A novel method for producing functionalized vesicles that efficiently deliver oligonucleotides *in vitro* in cancer cells and *in vivo in mice*[1,2]

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Abstract

Nano-based delivery systems have enhanced our ability to administer and target drugs and macromolecules to their targets. Oligonucleotide drugs have great therapeutic potential but often have off-target effects and stability issues. Therefore, they are often encapsulated in vesicles with targeting ligands such as antibodies (Ab) to deliver their cargo. Herein, we describe a novel, scalable and straightforward approach to produce functionalized vesicles called the “Functionalized Lipid Insertion Method.” This method differs from an older approach called the “Detergent-Dialysis Method.” The older method required excess detergent and extensive dialysis over many hours to produce the functionalized vesicles. With the “Functionalized Lipid Insertion Method,” only the functionalized lipid is detergent-solubilized during the engineering of the vesicle. The approach reduces the dialysis time, keeps the vesicles intact while orienting the targeting moieties of the functionalized lipid toward the outside of the vesicle. The dynamic light scattering (DLS) technique demonstrated that vesicle size is a lot more sensitive to the component mixture by the older method than our method. Increases of functionalized vesicle size by our method were consistent with the lipid inserted externally into the vesicle. *In vitro*, functionalized vesicles using our approach are able to efficiently deliver oligonucleotides and functionally affect liver cancer HepG2 cells. *In vivo*, functionalized vesicles achieve targeted delivery of oligonucleotides in mice without inducing a significant immune response through cytokine production or physical signs of the immune response such as inflammation. The industrial and therapeutic significance and implications of functionalized vesicles produced by our method are also discussed.

Keywords: Drug delivery, oligonucleotides, liposomes, extracellular vesicles, bioengineering

1 **Abbreviations:** Ab, antibody; C_t, cycle threshold; CMC, critical micelle concentration;
2 EMEM, Eagle's minimal essential medium (EMEM); EV, extracellular vesicle; FA, fatty
3 acid; FBS, Fetal Bovine Serum; HepG2, human liver cancer cell line; HCC, hepatocellular
4 carcinoma; LNP, liposomal nanoparticle; miR, microRNA; miRNA, microRNA; mEV,
5 modified extracellular vesicle; mLNP, modified liposomal nanoparticle; PBMCs,
6 peripheral blood nuclear cells; PBS, phosphate-buffered saline.

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Introduction

Nano-based delivery systems have been developed to deliver a wide range of molecules, including drugs, nucleotides, and proteins [1]. Several nano-based delivery systems are available, including liposomes, dendrimers, and carbon nanotubes [2]. To be effective *in vivo* carriers, they are designed to be biodegradable, biocompatible, and non-immunogenic [3–5]. Ideal nano-based delivery systems must overcome many challenges, including rapid clearance, instability, toxicity, and inefficient targeting [5–7].

Two major nano-based delivery systems recently employed are extracellular vesicles (EVs) and liposomal nanoparticles (LNPs) [e.g., 8,9]. EVs are naturally occurring vesicles with an average diameter around 200 nm excreted from various body fluids such as blood and urine [10]. Depending on their source, their sizes range from 20 nm to 10 μm in diameter [10]. In contrast, LNPs are artificially produced delivery systems and can be made into specific sizes through different techniques [e.g., 9,11]. EVs are formed by inward budding of the plasma membrane with other surface membrane invaginations from the Golgi apparatus [11]. These play an essential role in cell-to-cell communication and naturally carry RNA and proteins [11]. Naturally-occurring EVs are advantageous for drug delivery because of their inherent biocompatibility, long-circulating half-life, low toxicity, and tendency to be endocytosed into target tissues [12]. On the other hand, LNPs also have a lot of therapeutic potential because they can be designed with improved biocompatibility, stored lyophilized for relatively long periods, and produced on an industrial scale [13,14].

