pOPINE GFP nanobody sequence, a gift from Brett Collins (Addgene plasmid #49172), was PCR amplified and restriction cloned into Tf-R Ecto-mRFP AP180 CTD using primers containing MluI sites. All constructs were confirmed by DNA sequencing.

A plasmid coding for the model receptor (eGFPr) was constructed by modifying a Transferrin receptor GFP (Tf-R-GFP) construct (pEGFP-N1 backbone), kindly provided by Dr. Tomas Kirchhausen (Harvard Medical School), where the entire Tf-R sequence was

excised by digesting with EcoRI and BamHI, and inserting only the PCR amplified intracellular and transmembrane domains of Tf-R adjacent to the GFP fluorophore with EcoRI and BamHI sites. An intermediate construct lacking a stop codon was made by digesting Tf-R Ecto-GFP with BamHI and NotI to remove GFP, and inserting a mutated PCR amplified GFP with the stop codon (TAA) replaced by a glycine (GGA).

Targeted Connectosome donor cell line

Stably transfected, inducible tet-on HeLa cells expressing connexin 43 with a C terminal YFP modification (Cx43-YFP) were a gift from Matthias Falk^{22, 23}. To produce a targeted Connectosome donor cell line, these Cx-43YFP HeLa cells were then stably transfected with the plasmid coding for the targeting protein (GFPnb-mRFP) via lentiviral transfection. The GFP nanobody gene sequence was subcloned onto pLJM1 viral transfer vector (addgene #19319) with AgeI and EcoRI sites. Lentiviruses were generated by co-transfecting the transfer plasmid, packaging plasmid 8.9 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. Transduced cells were selected with 2 µg/mL puromycin for 7 days, before flow sorting to select for cells with both YFP and mRFP fluorescence. After selection, these cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin, 1% streptomycin, 1% L-glutamine (PSLG), 100 µg/ml geneticin and 0.4 µg/ml puromycin. To induce Cx43YFP expression, cells were incubated with 1 µg/mL doxycycline. All studies were conducted on the fifth day after plating, when cells reached approximately 85% confluency.

Target cell line

To produce a target cell line expressing the model receptor (eGFPr), wild type HeLa cells were stably transfected with the model receptor (eGFPr) plasmid via lentiviral transfection. The model receptor gene sequence was subcloned onto pLJM1 viral transfer vector (addgene #19319) with NheI and EcoRI sites. Lentiviruses were generated by co-transfecting the transfer plasmid, packaging plasmid 8.9 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 wild type HeLa cells with 8 µg/mL of polybrene. Transduced HeLa cells were selected with 2 µg/mL puromycin for 7 days. After selection, these cells were cultured in DMEM supplemented with 10% FBS and 1% PSLG. Cell media was changed every 48–72 hours. Cells were incubated at 37 °C with 5% CO₂. All studies were conducted on the fifth day after plating, when cells reached approximately 85% confluency.

Off-target cell line

Wild type HeLa cells were cultured in DMEM supplemented with 10% FBS and 1% PSLG. Cell media was changed every 48–72 hours. Cells were incubated at 37 °C with 5% CO₂. All studies were conducted on the fifth day after plating, when cells reached approximately 85% confluency.

eGFP purification

The pRSET vector containing the nondimerizable hexa-his-tagged eGFP (hisGFP A206K) was shared by Dr. Adam Arkin (University of California, Berkeley). Following published purification protocols²⁴, his-eGFP was expressed in BL21(DE3) pLysS cells overnight at 18 °C and purified from bacterial extracts by incubating with Ni-NTA agarose beads in 25 mM HEPES, 150 mM NaCl, 1 mM imidazole, 1 mM β -mercaptoethanol, pH 7.4. After extensive washing with 25 mM HEPES, 150–300 mM NaCl, 1–5 mM imidazole, 1 mM β -mercaptoethanol, pH 7.4, proteins were eluted via a gradient imidazole wash to a final concentration of 250 mM. Eluted proteins were concentrated and dialyzed in 2 L of 25 mM HEPES, 150 mM NaCl, 1 mM EDTA, and 1 mM β -mercaptoethanol, pH 7.4, at 4 °C overnight and again for 2 h in fresh buffer at 4 °C.

