

## Cyanine lipids promote the shedding of extracellular vesicles from cell membranes

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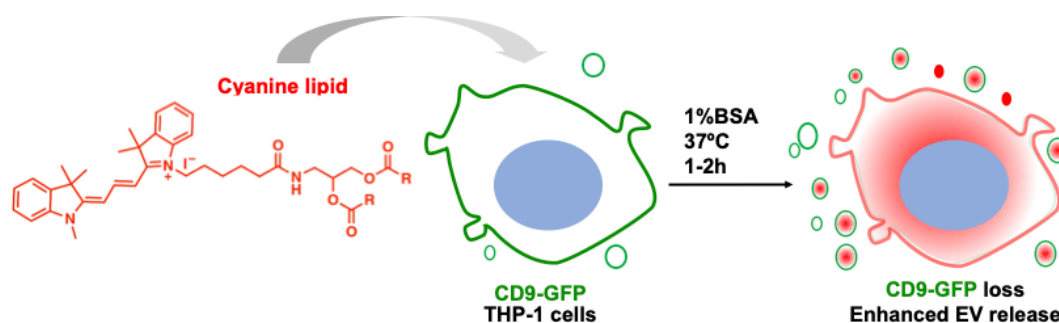
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### Graphical abstract



### Abstract

Extracellular vesicles (EV) have garnered significant attention in drug delivery, imaging, and immunotherapy. There is a need for methods to enhance the release of EV from cells. We found that at high labeling concentrations (100 $\mu$ M), indocarbocyanine lipids DiD and DiR that are commonly used for labeling cells, nanoparticles, and EV, promoting the shedding of cell membrane tetraspanins with concomitant release of EV in the medium. We screened a library of lipids and liposomal formulations to investigate this phenomenon further to release membrane marker CD63 from THP-1 cells and membrane red-enhanced nanolanthan (RNL) from 4T1 cells. We found a strong dependency of the EV release on lipid structure. In general, lipids that had a cyanine headgroup were more efficient than PEGylated phospholipids, neutral and cationic liposomes, with some lipids enhancing the release of CD63 up to 4-fold and of RNL up to 8-fold, over vehicle-treated control. A side-by-side comparison of cyanine lipid derivatives and corresponding precursor lipids confirmed that the cyanine headgroup significantly promoted the shedding of RNL. Mutation of an exosome biogenesis regulator UNC13D did not hinder the release. Lipid-released EV could be modified with anti-interleukin 13 receptor alpha 2 antibody and targeted to glioma cells, suggesting potential utility in drug delivery. Furthermore, the impact of extraneously added lipids should be carefully considered in cell labeling and drug delivery applications.

**Keywords:** extracellular vesicles, exosomes, cells, lipids, delivery, antibody, targeting

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## Rationale, Purpose, and Limitations

The current methods for EV production suffer from low yield and limited scalability. We studied the effect of extraneously added lipids on the release of EV from cells and found that cyanine headgroup enhances the release. The main limitation is the need for additional purification of released EV from excess lipids.

## Introduction

Virtually all animal cells release extracellular vesicles (EV).<sup>1,2,3,4</sup> Natural EV form through the secretion of intraluminal vesicles enclosed in multivesicular bodies/endosomes (exosomes) or through budding from the plasma membrane (microvesicles).<sup>5</sup> EV have shown promise as drug delivery vehicles due to biocompatibility, low immunogenicity, ability to cross biological barriers, and tissue tropism.<sup>6-8</sup> Several methods exist to obtain natural EV. Still, there are scalability and cost challenges.<sup>9,10</sup> There is a substantial interest in producing “forced” EV, such as by using physical methods such as sonication, extrusion, or high-pressure homogenization that disrupt cell membranes to release membrane vesicles.<sup>9-11</sup> Several groups explored chemically triggered release as an alternative to the aggressive energy-dependent physical disruption that may damage cellular proteins. Thus, using high throughput screening of engineered cells that express nano luciferase-tetraspanin fusion, chemicals that can promote the formation of EV via stimulation of natural biogenesis have been discovered.<sup>12-14</sup> While promising, adding chemicals and inhibitors may have regulatory challenges due to potential toxicity issues.

Lipids are the most biocompatible component of modern drug delivery vehicles, including liposomes, lipid nanoparticles, and lipid-drug conjugates.<sup>15-18</sup> Moreover, hybrid EV have been generated by fusion between lipids and pre-isolated EV, with additional targeting and biological properties.<sup>9,19-22</sup> We screened several cyanine lipid derivatives and common non-cyanine lipid formulations for the efficiency of EV release from THP-1 monocytic cells. We confirmed the release by selected cyanine lipids from bioluminescent 4T1 breast cancer cells. We found that lipids enhance the shedding of cell membranes, with cyanine lipids showing

significantly better release. Further understanding of this phenomenon can improve the methods of EV production.