EVs and LNPs can be designed to have a particular function by engineering them with a fluorescent fatty acid (FA) for tracking, or a FA added with a targeting molecule

such as an antibody (Ab) or a receptor ligand. They can also be loaded with macromolecular or a small molecule as cargo. These modified EVs (mEVs) and modified LNPs (mLNPs) fit into a general category of vesicles referred to as functionalized vesicles. Functionalized vesicles have been engineered using relatively simple methods [15,16]. EVs have been covered with antibodies against the exosomal transmembrane protein CD9 and have improved delivery of miRNA to effector T-cells [15]. Since the early 1980s, an approach called the “Detergent-Dialysis Method” has been employed that uses detergent and dialysis to produce functionalized vesicles [17–24]. Complete solubilization of the vesicular components by this approach leads to complete disruption of the vesicular structure [17–24]. These functionalized vesicles reassemble randomly by extensive and time-consuming dialysis to remove detergent [17–24]. Recently, a two-stage process for producing mEVs was developed where the EVs were PEGylated and covalently linked with antibodies [16]. The three-day process has a lot of potential for industrial scale-up [16,25,26]. Unfortunately, the mEVs undergo large temperature fluctuations between 4°C and 40°C, which may destabilize constitutive proteins of the mEVs and the attached antibodies (Abs) of the vesicles [16,25,26].

Functionalized vesicles can be loaded with oligonucleotide drugs. Oligonucleotide drugs can be from natural sources or manufactured synthetically [27,28]. They have shown a lot of potential for treating diseases, and several have been approved by the FDA [28]. Oligonucleotide drugs can be easily modified and produced, which is a major advantage over traditional small molecule drugs [29]. Most oligonucleotide drugs have sequences that complement their DNA or RNA targets [28]. Oligonucleotide drugs include small interfering RNAs (siRNA) [30,31], antisense oligonucleotides (ASOs) [32,33] and

microRNAs (miRNAs) [34]. Unfortunately, oligonucleotide drugs suffer from immunogenicity, enzymatic degradation, and off-target effects [27].

SiRNA is generally recognized as a short 21 to 23 base pair double-stranded RNA (dsRNA) molecule with two 3' nucleotide overhangs [30]. They play a vital role in RNA interference (RNAi), which is involved in cellular growth and differentiation [35]. They can also target several disease-causing genes [30,36]. Therefore, siRNA drugs represent viable drug candidates and are in different stages of drug development [30,36]. Like other oligonucleotide drugs, siRNAs are susceptible to enzymatic degradation, off-target gene silencing, and potential activation of the immune response [31]. To overcome these challenges, these nucleotide polymers have been chemically modified and delivered through nanoparticle delivery vehicles such as liposomes [31].

ASOs are short 15-25 base pair polynucleotides that can be designed to bind to disease-related mRNA targets [32,33]. Mechanistically, they can attach to a target mRNA molecule and induce mRNA degradation [33]. Chemically modified ASOs can inhibit mRNA degradation by causing steric hindrance with their mRNA targets [33]. They ultimately affect mRNA translation and subsequent protein expression of the target mRNA molecule [33]. However, ASOs suffer from similar challenges as other oligonucleotide drugs, so they have been delivered by various means, including liposomes, cationic amphiphiles, and dendrimers [33,37].

Finally, a small polynucleotide called microRNA (miRNA) represents another potential therapeutic delivered with functionalized vesicles [34]. These macromolecules are small non-coding polynucleotides that range in length between 17 and 25 nucleotides [38]. They play an essential role in physiology by modulating gene expression through

binding to mRNA [38]. MiRNAs have been associated with many diseases, including several cardiovascular disorders, cancer, and allergic responses [39]. They can also elicit anticancer drug resistance in cancerous tumors [40]. MiRNAs have served as biomarkers for various diseases, and they can modulate gene expression [34,39]. These miRNA-related therapeutics can be synthetic mimics of naturally occurring miRNAs or miRNA inhibitors called anti-miRs [34]. These polymers suffer from similar problems as other oligonucleotide drugs [34]. To compensate, both miRNA mimics and anti-miRs have been chemically modified to reduce degradation and improve their therapeutic efficacy [34]. They have also been encapsulated in liposomes, dendrimers, and polymers [34]. Currently, several miRNA therapeutics are going through different stages of drug development to treat cancer and liver diseases [34]. Therefore, targeted delivery of miRNA represents an excellent opportunity to test the effectiveness of our approach to produce functionalized vesicles.