Optical microscopy

Fluorescence and brightfield images have been optimized for contrast and brightness. A Zeiss AxioObserver microscope with 10x and 20x objectives was used for widefield imaging. A Zeiss AxioObserver Spinning Disk Confocal microscope with 100x oil immersion (numerical aperture, 1.4) and 63x oil immersion (numerical aperture, 1.4) objectives was used for both fluorescence and brightfield imaging. Three filters were used: an emission filter centered at 525 nm with a 50nm width, an emission filter centered at 629 nm with a 62 nm width, and a triple pass dichroic mirror designed to reflect laser illumination at 405 nm, 488 nm, and 561 nm excitation wavelengths. For spinning disk confocal and brightfield imaging, cells were cultured on 35 mm collagen-coated glass bottom dishes (MatTek).

Flow cytometry

A BD Accuri C6 Flow Cytometer was used for all flow cytometry studies and all flow cytometry data was analyzed using FlowJo software. Flow cytometry data was collected at a speed of 35 events per second. Gates were drawn to include at least 30% of the detected events, and to exclude electronic noise and cellular debris. In each experiment, once the appropriate gate was determined it was applied to all trials and all experimental conditions without modification.

Targeted Connectosome formation

Following established protocols for making giant plasma membrane vesicles (GPMVs)²⁵, targeted Connectosomes were formed by rinsing donor 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, 25 mM PFA, 2 mM DTT, 125 mM glycine). Next, the cells were incubated for 6 hours in active buffer. After 6 hours, active buffer containing the targeted Connectosomes was collected from the cells. To purify the targeted Connectosomes from any cell debris, the targeted Connectosomes were centrifuged at $300 \times g$ for 5 minutes. Then, to concentrate the targeted Connectosomes, the sample was centrifuged at $17,000 \times g$ for 20 minutes at 4 °C. Finally, the targeted Connectosome pellet was resuspended in fresh media.

Targeting protein function study

HeLa cells stably expressing the GFPnb targeting protein were co-incubated with 250 nM soluble eGFP for 5 min at 37 °C, and then imaged. Then, to test the functionality of the GFPnb targeting protein embedded in the targeted Connectosomes, HeLa cells stably expressing the GFPnb targeting protein undergoing the vesicle formation process were co-incubated with 250 nM soluble eGFP for 5 min at 37 °C, and then imaged. Finally, plasma membrane vesicles collected from HeLa cells expressing the GFPnb protein were incubated with 250 nM purified eGFP. Images were taken after 5 min of incubation at 37 °C. To analyze eGFP binding, the peak intensity of a line profile of fluorescence was recorded. A total of 45 vesicles were analyzed.

Binding study

Off-target and target cells were plated separately in 96 well plates at a density of 5,000 cells per well. Targeted Connectosomes were collected from donor cells as described above, and extruded to 1 μ m by passing through a 1 μ m pore filter 11 times. The media was aspirated away from confluent layers of receiving cells, and the extruded, targeted Connectosomes were added to the cells and incubated for 4 h at 37 °C. At the end of the incubation, recipient cell samples were then either imaged on the spinning disc confocal microscope or prepared for flow cytometry. To quantify the extent of binding, samples were prepared for flow cytometry analysis following established protocols¹. Briefly, after incubation, the recipient cells were rinsed with 200 μ L trypsin to remove unbound targeted Connectosomes. Then recipient cells were trypsinized with 200 μ L trypsin for 5 minutes at 37 °C, 5% CO₂, then quenched with 1 mL media and centrifuged for 5 minutes at 300 $\times$ g. The cell pellet was resuspended in 80 μ L PBS before flow cytometry. At least 6,000 cells were analyzed for each trial.

Competitive binding study

Off-target and target cells were co-plated in 96 well plates at a density of 5,000 total cells per well. Targeted Connectosomes were collected from donor cells as described above, and extruded to 1 μ m by passing through a 1 μ m pore filter 11 times. The media was aspirated away from a confluent layer of donor cells and the extruded, targeted Connectosomes were added to the cells and incubated for 4 h at 37 °C. At the end of the incubation, recipient cell samples were prepared for flow cytometry as described above. At least 4,000 cells were analyzed for each trial.

Calcein red-orange loading

A stock solution of calcein red-orange (CRO) acetomethoxy (AM) dye in DMSO was prepared at a concentration of 1.7 mg/mL and diluted to a final concentration of 17 ng/ μ L in GPMV buffer. To form CRO dye-loaded targeted Connectosomes, donor cells were incubated in the CRO AM dye solution for 20 minutes immediately before extraction of membrane blebs.