## Materials and Methods

### Materials

DiD (1,1'-Diioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine, 4-chlorobenzenesulfonate salt), DiI (1,1'-Diioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Perchlorate) and DiR (1,1'-Diioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide) were from Biotium (Hayward, CA, USA). DiI-C12, DiI-C16, and DiI-C18:2 were obtained from ThermoFisher (Waltham, MA, USA). The synthesis of Cy3 lipids was described before.<sup>23</sup> All lipids were prepared in ethanol (10mM) and stored at 4°C. Lipid stock solutions were warmed to 37°C immediately before use. Bovine serum albumin and ethyl alcohol were from Sigma-Aldrich (St. Louis, MO, USA). The nitrocellulose membrane (0.45 µm) was from Bio-Rad (Hercules, CA, USA). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-2000), DSPE-PEG-1000, DSPE-PEG-750, DSPE-PEG-350, 1,2-dioleoyl-sn-glycero-3-phosphocholine DOPC, cholesterol, N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were from Avanti Polar Lipids (Alabaster, AL, USA). Nuclear staining reagent Hoechst 33342 trihydrochloride trihydrate was purchased from ThermoFisher. Anti IL13Rα2 antibody was described before.<sup>24</sup> The mouse anti-human anti-CD63 antibody was from BioLegend (#353014), and the mouse anti-human anti-CD81 antibody was from Santa Cruz Biotechnology (#sc-166029). The secondary antibody used in Western blotting was HRP-conjugated anti-mouse IgG (Sigma-Aldrich, #A6782). Anti-mouse IRDye800 was from Li-COR Biosciences (Lincoln, NE, USA).

### Methods

**EV preparation and purification:** THP-1 cells were resuspended in 200 µL of 1% BSA in phosphate-buffered saline (PBS) at 3 million cells per mL concentration. The cells were then incubated with lipids at 100 µM under rotation at 37°C in a cell culture incubator for 2 hours.

After the incubation period, the cells were centrifuged at 500g for 5 minutes, and this centrifugation step was repeated three times. The supernatant containing the EV was collected. EV were purified using a Beckman Optima ultracentrifuge with a TLA-100.3 rotor at 60,000 rpm (150,000g) for 20 minutes. The resulting EV particles were resuspended in PBS and stored at -80°C for future use.

**Liposome preparation:** Lipids dissolved in chloroform were mixed and dried under a stream of nitrogen gas. The resulting dry lipid cake was then resuspended in PBS to achieve a final lipid composition of 20 mM. The suspension was incubated at 60°C for 30 minutes to facilitate lipid hydration. Subsequently, the solution was vortexed for 2 minutes to ensure proper mixing, followed by extrusion using an Avestin manual extruder (Avestin, Ottawa, Canada) through Whatman Nucleopore Track-Etch Membranes with a pore size of 100 nm. The extrusion process was repeated 15 times to obtain homogeneous liposomes. Finally, the liposomes were stored at 4°C at a final concentration of 20 mM lipid for further use.

**Western blot and dot blot:** EV were lysed in an SDS protein sample buffer for tetraspanin Western blot. EV lysates were resolved on 8% bis-tris SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and probed using primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. For dot blotting analysis of released CD63, EV in the cell supernatant (after pelleting the cells but without ultracentrifugation) were directly spotted onto a nitrocellulose membrane in triplicate using 2 µL of each sample. To quantify the percentage of EV release, an equivalent number of THP-1 cells was probe sonicated for 30 seconds and were used as a standard curve of CD63 spotted on the membrane alongside the EV samples. The membrane was dried and blocked with 5% nonfat milk for 2 hours. Following blocking, the membrane was washed three times with 0.1% Tween-20/PBS and incubated with a primary anti-human CD63 antibody at a dilution of 1:500 in 5% milk overnight at 4°C. After the overnight incubation, the membrane was washed three times with 0.1% Tween-20/PBS and subsequently incubated with a corresponding IRDye800 labeled secondary antibody at a dilution of 1:5000 at room temperature for 2 hours. Following another round of washing

(three times with 0.1% Tween-20/PBS), the membrane was scanned at 800 nm using a Li-COR Odyssey imaging system. The integrated density of the dots in the 8-bit TIFF images was measured using ImageJ software to analyze the dot blot results.

**Cell culture:** The human monocyte cell line THP-1 was from ATCC (Manassas VA). 4T1 mouse mammary cells stably transfected with palmitoylated red-enhanced Nano-lantern<sup>25</sup> (PalmReNL) were generated in the Kanada lab. THP-1 CD-9 GFP and WT and KO cell lines were generated in the Shen lab. GL261 IL13Rα2<sup>+</sup> was generated in the Balaysnikova lab. THP-1 cells were grown in the RPMI-1640 medium (Corning Life Sciences, Corning, NY, USA) and GL261 and PalmReNL-4T1 cells in the DMEM medium (Corning Life Sciences). Both media contained 10% (v/v) fetal bovine serum (Gibco/Life Technologies, Carlsbad, CA), 100 U/mL penicillin, and 100 ng/mL streptomycin (Corning Life Sciences). Cells were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

**Confocal microscopy:** Cells were examined by Nikon Eclipse AR1HD inverted confocal microscope with 405 nm, 488 nm, 561 nm, and 640 nm lasers). CD9-GFP THP-1 cells (3×10<sup>6</sup> cells/ml) were treated with lipids in complete media at 37°C for the indicated times. Cells were then collected and pelleted at 500 rpm×1 min. The cell pellet was incubated with 2 mL of HBSS (minis calcium/magnesium and phenol red (Corning Life Sciences) containing 2 µg/mL Hoechst 33342 for 3 minutes at room temperature. Cells were re-pelleted at 500 rpm × 1 min, then washed again with 2 mL HBSS. Cells were re-pelleted at 500 rpm × 1 min then resuspended in 20 µL of mounting media (0.5% (w/v) carboxymethyl cellulose in HBSS containing 1% (v/v) fetal bovine serum). For confocal analysis, 15 µL of cell suspension in mounting media was applied to a microscope slide and then covered with a glass coverslip (22 × 30 mm). Cells were allowed to adhere for 5 minutes before confocal analysis using a 60× (oil) objective at room temperature.