In this work, a straightforward, efficient, and non-disruptive approach for producing functionalized vesicles called the “Functionalized Lipid Insertion Method” is presented and compared to the “Dialysis-Detergent Method” [17–24]. The functionalized vesicles can be produced much more efficiently than [16] and the older method [17–24]. Our innovative approach maintains the temperature where vesicles, proteins, and Abs are stable [25,26,41]. Functionalized vesicles can be produced with any targeting ligand or protein, such as receptor ligands, antibodies, and affibodies. These vesicles will give them the potential ability to target any cell, tissue, or organ. The *in vitro* targeting and functional effects of mEVs and mLNPs by our procedure are demonstrated with liver cancer HepG2 cells. In mice, mEVs and mLNPs were designed with different Abs to target specific

organs. The potential commercial advantages of targeted therapeutic delivery using our approach are discussed.

Materials and Methods

Materials

The conjugating fatty acid (FA) label, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-maleimide), and fluorescent 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-DSPE) were purchased from Avanti Polar lipids (Alabaster, AL). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), *n*-dodecyl-β-*D*-maltoside (DDM) detergent, and Histopaque 1077 Reagent were purchased from Sigma Aldrich (St. Louis, MO). For the construction of liposomal nanoparticles (LNPs), *Escherichia (E.) coli* polar lipid extract was ordered from Avanti Polar lipids (Alabaster, AL), and chloroform was acquired from Sigma Aldrich (St. Louis, MO). Asialoglycoprotein receptor 1 (ASGPR1)/HL-1 polyclonal antibody ($ASGR1_{PAB}$) (ab49355) and recombinant ACE2 monoclonal antibody ($ACE2_{MAB}$) (ab108252) were purchased from Abcam (Cambridge, MA). NPHS2 (podocin) polyclonal antibody ($NPHS2_{PAB}$) (MBS3013144) was acquired from MyBioSource (San Diego, CA). GFP monoclonal antibody (GF28R) (GFP_{MAB}) (MA5-15256) was obtained from Thermo Fisher (Waltham, MA).

MiRNA mimics (mmu-miR-298-5p (0.38 mg/ml), hsa-miR-26a-5p (0.28 mg/ml)), and TaqMan™ MicroRNA assay were ordered from Thermo Fisher Scientific (Waltham, MA). Six ml Becton, Dickinson, and Company (BD) (Franklin Lakes, NJ) hematological tubes spray-coated with 1.8 mg/ml of dipotassium ethylene diamine tetraacetic acid (EDTA), and the blood separation agent Histopaque® 1077 Reagent, which is a solution of polysucrose and sodium diatrizoate (1.077 g/mL), was obtained from Sigma-Aldrich (St. Louis, MO). DharmaFECT™ 4 transfection reagent was bought from Horizon

Discovery (Cambridge, UK). The LegendPlex™ 8-panel Th1/Th2 Bio-plex cytokine assay kit was obtained from BioLegend (San Diego, CA).

Cell isolation and culture

Human peripheral blood mononuclear cell (PBMC) donors were enrolled for blood collection in compliance with the World Medical Association's Declaration of Helsinki and the Human Research Protection Program and Institutional Review Board guidelines for human subject research at the University of Georgia. Enrolled healthy volunteers signed consent forms to inform them about the study. The human blood protocol (University of Georgia Institutional Review Board (IRB) no STUDY00006632) and the consent form were reviewed and approved by the IRB of the University of Georgia.

The human liver cancer HepG2 cells were purchased from American type culture collection (ATCC, Maryland, MD). These were grown and maintained in Eagle's minimum essential medium (EMEM) (Corning), which was supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) and 5% penicillin/streptomycin (Thermo Fisher, Waltham, MA). These cells were incubated in a humidified atmosphere of 5% CO_2 at 37ºC in Thermo Fisher Scientific Napco Series 8000 WJ CO_2 incubator (Waltham, MA).

Isolation of extracellular vesicles (EVs)

Approximately 10 ml of human blood were put into EDTA-coated BD hematological tubes to declot them. The peripheral blood nuclear cells (PBMCs) were isolated as described with some modifications [42,43]. Five ml of EDTA-treated blood samples were layered onto an equal amount of Histopaque® 1077 Reagent in a 15 ml conical tube. The tube was centrifuged at approximately 400 g (1478 rpm) for 30 minutes