Dye delivery study

Recipient target cells were plated in a 48 well plate at a density of 5,000 cells per well and a total media volume of 1 mL per well. The recipient cells received fresh media seven hours before addition of the targeted Connectosomes. The media for the blocked condition was supplemented with 300 μ M carbenoxolone, prepared from a 600 μ M carbenoxolone stock in media. Concentrated, targeted Connectosomes were resuspended in fresh media after formation following dye loading as described above. Targeted Connectosomes were added to recipient cells and incubated in the dark at 37 °C and 5% CO₂ for 2 hours. After incubation, the recipient cells were rinsed with 200 μ L trypsin to remove the targeted Connectosomes and then prepared for flow cytometry. For flow cytometry, the recipient cells were incubated with 200 μ L trypsin for 5 minutes at 37 °C, 5% CO₂, and then quenched with 1 mL media and centrifuged for 5 minutes at 300 $\times$ g. The cell pellet was resuspended in 80 μ L PBS before flow cytometry. At least 6,000 cells were analyzed for each trial.

Doxorubicin loading

A 300 μ M stock solution of doxorubicin was prepared in media (3% DMSO). To form doxorubicin-loaded Connectosomes, concentrated, targeted Connectosomes were resuspended in the doxorubicin solution and then extruded by passing through a 1 μ m pore filter 11 times. To remove unencapsulated doxorubicin, targeted Connectosomes were then centrifuged for 30 minutes at 100,000 $\times$ g, followed by resuspension in fresh media.

Equivalent doxorubicin encapsulation measurement

To estimate the amount of doxorubicin encapsulated within the Connectosomes, free doxorubicin was serially diluted in media to generate a calibration concentration curve (n=3). The doxorubicin fluorescence of the calibration curve was measured in a BioTek Cytation 3 fluorimeter (excitation wavelength 485 nm). To determine the average molar doxorubicin concentration of the targeted Connectosome samples, the fluorescence of each sample of targeted Connectosomes was also measured in the fluorimeter and then compared to the calibration curve.

Doxorubicin cytotoxicity study

Recipient target and off-target cells were co-plated in a 48 well plate at a density of 5,000 total cells per well and a total media volume of 1 mL media per well. Just before addition of the Connectosomes, the recipient cells were incubated in fresh media. For each trial, a sample of doxorubicin-loaded Connectosomes was formed as described above, and the doxorubicin encapsulated within each sample of Connectosomes was determined as described above. Connectosomes were added to recipient cells at increasing equivalent doxorubicin doses and incubated in the dark at 37 °C with 5% CO₂ for 2 hours. After incubation, the recipient cells were rinsed three times with 1 mL fresh media to remove unbound targeted Connectosomes, and then incubated in 1 mL fresh media at 37 °C with 5% CO₂. After 24 hours, cell viability was analyzed using a 7-AAD assay. For the 7-AAD assay, the cells were trypsinized with 500 μ L trypsin solution for 5 minutes at 37 °C, 5% CO₂. Trypsinized cells were then quenched with 1.5 mL media and centrifuged for 5 minutes at 300 $\times$ g. The cell pellet was resuspended in 100 μ L PBS. Five microliters of 7-AAD solution

was added to 45 μ L of the resuspended cells. The cells were analyzed using flow cytometry three minutes after 7-AAD addition. At least 2,000 cells were analyzed for each trial. To determine the percentage of non-viable cells in each sample, a threshold was drawn on the flow cytometry fluorescence histograms at the minimum point between the population of cells excluding the dye and the population of cells including the dye (See fig. S4). The percentage of cells with fluorescence above these thresholds were considered non-viable. The relationship between the concentration of doxorubicin added via solution and off-target cell viability was previously published¹. The relationship between the concentration of doxorubicin added via solution and target cell viability was determined similarly. In brief, recipient target cells were incubated with the specified doxorubicin concentration diluted in media from a 10 mM stock in DMSO for 24 hours. Cell viability was measured using both the 7-AAD viability assay described above, as well as a trypan blue viability assay. For the trypan blue assay, the cells were detached with 200 μ L trypsin solution for 5 minutes at 37 °C, 5% CO₂. Trypsinized cells were then quenched with 1 mL media and pelleted for 5 minutes at 300 $\times$ g. The cell pellet was resuspended in 100 μ L PBS, and trypan blue was added to the cells at a volume ratio of 1:1. At least 100 cells were counted for each trial using a hemocytometer. Cells including the trypan blue stain were considered non-viable and cells excluding the trypan blue were considered viable.