**EV labeling with antibodies and uptake studies:** THP-1 WT cells were prepared at a concentration of 6 million cells per mL for cell modification. The cells were combined with DiI-PEG3400-anti-IL13Rα2 or DiI-PEG3400-IgG

at a concentration of 2  $\mu$ M in 1% BSA/PBS. The mixture was then incubated at 37°C for 30 minutes, followed by three washes with 1% BSA/PBS to remove unbound antibodies.

Next, the labeled THP-1 cells were incubated with DiR (100  $\mu$ M) at 37°C for 2 hours. After the incubation, the cells were centrifuged at 0.5 g for 5 minutes, and this centrifugation step was repeated three times. The supernatant was collected, and EV was purified from the supernatant.

IL13R $\alpha$ 2+ GL261 cells were grown on coverslips in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 ng/mL streptomycin for the uptake studies. The coverslips with the cells were then incubated with DiI-PEG3400-IL13R $\alpha$ 2 or DiI-PEG3400-IgG at a concentration of 3  $\mu$ g/mL, along with the corresponding EV samples in individual wells. The incubation was carried out for 24 hours at 37°C with 5% CO<sub>2</sub>.

After 24 hours, the coverslips were transferred to a new 6-well plate containing 2 mL/well of HBSS with 2  $\mu$ g/mL Hoechst 33342. After a 2-minute incubation, the HBSS/Hoechst solution was aspirated, and the coverslips were washed with 2 mL of HBSS. The coverslips were then inverted and mounted in pairs on a glass microscope slide using 15  $\mu$ L of mounting media (0.5% carboxymethyl cellulose in HBSS + 1% FBS). Images were acquired using a 60 $\times$  oil immersion objective with identical laser power and detector gain settings.

## Results

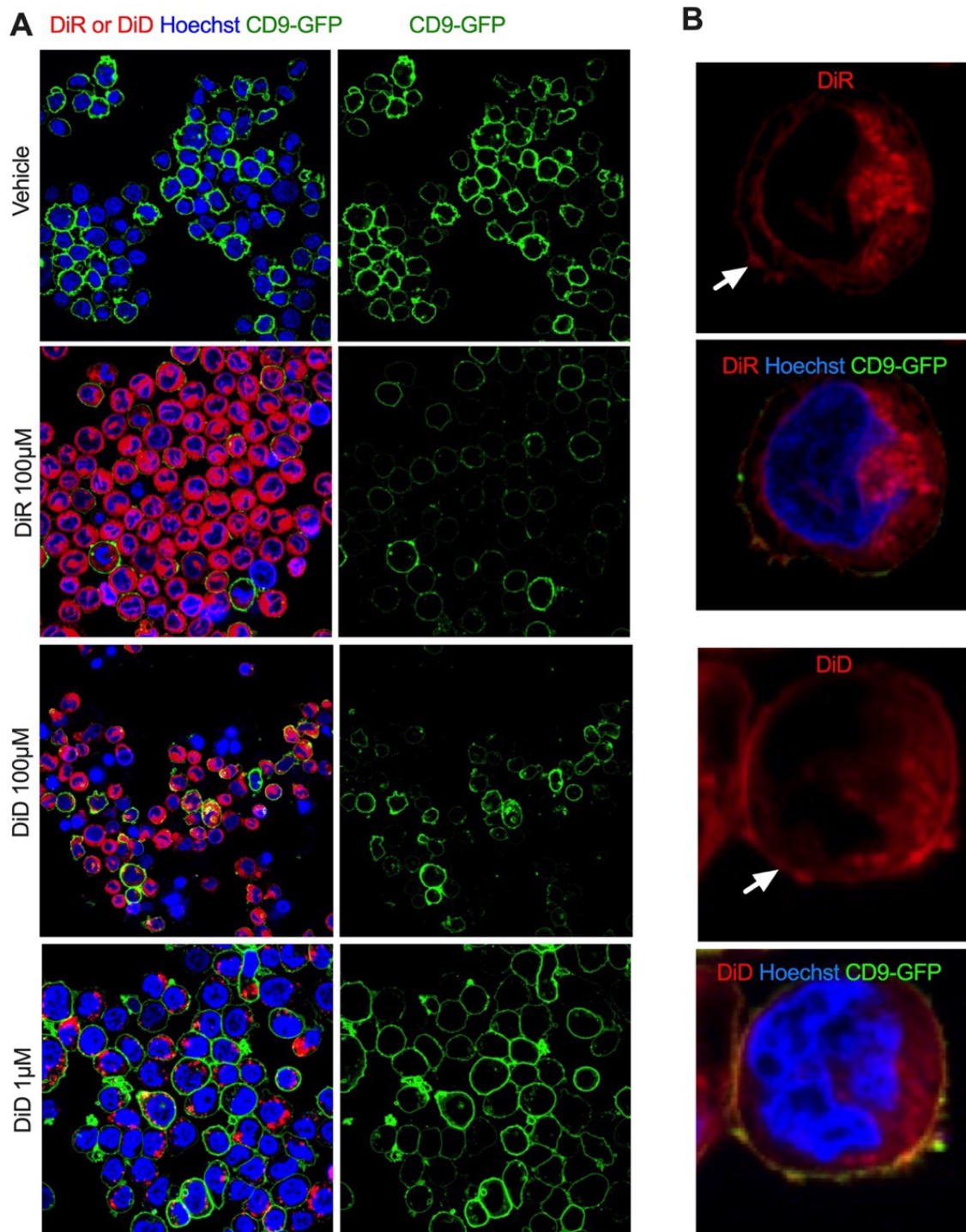
Indocarbocyanine lipids (ICLs: DiI, DiD, and DiR) are fluorescent dyes with cyanine fluorophore headgroups covalently linked to two alkyl chains. ICLs have been successfully used to label EV,<sup>26,27</sup> cells, nanoparticles, and liposomes.<sup>26,28-31</sup> While labeling a THP-1 monocytic cell line that stably expresses membrane tetraspanin CD9-GFP with high concentration (100  $\mu$ M of DiR or DiD, we serendipitously observed an almost complete loss of CD9-GFP from the membrane (Figure 1A). For comparison, cells treated with 1  $\mu$ M DiD did not show any loss of CD9-GFP. Notably, even after the CD9-GFP loss, the membrane showed DiR and DiD accumulation (Figure 1B, arrows). We further isolated EV from DiR-treated CD9-GFP THP-1 cells by differential centrifugation and