and 4°C in an Eppendorf 5810R centrifuge (Hamburg, Germany), which was separated the blood into plasma PBMCs, and erythrocyte layers. The top plasma layer was removed and discarded. The turbid middle layer of PBMCs was removed and put into a clean 15 ml conical tube. The tube was centrifuged at approximately 450 g (1917 rpm) for 10 minutes at 4°C. The supernatant was carefully removed using a transfer pipette to avoid disrupting the PBMC pellet. The pellet was then washed with 5 ml of an isotonic phosphate-buffered saline (PBS) (137 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) solution and centrifuged twice at 300 g (1278 rpm). This pellet was suspended in RPMI growth media without glutamine and phenol red (Corning, NY, USA) and added to a 1M HEPES buffer solution (pH 7.4) to a final concentration of 25 mM HEPES to provide additional buffering capacity for the media. Cells were then transferred to a sterile Cellstar T-75 culture flask with a red filter screw cap (Greiner Bio-One, Monroe, NC) containing RPMI 1640 (with glutamine and phenol red) supplemented with 10% w/v fetal bovine serum (FBS) with 100 U/ml penicillin and 100 μg/ml streptomycin. This solution was incubated for about ~12 hours (overnight) in a humidified atmosphere at 37°C and 5% CO_2 in Thermo Fisher Scientific Napco Series 8000 WJ CO_2 incubator (Waltham, MA). Afterward, the media was transferred with a sterilized transfer pipette into 2 ml microcentrifuge tubes. The microcentrifuge tubes were centrifuged at 10,000 g (~14,000 rpm) for 5 min. on a tabletop centrifuge at room temperature. About 1 ml of supernatant from each microcentrifuge tube was transferred to a new 2 ml microcentrifuge tube. Four hundred microliters of precipitation buffer B from the Qiagen (Formerly, Exiqon) miRCURY Exosome Isolation Kit (Qiagen, Germantown, MD) was added to the supernatant in each tube. The microcentrifuge tubes were inverted and vortexed and

allowed to incubate for ~12 hours (overnight) at 4ºC. The longer incubation time improved the EVs yield from the PBMCs. The remaining steps follow the manufacturer's instructions for the Qiagen miRCURY Exosome Isolation Kit (Qiagen, Germantown, MD). The EVs were resuspended in 300 µl of the resuspension buffer supplied by the kit, were combined in one tube, and were stored at -80ºC until needed. The total protein concentration of the EVs was measured using the Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) to estimate the concentration and the yield, which was typically around ~10µg.

Preparation of liposomal nanoparticles (LNPs)

Unilamellar liposomal nanoparticles (LNPs) were prepared using the filter extrusion method [44]. The LNPs had 80% w/v *E. coli* Avanti polar lipids and 20% w/v cholesterol as described previously [45]. Briefly, lipids and cholesterol were mixed in 10 ml of chloroform to get a final concentration of 10 mg/ml. This solution was evaporated to dryness in a Rotavapor Model R-114 (Buchi). After evaporation, the film was reconstituted in 0.1 mM EGTA and 50 mM Tris/HCl. This suspension was freeze-thawed at least ten times using liquid nitrogen and extruded 11 times through a LIPEX extruder (Northern Lipids, Burnaby, B.C., Canada) with a 400 nm cutoff Millipore filter (Millipore Sigma, Burlington, MA).

Functionalized vesicles produced by the "Functionalized Lipid Insertion Method"

A 200 µl fatty acid (FA) solution was made with 100 µM fluorescent NBD-DSPE and 100 µM DSPE-PEG2000-maleimide, and 0.1% w/v DDM detergent (10X the critical micelle concentration (CMC)) in an isotonic PBS (pH 7.4) buffer. The NBD-DSPE fluorescence was monitored at 550 nm by exciting at 445-460 nm using a SpectraMax