Statistical analysis

As noted throughout the main text and methods sections, at least three independent trials are reported for all experimental results. In each case where a statistically significant comparison is reported, an unpaired, two-tailed student's t-test, with unequal sample variance was performed. The resulting probabilities are listed in the captions of each figure in which comparisons are reported.

Results and Discussion

Development and production of targeted Connectosomes

To achieve targeted cytoplasmic delivery, we began by incorporating a chimeric targeting protein into Connectosomes. To demonstrate this general strategy, we utilized a previously developed multi-functional targeting protein that displays a single domain antibody against green fluorescent protein (GFP) on the cell surface²¹. Incorporation of this targeting protein is expected to enhance Connectosome interactions with cells that express any GFP-tagged cell surface receptor. Notably, we took this approach to demonstrate the capacity to target Connectosomes on the basis of receptor expression. For specific biomedical applications, the antibody domain would be replaced with a protein domain that binds selectively to a disease-relevant receptor of interest²⁶. The targeting protein (GFPnb-mRFP) consisted of four domains from its N terminus to its C terminus: (i) the intracellular and transmembrane domains of transferrin receptor, (ii) an mRFP fluorophore domain for visualization, (iii) an intrinsically disordered linker domain to prevent steric inhibition of binding, and (iv) an affinity domain consisting of GFPnb, a single domain camelid antibody against GFP²⁷. For these studies our target cell line consisted of HeLa cells that expressed a chimeric transmembrane eGFP receptor protein (eGFP_r). This model receptor was comprised of the intracellular and transmembrane domains of the transferrin receptor, and an eGFP

ectodomain. Based on fluorescence intensity measurements made on vesicles derived from cells expressing a similar model receptor²¹, we estimate that the density of the model receptor on the plasma membrane was 40,000–200,000 receptors per cell, comparable to that of commonly targeted disease-relevant receptors^{28, 29}. For example, the concentration of EGFR in certain cancers ranges from 15,000–1,300,000 receptors per cell²⁹, depending on cell type. By using eGFP as a model target receptor, we were able to isolate the absolute binding specificity of the targeted Connectosomes, as cells do not endogenously express eGFP.

To produce targeted Connectosomes, we stably expressed both the targeting protein and a fluorescently tagged connexin 43 protein (Cx43-YFP) in HeLa cells (Figure 1a–c). By extracting membrane blebs from these donor HeLa cells^{1, 21, 25}, we produced targeted Connectosomes, cell-derived plasma membrane vesicles that incorporated both connexin 43 and the targeting protein (Figure 1d–e). Importantly, transmembrane proteins embedded in the plasma membrane vesicles that form during blebbing maintain their function and orientation in the membrane³⁰. Work from our lab and others has utilized these plasma membrane vesicles in order to develop therapeutic materials that depend on the functionality of embedded transmembrane proteins^{1, 21, 31}. It is important to note that exosomes are similar cell-derived vesicles with emerging therapeutic potential³². However, controlled incorporation of transmembrane proteins into plasma membrane vesicles is facilitated by detailed knowledge of the membrane trafficking pathways that deliver proteins to the cell surface³³. In contrast, little is understood about protein trafficking to exosomes at present³⁴, making it a challenge to control their protein content.

To test the accessibility and functionality of the GFP nanobodies displayed by Connectosomes, we exposed HeLa donor cells expressing the targeting protein to soluble eGFP. Fluorescence imaging showed that soluble eGFP bound significantly to membranes that displayed the GFP nanobody at each stage of vesicle extraction, but did not bind to membranes lacking expression of the targeting protein (Figure S1a–c).

Display of targeting proteins promotes selective binding of Connectosomes to target cells

Having demonstrated the display of functional targeting proteins on Connectosome surfaces, we next examined the ability of the targeted Connectosomes to bind to cells expressing the model receptor. After blebbing, Connectosomes had an average diameter of approximately 10 μm ¹. To minimize gravitational settling²¹ and increase therapeutic relevance, targeted Connectosomes were extruded using a 1 μm filter. To measure the baseline level of nonspecific interactions of the targeted Connectosomes with cells that did not express the model receptor, extruded, targeted Connectosomes were incubated with confluent monolayers of wild type HeLa cells. Using established flow cytometry protocols²¹, binding of targeted Connectosomes to HeLa cells was quantified by measuring the increase in mRFP fluorescence of the recipient cells after four hours of incubation with the vesicles (Figure 2a, left). As described above, mRFP is encoded by the targeting protein displayed by the targeted Connectosomes. Addition of the targeted Connectosomes increased the average mRFP fluorescence of the recipient off-target cells by approximately 3,000 arbitrary fluorescence units (a.u.), only slightly above the cellular autofluorescence (Figure 2b–c). In