ultracentrifugation (Figure 2A). High magnification confocal imaging showed colocalization of DiR and CD9-GFP and some DiR-only particles (Figure 2B-C), presumably due to the excess of unincorporated dye. NanoSight measurements showed the size of DiR particles to be less than 200 nm, whereas EV purified from cell medium were up to 320 nm (Figure 2D).

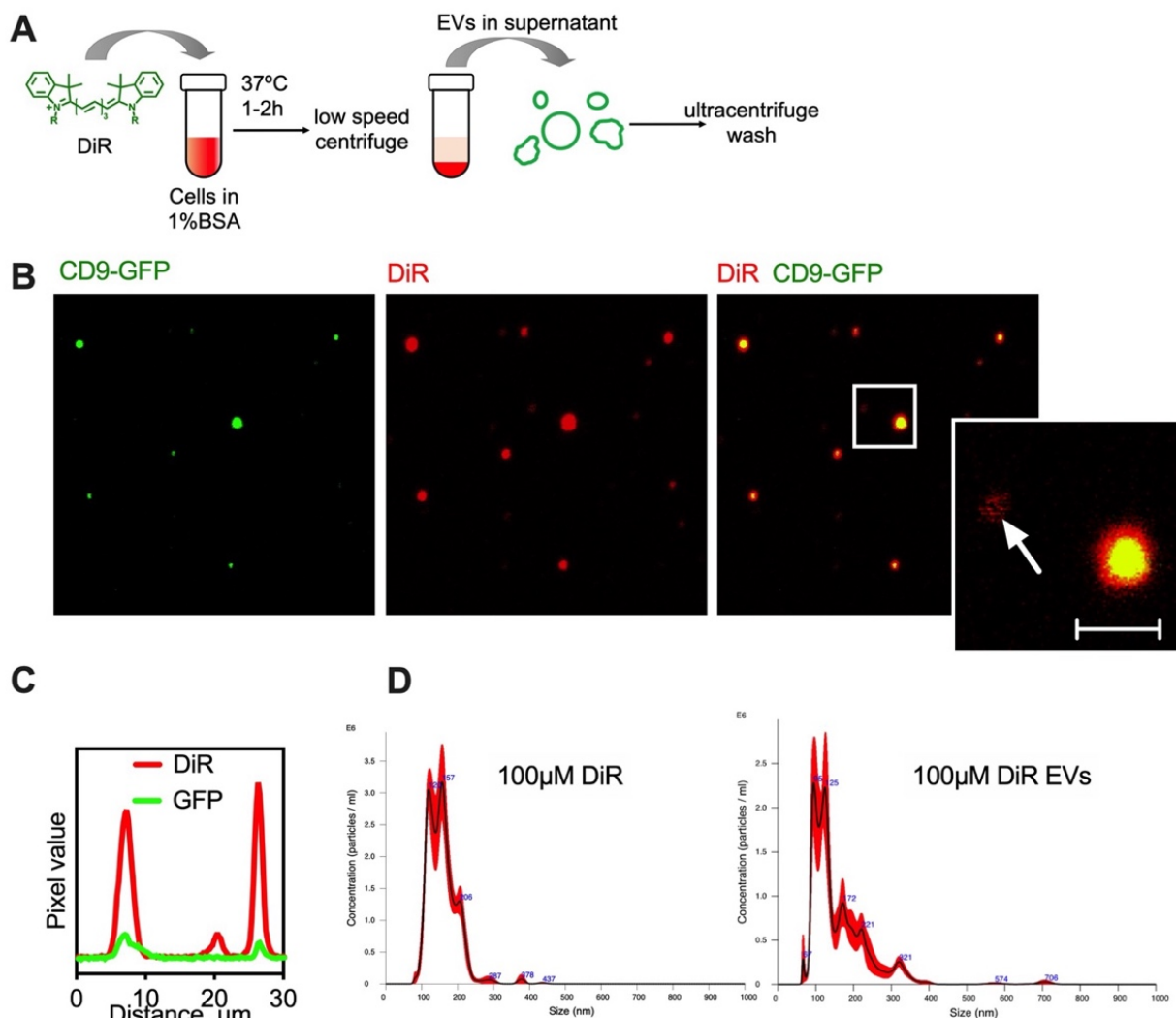
Recently, it was described that adding liposomal lipids, including cationic DOTAP, enhanced EV release from intact cells<sup>32</sup>, but the role of lipid structure in EV release is unclear. We used the previously described library of cyanine 3 lipid analogs with different lipid tails and linkages.<sup>23</sup> We compared 14 lipophilic Cy3 derivatives including DiI-C18 (Figure 3 and Methods) and non-cyanine lipids, including DSPE-PEG with different PEG lengths (350 to 2000), DOPC, DOPC/DOPE (1:1) and DOPC/cholesterol (3:1) liposomes, and cationic DOTAP and DOTAP/DOPE (1:1) liposomes. DOPE is especially interesting because it promotes non-bilayer inverted hexagonal structures (e.g., in DOTAP/DOPE lipoplexes<sup>33</sup>). As the primary assay, we quantified EV release from THP-1 cells via immunoblotting for tetraspanin CD63, one of the markers of EV.<sup>34</sup> Most Cy3-based lipids enhanced the release 2-4 fold, except for DiI-C18 (Figure 4A), which showed minimal efficacy. DOTAP liposomes, neutral DOPC liposomes, and PEGylated lipids also enhance EV release (Figure 4A).