M2 Plate Reader (Molecular Devices, Sunnyvale, CA) to monitor EVs and LNPs assembly. The FA solution was dialyzed using 0.5 mL Slide-A-Lyzer MINI Dialysis units with a 10 KD cut-off filter (Thermo Fisher Scientific, Waltham, MA) against 2 L isotonic PBS for 2 h at 4°C to remove excess DDM detergent. Almost identical fluorescence for the NBD-DSPE FA was measured after dialysis showing that the lipids remained in solution after dialysis (data not shown). To ensure that all DSPE-PEG2000 maleimide were conjugated, two-fold excess of the targeting antibody (~200 μM) was added to the dialyzed FA solution and incubated at room temperature for 1 hour. A 100 μl of purified 10 mg/ml EVs or LNPs was added to this 200 μl of the NBD-DSPE, and DSPE-PEG2000-Antibody solution was briefly centrifuged to remove any large aggregates. The molar ratio of vesicle lipid to NBD-DPSE and DSPE-PEG2000-Ab was approximately 75:1:1. This 300 μl solution was incubated for 1 hour at room temperature to allow slow mixing and prevent any potential disruption of the vesicles. After incubation, the supernatant was dialyzed in a 0.5 mL 10 KD cut-off Slide-A-Lyzer MINI Dialysis unit against 2 L of isotonic PBS buffer for two hours at 4°C. Dialysis slowly removes the DDM detergent that is surrounding the FAs. The dialysis process exposes hydrophobic surfaces of the derivatized or functionalized FA and entropically drives the FA tail into the vesicle bilayer to minimize exposure to water to form the functionalized vesicle. The inserted lipids, particularly the DSPE-PEG2000-Ab, remain embedded on the outer leaflet of the vesicle bilayer because a large thermodynamic barrier prevents them from flip-flopping to the inner leaflet of the bilayer [46]. A similar procedure has been used for directionally inserting a membrane protein transporter into a liposomal bilayer [52,53]. The orientation was confirmed enzymatically and through atomic force microscopy (AFM) [52,53]. Afterward, the mEV solution was

incubated with 100 µl of precipitation buffer B from the Qiagen miRCURY Exosome Isolation Kit (Qiagen, Germantown, MD) for ~12 hours (overnight) at 4 °C (Final volume = ~250 µl). The mLNPs solution was then centrifuged at 14,000 rpm for 30 minutes to produce a pellet. To pellet the mEVs, the solution was centrifuged at 104,000 g (30,472 rpm) in a Beckman TLA 110 rotor for one hour at 20ºC in a Beckman TLX ultracentrifuge. The supernatant was carefully removed, and the functionalized vesicle pellet was resuspended in 100 µl of isotonic PBS, which will be called the functionalized vesicle solution. The purpose of centrifuging the functionalized vesicles and removing the supernatant is to remove any remaining antibodies that were not cross-linked to the DPSE-PEG2000-Maleimide FA. The concentration of mEVs was measured by protein quantification using Pierce Bicinchoninic Acid (BCA) assay. The concentration of mLNPs was determined by tracking the amount of the lipid that was used throughout the experiments.

Functionalized vesicles produced by the "Detergent-Dialysis Method."

The production of modified vesicles by the "Detergent-Dialysis Method" was done as previously described [17–24]. All the components, the lipids, the proteins if present, and the derivatized lipids, were dissolved in detergent several times higher than the detergent's CMC [17–24]. These solutions were then extensively dialyzed over many hours to remove the detergent [17–24]. The dialysis-driven detergent removal process causes the lipids, the proteins, and the derivatized lipids to randomly coalesce into modified vesicles of indeterminate sizes [17–24]. Differences between this method and the "Functionalized Lipid Insertion Method" are described in the text and Fig. 1.

Lipids functionalized with antibodies (i.e., FA-PEG2000-Antibody) were made as described above. About a milligram of the following mixtures was dissolved in 0.03% DDM by a similar procedure as in [17]: 1) *E. coli* Avanti polar lipids, 2) *E. coli* Avanti lipids, NBD-DSPE, and DSPE-PEG2000-Antibody with molar ratios of 75:1:1, 3) PBMC-derived EVs and 4) PBMC-derived EVs, NBD-DSPE, and DSPE-PEG2000-Antibody with molar ratios of 75:1:1. These detergent-solubilized solutions in 200 μl microcentrifuge tubes were sonicated in a Kendal ultrasonic cleaner HB23 (Kendal) at 25°C until the solutions clarified, indicating complete dissolution. The mixtures were then extensively dialyzed as described in [17] by putting 50 μl of them into 0.2 mL Slide-A-Lyzer MINI Dialysis units with a 10 KD cut-off filter (Thermo Fisher Scientific, Waltham, MA) against 2 L isotonic PBS for at least 45 hours at room temperature.