contrast, the same concentration of targeted Connectosomes increased the fluorescence of HeLa cells that expressed the model receptor by approximately 32,000 arbitrary units (Figure 2a, right; 2b–c). This ten-fold increase in the shift in average mRFP fluorescence upon vesicle addition demonstrates that displaying the targeting protein on the surfaces of Connectosomes substantially enhanced their interaction with cells expressing the model receptor. Fluorescence imaging of the cells further confirmed that targeted Connectosomes bound to the surfaces of cells expressing the model receptor in greater density than they bound to the surfaces of wild type HeLa cells (Figure 2d–e). Taken together, these data demonstrate that the display of targeting proteins on the surfaces of Connectosomes selectively enhanced the binding of Connectosomes to cells that expressed a model receptor.

To better elucidate the specificity of targeting, we next analyzed the correlation between targeted Connectosome binding and the receptor expression level of the target cells. Specifically, using the eGFP-channel fluorescence of the untreated target cells as an indicator of their level of expression of the model receptor, target cells were divided into four groups of increasing fluorescence, each of which contained the same number of cells, about 20% of the total cell population (Figure 3a). Notably, the top 20% of cell in terms eGFP fluorescence were not included in the analysis because the additional fluorescence associated with targeted Connectosome binding caused many of these cells to saturate the fluorescence detector. The average eGFP fluorescence for the four groups we analyzed increased from 150,000 (a.u.) for the group with the lowest eGFP receptor expression, to nearly 1,500,000 (a.u.) for the group with the highest eGFP receptor expression (Figure 3b). For each cell group, binding of targeted Connectosomes was quantified by measuring the increase in average mRFP fluorescence after incubation with targeted Connectosomes (Figure 3c–e). As expected, we found that targeted Connectosome binding increased significantly with increasing eGFP receptor expression. For example, the group of target cells with the lowest eGFP receptor expression (group 1) had an increase in mRFP fluorescence of only 6,000 (a.u.) after incubation with targeted Connectosomes. However, for the group of target cells with the highest eGFP receptor expression (group 4), the increase in mRFP fluorescence after addition of the targeted Connectosomes was greater than 40,000 (a.u.). Plotting the increase in mRFP fluorescence owing to binding versus the increase in eGFP fluorescence owing to receptor expression reveals a strong, approximately linear increase, clearly illustrating that the extent of binding is well controlled by the level of the model receptor expression.

Next, to assess the ability of targeted Connectosomes to select target cells out of a heterogeneous population, we performed a competitive binding experiment. Specifically, confluent monolayers of recipient target and off-target cells co-cultured in the same dish were incubated with targeted Connectosomes. After incubation, the heterogeneous population of recipient cells was washed to remove unbound vesicles, and binding was quantified using flow cytometry (Figure 4). During the analysis of these data, target cells were separated from off-target cells based on their eGFP fluorescence intensity, so that binding for each cell type could be analyzed separately (Figure 4a). The average mRFP fluorescence of off-target cells increased slightly upon exposure to targeted Connectosomes (2,000 a.u.), presumably owing to nonspecific binding (Figure 4b–d). In contrast, the average mRFP fluorescence of target cells increased substantially upon exposure to targeted

Connectosomes (50,000 a. u.) (Figure 4b–d). These data illustrate that in a competitive scenario, the display of targeting proteins enhanced Connectosome binding to target cells by more than 25-fold (Figure 4d). Interestingly, this increase in binding is more than twice the ten-fold increase seen when the target and off-target cells were exposed to targeted Connectosomes separately (Figure 2c). Importantly, because the number of Connectosomes and the total number of recipient cells was held constant for both the noncompetitive and competitive studies, the number of Connectosomes per target cell was greater in the competitive study. Therefore, the apparent enhancement in selectivity in the competitive study suggests that the interactions of targeted Connectosomes with cells are somewhat transient, allowing particles that initially encounter off-target cells to repartition to target cells, for which they have a higher binding affinity.