Still, on average, these lipids were less efficient than cyanine 3 lipids (Figure 4B). We further tested the enhancement of EV release from 4T1 murine breast cancer cells stably modified with membrane-anchored palmitoylated Red enhanced Nano-lantern (palmReNL, hereafter RNL).<sup>35</sup> We used Cy3-C18, Cy3-C16, Cy3-C14, Cy3-C12, DiR, DiD and non-cyanine lipid DOTAP. The reason for choosing this assay is for more robust measurements due to the lower baseline release of EV than THP1, luminescent output, and compatibility with far-red and near-infrared lipids DiD and DiR. Cy3 lipids at 100  $\mu$ M promoted an 8-fold increase in the release of RNL compared to non-treated cells (Figure 4C). Cy3-C12 diester lipid was significantly more efficient than DiR, DiD, and DOTAP, which enhanced the release by 1.5-2-fold. Calculations suggested that up to 80% of RNL was released by Cy3-C12 diester lipid (Figure 4D).





*Figure 1. Loss of membrane tetraspanins after treatment with DiR or DiD. CD9-GFP THP-1 cells were treated with 100 µM dye for 1 hour, and the cells were washed and imaged with a confocal microscope. A) Cells treated with 100 µM DiR or 100 µM DiD show significant loss of membrane CD9-GFP, whereas cells treated with 1 µM DiD showed mostly intact membrane CD9-GFP; B) Cells treated with 100 µM DiR (upper panel) or 100 µM DiD (lower panel) show membrane and perinuclear localization of the dye. Arrows point to parts of the plasma membrane that have lipid staining*



**Figure 2.** Tetraspanins are released in EV and colocalize with DiR; *A*) Lipid incubation and EV isolation by differential centrifugation followed by ultracentrifugation. Note that the EV isolation protocol does not remove the excess of free dye particulates; *B*) High magnification confocal imaging of purified EV showing colocalization of CD9-GFP and DiR. The cropped image shows a large DiR/CD9-GFP-positive particle and a faint DiR-only particle-size bar 2  $\mu$ m; *C*) Line profile drawn across several particles shows colocalization of GFP and DiR, but also DiR-only particles that are hard to remove by ultracentrifugation; *D*) Nanosight measurement of DiR-only (left) and isolated EV (right) show a difference in size.

To confirm that the cyanine group enhances the release of EV compared to non-cyanine lipids, we measured RNL by Cy3 diester lipids and their corresponding aminated precursor lipids. The presence of the Cy3 group significantly enhanced the release, while free Cy5-COOH did not show any effect (Figure 4E). To test whether cyanine lipids trigger EV release

due to stimulation of exosome biogenesis, we used THP1 cells with CRISPR/Cas9 knockout of the *UNC13D* gene, a central regulator of exosome secretion but not microvesicle release.<sup>36</sup> Cells were treated with 100  $\mu$ M Cy3-C18 diester or Cy3-DSPE, and the released EV were purified and analyzed for CD63 and CD81 release with Western blotting (Figure 5).