Characterizing functionalized vesicles

Dynamic light scattering (DLS) was used to characterize the vesicle sizes by the "Detergent-Dialysis Method" and the "Functionalized Lipid Insertion Method" (Fig. 3), which is well established for this purpose [47–51]. Before the DLS experiments, all the samples were centrifuged at ~8000 g (5,000 rpm) for 30 min. using a Microfuge™ 22R (Beckman Coulter, Brea, CA) at 4°C. The purpose of the centrifugation was to remove large particulates such as dust from the samples, which can interfere with the DLS measurement. Although not visible by the naked eye, all centrifuged samples were assumed to have a pellet, so only the aqueous portion on the top was carefully removed before DLS analysis. The DLS experiments were performed on a Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK) using a Malvern 45 μl ultra-micro cuvette (ZEN2112). The DLS experiments were analyzed using the Zetasizer Software Version

8 (Malvern Panalytical, Worcestershire, United Kingdom), assuming a refractive index of 1.330 and a viscosity of 0.8872, which are parameters typically used for lipid-containing vesicles [52]. The size distribution curves in this manuscript were rendered on Igor Pro 6.3 (WaveMetrics, Portland, OR). Protein concentrations of the EVs were determined using the Pierce Bis-cinchonic assay kit (BCA), and LNPs were determined by tracking the lipid concentration throughout the experiments.

Loading functionalized vesicles with miRNA

Electroporation was used to load miRNA in the vesicles, as it is an efficient method to load oligonucleotides into vesicles [48,53]. Equal amounts of mEVs or mLNPs were added with an equal amount of miRNA mimics (Thermo Fisher, Waltham, MA) in SFM to a total volume of 400 μl. The solution was put into 0.4 cm gap Bio-Rad (Hercules, CA) electroporation cuvettes. The miRNA in the *in-vitro* experiments with the HepG2 cells varied between 1 and 4 μg, while the miRNA varied between 80 and 110 μg for the *in-vivo* mouse experiments. Unmodified and modified vesicles were electroporated at 150 V and 10-15 ms with an exponential wave pulse in a Bio-Rad Gene Pulser X-Cell electroporator (Hercules, CA). Afterward, the samples were incubated at room temperature and at 4°C for 30 minutes to allow the vesicles to recover.

miRNA delivery to HepG2 cells by functionalized vesicles

The HepG2 cells were treated in various ways to determine their relative uptake of miRNA. The cells were counted using a hemocytometer on a Zeiss Invertoskop 40 C inverted microscope (Zeiss, Oberkochen, Germany). The cells were plated to approximately 200,000 cells per well in a 6-well VWR tissue culture-treated plates (VWR, Suwanee, GA) and incubated ~12 hours (overnight). Afterward, the media was

replenished with 2.7 mL of fresh 10% Eagle's minimal essential medium (EMEM) media. The cells were FBS-starved for ~12 hours before treatment to make proliferating HepG2 cells behave homogenously [54]. For experiments with unmodified and modified vesicles, 400 μl of them were electroporated with ~4 μg miRNA and then they were added wells in the cell-containing 6-well plates. For the DharmaFECT™ 4 transfection reagent experiments, the reagent was added with ~4 μg miRNA in a 400 μl solution, and they were added to other cell-containing wells. The final volume for all the wells after adding the solutions was approximately 3 ml. Before RNA extraction, the treated plates were incubated for 72 hours under humidifying conditions at 37ºC with 5% CO_2 in a Thermo Fisher Scientific Napco Series 8000 WJ CO_2 incubator (Thermo Fisher, Waltham, MA).

RNA extraction and quantitative RT-PCR (qRT-PCR)

TRIzol Reagent (Invitrogen, Carlsbad, CA) was used to isolate intracellular RNA as per the manufacturer's protocol. All the isolated RNA was stored at -80ºC. Complementary DNA (cDNA) was prepared from intracellular RNA (i.e., microRNA and the housekeeping U6 small nuclear (snRNA)) using the TaqMan™ microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The cDNA was analyzed on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) in a 384-well microplate with the TaqMan™ Universal PCR MasterMix (Thermo Fisher Scientific, Waltham, MA). For measurements to determine miRNA transfer efficiency, TaqMan™ MicroRNA assay (Thermo Fisher Scientific, Waltham, MA) kits with specific fluorescent cDNA primers for the miRNA and fluorescent cDNA primers for the U6 snRNA reference. The DNA was quantitated on the qRT-PCR instrument using the threshold cycle (C_T) method with the U6 snRNA expression as a reference to calculate

$\Delta\Delta C_T$ values, which correlates to the relative miRNA uptake [55–58]. These values were exported into Microsoft Excel format to calculate fold-difference and analyzed in GraphPad Prism 7 (GraphPad, San Diego, CA). The $\Delta\Delta C_T$ values were normalized against a condition of interest, or U6 snRNA indicates the relative amount of miRNA delivered to the cells.