Molecular delivery by targeted Connectosomes is connexin-dependent

Having established the selectivity of targeted Connectosome binding, we next verified the dependence of molecular delivery on gap junction channels. Our previous work using untargeted Connectosomes demonstrated the ability of Connectosomes to deliver dye directly to the cytoplasm of recipient cells using fluorescence imaging¹. We have previously shown that these particles interact with recipient cells primarily at the plasma membrane surface³⁵. During the biogenesis of connexin, unpaired connexin channels are trafficked to the plasma membrane from the endoplasmic reticulum³⁶. These unpaired channels diffuse on the plasma membrane and are thought to play critical roles in intercellular signaling³⁷. Typically, it is only after reaching a gap junction plaque, which is a region of the of the plasma membrane that contains hundreds to thousands of connexin channels, that the connexin channels dock with channels from neighboring cells. Therefore, it is likely that the Connectosomes interact with unpaired connexin channels that have not yet reached a gap junction plaque.

Our previous work also established that delivery was gap junction-dependent¹. In particular, while dye-loaded Connectosomes released encapsulated dye when gap junction channels were opened via calcium removal, vesicles lacking gap junction channels did not release encapsulated dye under the same conditions. Additionally, transfer of dyes from cell-derived vesicles to cells was significantly reduced when vesicles harvested from gap junction-deficient cell lines were used as vehicles for delivery¹.

Building on this work, here we sought to evaluate the extent to which targeted Connectosomes require gap junction functionality to deliver their cargos to cells (Figure 5). In these experiments, targeted Connectosomes were loaded with calcein red orange (CRO) dye by treating donor cells with CRO-acetomethoxy (CRO-AM) dye prior to Connectosome production. The AM group renders the CRO-AM dye membrane permeable. However, as the CRO-AM dye accumulates in the cytoplasm, the AM group is metabolized by intracellular esterases, such that the resulting CRO dye is trapped in the cytoplasm and permeable only to gap junctions³⁸.

Confluent monolayers of recipient target cells were incubated with unprocessed, intact, CRO dye-loaded targeted Connectosomes. To examine the dependence of molecular delivery on functional gap junctions, targeted Connectosomes loaded with CRO were also added to

confluent monolayers of recipient target cells that were pretreated with 300 μ M of the gap junction blocker, carbenoxolone³⁹. Carbenoxolone, a commonly used gap junction inhibitor, uncouples cellular gap junctions and prevents the exchange of signals between cells⁴⁰. Therefore, pretreating recipient cells with this inhibitor blocks the ability of Connectosomes to form junctions with and transfer materials to recipient cells¹.

Dye delivery was quantified by using flow cytometry to measure the fluorescence of cells in the CRO dye channel after a two-hour incubation with the vesicles (Figure 5A–B). Treatment with carbenoxolone reduced the increase in CRO dye fluorescence after treatment with the targeted Connectosomes by almost half, demonstrating the substantial role of gap junctions in dye delivery. The residual CRO fluorescence transfer that is not lost in the presence of carbenoxolone may arise from several sources including targeted Connectosomes adhering to the surfaces of cells, cellular uptake of Connectosomes, or incomplete blockade of gap junction channels by carbenoxolone.

Targeted Connectosomes deliver chemotherapeutics selectively to target cells

We next evaluated the extent to which displaying targeting ligands on the surfaces of Connectosomes enabled selective delivery of chemotherapeutics to cells expressing the model receptor. In our previous work, doxorubicin-loaded Connectosomes were formed by loading donor cells with the drug before harvesting the vesicles. However, in the present study, to increase the therapeutic relevance of the Connectosomes, we extruded them to smaller diameters. Because extrusion would significantly dilute the doxorubicin encapsulated within Connectosomes loaded using the above method, we developed a new method of loading. Specifically, we formed doxorubicin-loaded targeted Connectosomes by extruding unprocessed, targeted Connectosomes through a filter of 1 μ m pore size in the presence of media containing 300 μ M doxorubicin. The resulting doxorubicin-loaded, targeted Connectosomes were pelleted and washed to remove the unencapsulated doxorubicin. Then, the Connectosomes were resuspended in fresh media, and their doxorubicin content was quantified by measuring the doxorubicin fluorescence emission of the vesicles in comparison to a standard curve (Figure S2). The targeted Connectosomes were then incubated with co-cultured target and off-target cells at equivalent doxorubicin doses increasing from 40 nM to 160 nM. These equivalent concentrations are calculated by dividing the total molar amount of encapsulated doxorubicin by the total volume of the culture media during the doxorubicin treatment. After two hours, unbound targeted Connectosomes were washed away. After 24 hours, cell viability was measured using a 7AAD viability stain (Figure 6, S3, S4, and S5). As expected, for both target and off-target cells, the viability decreased with increasing doxorubicin dose. However, we found that at each equivalent doxorubicin dose, target cells were significantly less viable than off-target cells. For example, at 80 nM doxorubicin, only 3% of off-target cells were nonviable, while approximately 23% of target cells were nonviable. Further, at 160 nM doxorubicin, only 10% of off-target cells were nonviable, while 62% of target cells were nonviable. Therefore, at the lowest equivalent doxorubicin concentration at which a majority of target cells were nonviable, 160 nM, more than six times fewer off-target cells were nonviable, demonstrating that the display of targeting proteins on the surfaces of Connectosomes enhances the therapeutic efficacy of doxorubicin delivery to cells that express the model receptor, while