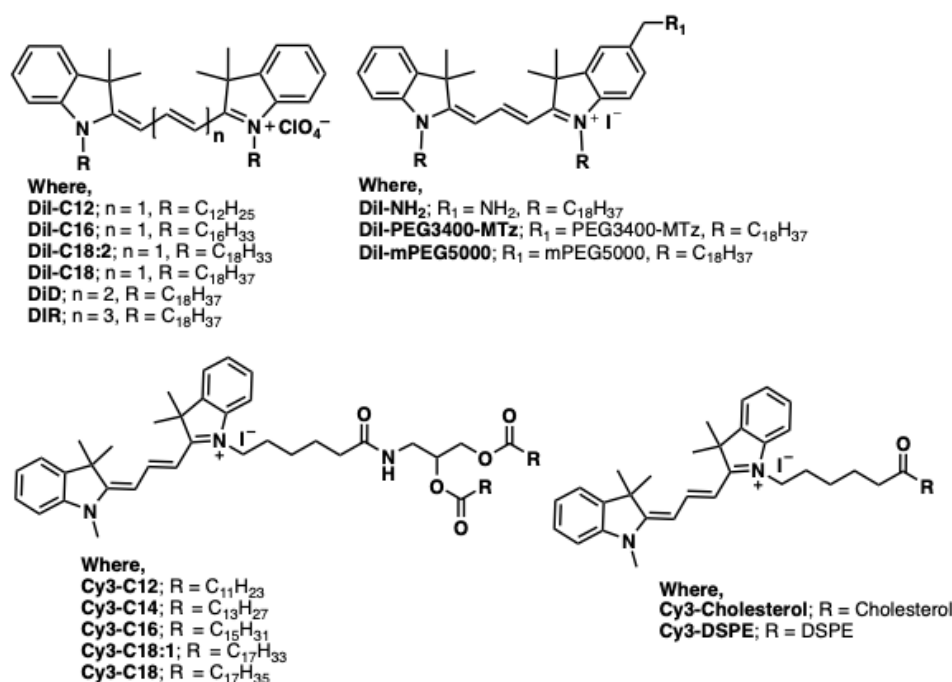


Figure 3. Library of cyanine lipids. Most lipids possess cyanine 3 headgroup, except indocarbocyanine lipids DiD and DiR.

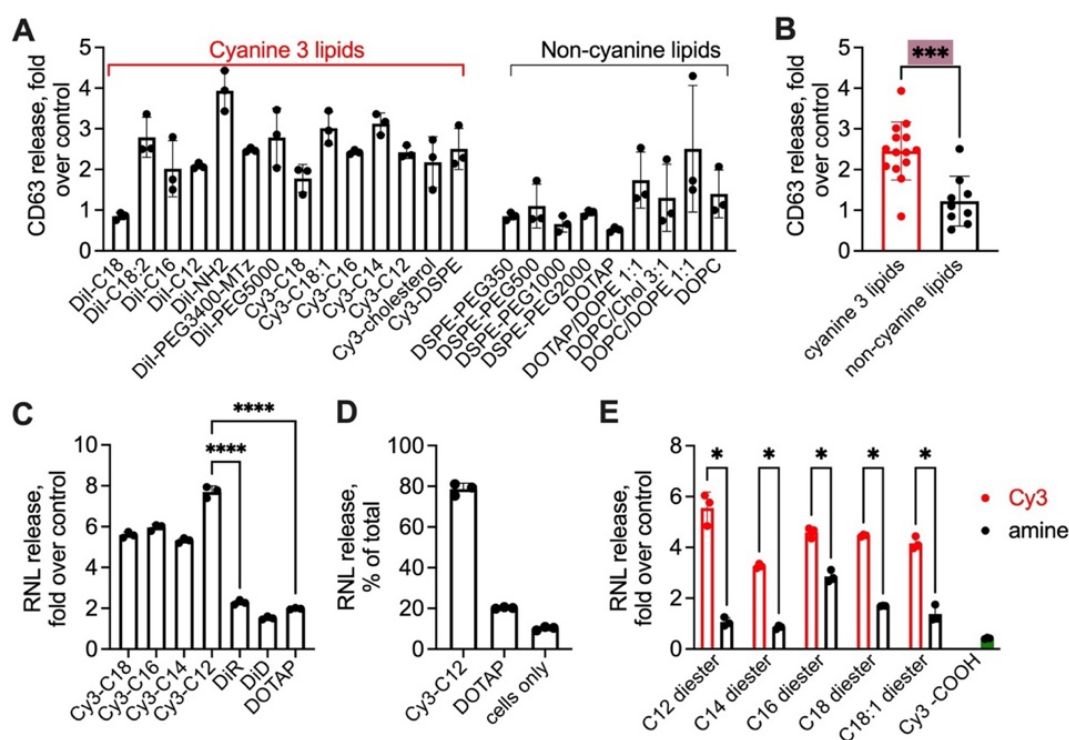
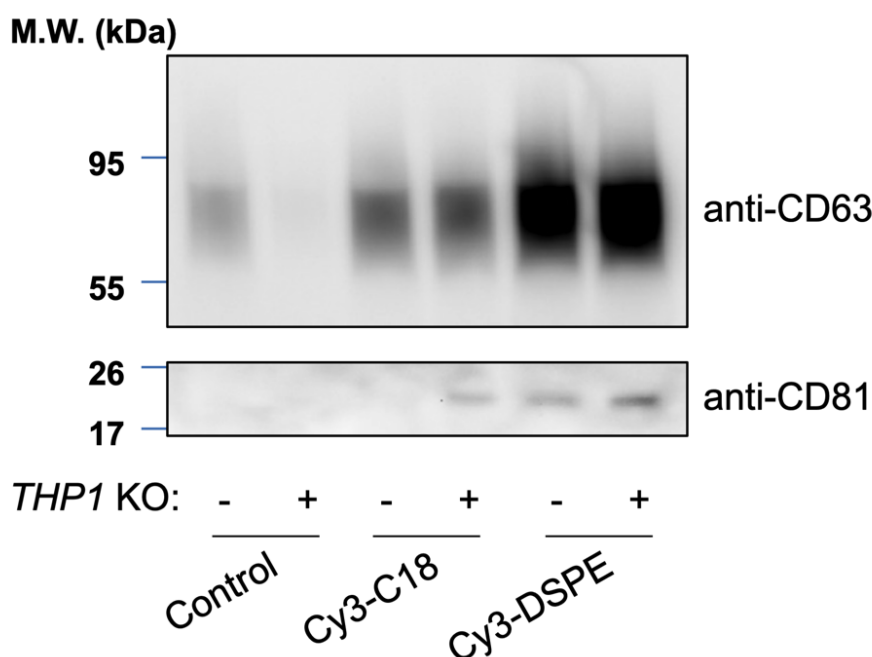


Figure 4. Cyanine headgroup-containing lipids are more efficient at EV release than non-cyanine lipids. A) Screening of cyanine 3 lipids described in Figure 3, as well as non-cyanine PEGylated phospholipids, neutral and cationic liposomes for CD63 shedding from THP-1 cells; B) Cyanine 3 lipids show significantly better release of CD63 than non-cyanine lipids (2-tailed  $t$ -test;  $p^{***} < 0.001$ ); C) In PalmReNL 4T1 cells, Cy3 lipids show better release than DOTAP, whereas DiR and DiD show similar release to cationic DOTAP liposomes; D) Percent release of RNase L vs the total cell content. Cy3-C12 diester releases almost 80% of the RNase L; E) Comparison of Cy3 diester lipids and parent amino lipids and free Cy3-COOH ( $n=3$ , 2-tailed  $t$ -test,  $p^{****} < 0.0001$ ;  $p^{***} < 0.001$ ).



*Figure 5. Effect of knockdown of exosome biogenesis. Wild-type and UNC13D knockout THP-1 cells were incubated with 100 $\mu$ M lipids for 1 hour in 1% BSA/PBS. EV were separated by differential centrifugation and ultracentrifugation and analyzed with Western blotting for CD63 and CD81. Cy3-C18 and Cy3-DSPE showed enhanced release of EV compared to vehicle-treated cells. There was inhibition of natural EV but no effect on lipid-triggered EV release by knockdown of the exosome biogenesis pathway.*

While UNC13D knockout prevented EV release from vehicle-treated cells, it did not prevent lipid-mediated shedding, suggesting that the primary mechanism involves a physical effect on the plasma membrane-derived vesicle production rather than on exosome biogenesis.

While exosomes and EV exhibit some tropism for tissues, adding a targeting functionality may improve the specificity. Interleukin-13 receptor alpha 2 is a well-established tumor-associated antigen of glioma and other brain tumor cells<sup>37-39</sup>. We previously developed an anti-IL13R $\alpha$ 2 antibody,<sup>24</sup> which binds to IL13R $\alpha$ 2 but not IL13R $\alpha$ 1. This antibody, or control mouse IgG, was conjugated to DiI via the PEG3400 linker

(DiI-PEG-anti-IL13R $\alpha$ 2, Figure 6A) using previously described Mtz-TCO copper-free click chemistry.<sup>35</sup> To incorporate the antibody in EV, we employed a 2 step strategy (Figure 6B). First, we painted THP-1 cells with the DiI-PEG-antibody conjugate, washed and then incubated them with 100  $\mu$ M DiR to induce EV release. The targeted EV were collected, washed, and incubated with IL13R $\alpha$ 2-overexpressing murine GL261 glioma cells. According to confocal imaging, DiI-PEG-anti-IL13R $\alpha$ 2 EV showed better binding and accumulation in the cells than control DiI-PEG-IgG EV (Figure 6C).