protecting off-target cells cultured in the same dish. Notably, target cells and off-target cells had equivalent dose-response behavior when treated with free doxorubicin (Figure S6 and S7).

Conclusion

Our previous work demonstrated that Connectosomes are capable of efficiently transporting therapeutics to the cytoplasm through gap junctions¹, reducing the effective dose of doxorubicin by an order of magnitude compared to free doxorubicin, and by more than 100-fold in comparison to conventional liposomal doxorubicin. However, since nearly all tissues express gap junction channels, the translational relevance of Connectosomes was limited by their inherent lack of specificity for diseased tissues. Towards addressing this issue, here we report the development of targeted Connectosomes, which use a combination of gap junction channels and targeting proteins to deliver molecular cargos selectively to the cytoplasm of cells that express a model receptor.

Displaying targeting proteins on the surfaces of Connectosomes enhanced binding to cells expressing the target receptor by 25 times in comparison to off-target cells in a competitive binding assay. This increase is comparable to increases in binding exhibited by synthetic targeted particles¹³. Further, our data demonstrate that targeting substantially enhances the therapeutic efficacy of doxorubicin delivery to cells that express a model receptor. In sum, our data demonstrates that harnessing the cell's machinery to display targeting proteins on the surfaces of Connectosomes is a promising strategy for overcoming the inherent non-specificity of gap junction channels.

Ultimately, the development of a chemotherapeutic delivery system that both efficiently and selectively delivers therapeutics to diseased cells represents a critical step towards realizing the therapeutic potential of Connectosomes and other particles which employ gap junctions for delivery. In the future, our targeting strategy could be utilized to target Connectosomes to a broad range of cell types simply by replacing the targeting moiety with the desired ligand or single-domain antibody²¹. In this way, targeted Connectosomes could be used to deliver a range of membrane impermeable drugs and reagents, such as siRNA, peptides, and other macromolecules, to diverse populations of target cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Targeted Connectosomes were harvested from donor cells. (a) Schematic of the targeting protein and hexameric connexin 43-YFP channel. Hexamer appears as a dimer in side view. (b) Schematic of the targeted Connectosome formation process including establishment of the donor cell line (left); inducing the donor cells to undergo membrane blebbing (center); and harvesting membrane blebs containing both targeting proteins and connexin channels (right). (c-e) Giant plasma membrane vesicles were harvested from donor cells co-expressing connexin 43-YFP (green) and an mRFP-tagged GFP nanobody targeting protein (magenta) (c), through the process of membrane blebbing (d) to produce targeted Connectosomes (e). Confocal fluorescence images. Scale bar in (c) and (d), 10 μ m. Scale bar in (e), 2 μ m. Images intentionally saturated to show membrane expression of the connexin and targeting proteins.

Figure 2.

Display of targeting proteins enhances Connectosome binding to cells that express a model receptor. (a) Flow cytometry histograms showing mRFP fluorescence of recipient cells that do not express the model receptor (off-target cells, left) and cells that do express the model receptor (target cells, right). The green and magenta curves represent cell fluorescence before and after incubation with extruded, targeted Connectosomes, respectively. Each curve represents 3 independent, concatenated trials. 6,000 cells were analyzed per trial. Notably, the baseline, untreated mRFP fluorescence for the target cells is greater than the baseline, untreated mRFP fluorescence for the off-target cells due to bleed-through of the eGFP fluorescence from the model receptor into the mRFP channel on the flow cytometer. (b) Summary of (a) showing the average recipient cell mRFP fluorescence for each condition in (a). The error bars represent the standard deviations of 3 independent trials. (c) Subtraction of averages in (b) (after incubation – before incubation), yielding the average increase in mRFP fluorescence upon incubation with targeted Connectosomes for off-target and target cells. The error bars represent the standard deviations of 3 independent trials. (d) Confocal fluorescence images showing that targeted Connectosomes bound to target cells. (e) Confocal fluorescence and brightfield images showing that off-target cells recruit far fewer targeted Connectosomes in comparison to target cells in (d). Notably, brightfield images are provided in (d) to show the overall cell shape instead of eGFP fluorescence images because the off-target cells do not express green fluorescent protein. All scale bars 10 μ m. Legend in (a) applies to (b). Asterisks represent statistically significant differences (two-tailed t test, $p < 0.008$ (b, left), $p < 0.003$ (b, right), and $p < 0.006$ (c)).