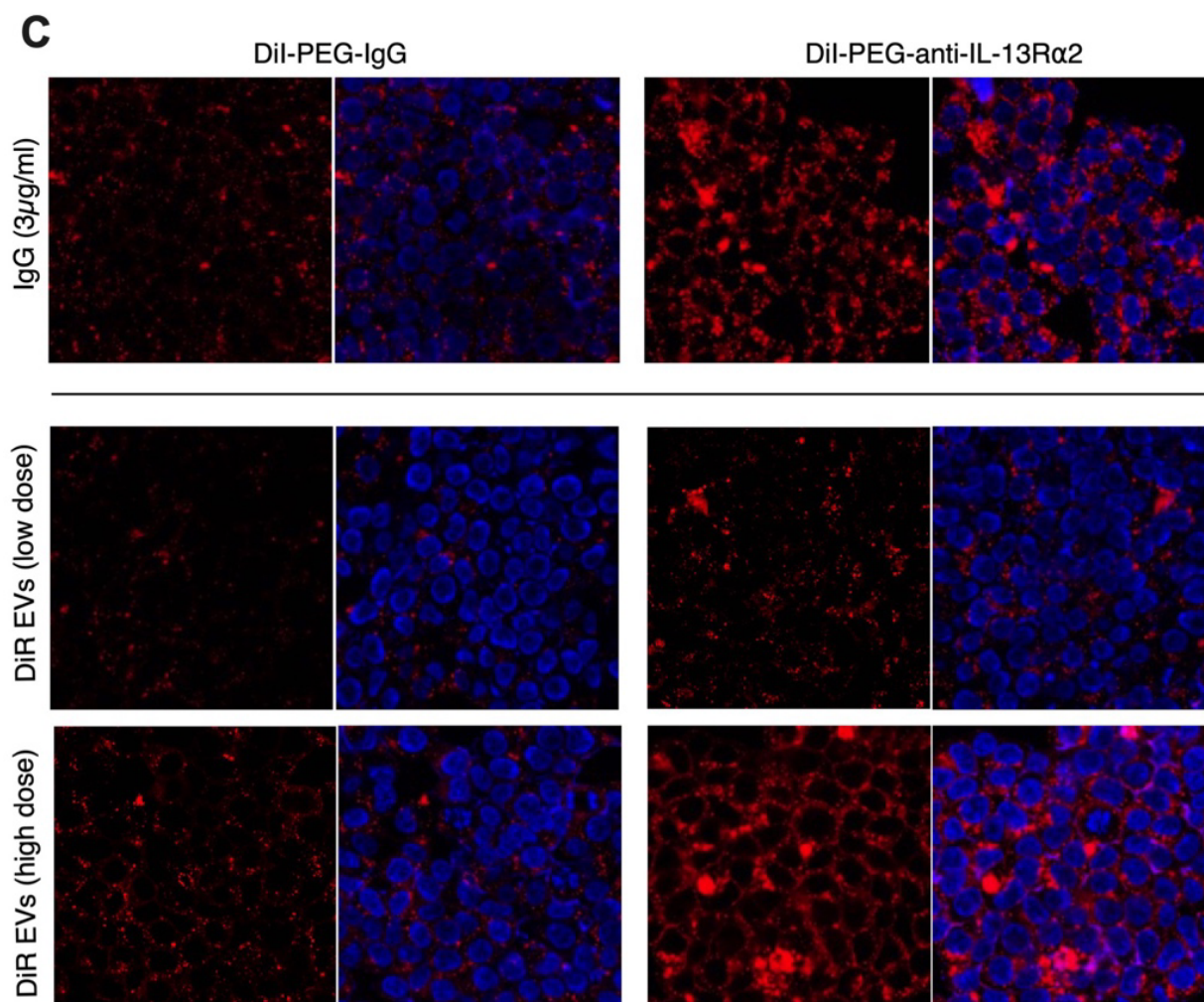
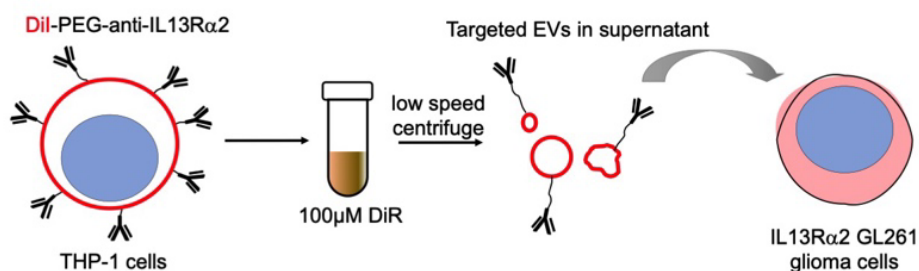
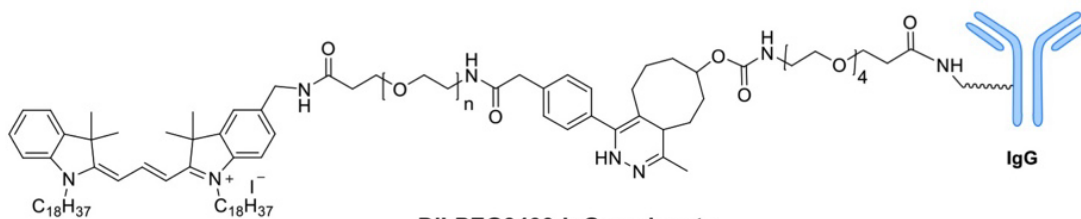


Figure 6. Targeting of EV to glioma cells. A) DiI-PEG3400-IgG conjugate; B) Strategy to produce and test IL13R $\alpha$ 2 targeted THP-1 EV in GL261 glioma cells expressing engineered IL13R $\alpha$ 2; C) Confocal imaging of DiI-PEG-antibody. A low dose corresponds to EV derived from 60,000 THP-1 cells; a high dose corresponds to EV derived from 300,000 THP-1 cells. There is a higher uptake of targeted DiI conjugates and targeted EV by GL261 cells at both concentrations.

## Conclusions

We demonstrate that cyanine lipids significantly enhance the shedding of tetraspanins and the release of EV from two different cell lines. The molecular mechanism is not entirely clear, but our hypothesis is the efficient incorporation of the cyanine group in the plasma membrane,<sup>26,27</sup> and inverted cone-shaped lipid geometry lead to membrane curvature strain and ejection of the plasma membrane in an exosome biogenesis-independent fashion. Intriguingly, this process leaves the membrane intact, and the cells remain viable. These resulting EV can be tailored further with antibodies to confer specific targeting capabilities. It is important to note that the presence of dye-derived particles has hindered the accurate characterization of EV, such as through electron microscopy.<sup>40</sup> Further research efforts are warranted to refine purification and characterization techniques. This includes a comprehensive examination of vesicle morphology, protein composition, and miRNA content. Since lipids can be made into prodrugs,<sup>41</sup> exploring the potential of incorporating lipid-drug conjugates into EV would be intriguing. Furthermore, the capacity of intracellular lipophilic dyes like DiD and DiR to promote EV release should be taken into account in biological applications involving cell labeling. Lastly, it is important to investigate the impact of EV on intratumoral transfer when utilizing lipid formulations for drug and gene delivery. Understanding these dynamics will be essential for advancing the field of targeted therapeutics and optimizing the use of lipids in biomedical applications.

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## Conflict of Interest

The author is an editorial board member of the journal and declares no other conflicts of interest. For a signed statement, please contact the journal office at [editor@precisionnanomedicine.com](mailto:editor@precisionnanomedicine.com).

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