Figure 3.

Targeted Connectosome binding was correlated with target cell eGFP receptor expression. (a) Example scatterplot from flow cytometry analysis showing the four gates used to analyze recipient target cells based on their eGFP receptor expression, as represented by their eGFP fluorescence. (b) Average recipient cell eGFP fluorescence for each group. The error bars represent the standard deviations of 3 independent trials, 6,000 total cells were analyzed per trial. (c) Flow cytometry histograms showing mRFP fluorescence for each group of recipient target cells before (green) and after (magenta) incubation with extruded, targeted Connectosomes. (d) Average increase in mRFP fluorescence after incubation with targeted Connectosomes for each cell group. (e) Targeted Connectosome binding as a function of GFP receptor expression. Asterisks represent statistically significant differences (two-tailed t test, all $p < 0.005$ (b) and all $p < 0.04$ (d)).

Figure 4.

Targeted Connectosomes bound selectively to target cells in a competitive binding study. (a) Flow cytometry histogram showing eGFP fluorescence for co-cultured target and off-target cells. Gates shown were used to analyze each group of cells separately. The curve represents 3 independent, concatenated trials. 4,000 total cells were analyzed per trial. (b) Flow cytometry histograms showing mRFP fluorescence of recipient cells that do not express the model receptor (off target cells, left) and cells that do express the model receptor (target cells, right). The green and magenta curves represent cell fluorescence before and after incubation with extruded, targeted Connectosomes, respectively. Each curve represents 3 independent, concatenated trials, 4,000 total cells analyzed per trial. Notably, the baseline, untreated mRFP fluorescence for the target cells is greater than the baseline, untreated mRFP fluorescence for the off-target cells due to bleed-through of the eGFP fluorescence from the model receptor into the mRFP channel on the flow cytometer. (c) Summary of (b) showing the average recipient cell mRFP fluorescence for each condition in (b). The error bars represent the standard deviations of 3 independent trials. (d) Subtraction of averages in (c) (after incubation – before incubation), yielding the average increase in mRFP fluorescence upon incubation with targeted Connectosomes for off-target and target cells. Legend in (b) applies to (c). Asterisks represent statistically significant differences (two-tailed t test, $p < 0.0002$ (b, left), $p < 0.0005$ (b, right), and $p < 0.002$ (c)).

Figure 5.

Dye delivery using targeted Connectosomes was dependent on functional gap junctions. (a) Schematic of a targeted Connectosome binding and release encapsulated CRO dye into a recipient cell that expresses the model receptor. (b) Flow cytometry histogram showing CRO-channel fluorescence for untreated target cells (green), for target cells after treatment with targeted, CRO-loaded Connectosomes (magenta), and for target cells after treatment with targeted, CRO-loaded Connectosomes and channel blocker, carbenoxolone (black). 6,000 cells were analyzed per trial. (c) Average recipient cell CRO-channel fluorescence for each condition in (b). Legend in (b) applies to (c). Asterisks represent statistically significant differences (two-tailed t test, $p < 0.02$ (b, left), $p < 0.04$ (b, right)).

Figure 6.

Targeted Connectosomes enhanced the specificity of doxorubicin delivery to cells expressing a model receptor. (a) Percentage of nonviable cells that did not express the model receptor (off-target cells, green) and nonviable cells that did express the model receptor (target cells, magenta) after treatment with doxorubicin-loaded, targeted Connectosomes. All measurements were made using a 7-AAD viability assay. The error bars represent the standard deviations of 3 independent trials. 2,000 total cells were analyzed per trial. (b) Fluorescence (top) and brightfield (bottom) image showing that many of the rounded up dead cells appear to express the model receptor at higher levels (ie. have higher green fluorescence) in comparison to well-spread viable cells. Scale bar 250 μm. Asterisks represent statistically significant differences (two-tailed t test, target cells from left to right $p < 0.04, 0.05, 0.003$, off-target cells $p < 0.02$).