***In-vitro* time course for miRNA uptake**

Six-well VWR culture-treated plates (Suwanee, GA) were plated with HepG2 cells to a density of 100,000 cells/well. The HepG2 cells were counted using a hemocytometer on a Zeiss Invertoskop 40 C inverted microscope (Zeiss, Oberkochen, Germany). After the cells adhered, the cells were serum-starved for one day. The next day, the cells were treated with functionalized vesicles containing 4 μg of mmu-miR-298-5p. Before extracting RNA, the cells were incubated from 12 to 72 hrs in a humid atmosphere at 37°C with 5% CO_2 in a Thermo Fisher Scientific Napco Series 8000 WJ CO2 incubator (Waltham, MA). Then, the RNA was extracted and analyzed by qRT-PCR using the procedures described above.

MiRNA effect of HepG2 cell wound healing delivered by the functionalized vesicles

For quantifying the functional effect of miRNA delivered by mEVs and mLNPs, the functionalized vesicles were loaded with a tumor suppressor miRNA, miR-26a-5p [47,48]. The HepG2 cells were plated in a 24-well plate at a cell density of 300,000 cells/well. A wound was created in each well using a 200 μl pipette tip followed by gentle washing with PBS buffer. The cells were then treated with mEVs and mLNPs containing miR-26a-5p at a dosage of 0.35 mg per well (50 ml/well) in 500 ml of fresh 10% EMEM media and incubated for 72 hours. The controls for this experiment were untreated HepG2

cells (abbreviated Cells), cells treated with empty EVs (abbreviated Cells + EVs()), and cells treated with empty mEVs with the $ASGR1_{PAB}$ (abbreviated mEVs($ASGR1_{PAB}$)) under similar conditions. The cells were imaged from 0 to 72 hours on an Olympus IX71 inverted Microscope with a TH4-100 power source (Tokyo, Japan). The images were analyzed using the Fiji image processing software and the MRI wound healing tool in ImageJ Software (National Institutes of Health, Rockville, MD).

Mouse Studies

Animal welfare at the University of Georgia is covered by the NIH Animal Welfare Assurance #: D16-00276/A3437-01. Our animal studies were performed under two animal use protocols (AUPs): A2018 03-025-R2 and A2020 03-014-A3. These studies were carried out at the animal facilities at the David Life Sciences Building and the Paul D. Coverdell Center at the University of Georgia in Athens, GA. We greatly appreciate the generosity of Drs. Mandi M. Murph and Yao Yao for providing some mice used in the studies and sharing their spaces for our animal studies.

Eighteen Nu/Nu male nude mice, 48 Nu/Nu female nude mice for targeting studies, and 18 female C57/BL6 mice for immune reactivity studies were ordered from Jackson labs (Maine). All mice were intraperitoneally (IP) injected with a 25-gauge needle and 200 μl or 400 μl of solutions containing SFM or functionalized vesicles in SFM. Unless otherwise specified, each of the functionalized vesicles had 114 μg of mmu-miR-298 microRNA. All the mice were anesthetized using isoflurane (Millipore Sigma, Burlington, MA).

As a negative control, mEVs and mLNPs were bioengineered with the GFP_{MAB} since they are not known to interact with any mouse proteins (Fig. 7). The functionalized

vesicles are abbreviated mEV(GFP_{MAB}) and mLNP(GFP_{MAB}) to denote their Ab modifications. In the experiment, 12 male Nu/Nu nude mice aged 5-7 weeks were divided into three groups (n=3). One group of mice were IP-administered 200 µl of SFM. The next two groups were IP-administered 200 µl mEVs($GF28R_{MAB}$) and mLNPs($GF28R_{MAB}$) electroporated with miRNA (i.e., mmu-miR-298). These functionalized vesicles are abbreviated mEV($GF28R_{MAB}$, mmu-miR-298) and mLNP($GF28R_{MAB}$, mmu-miR-298) to denote their Ab modifications and their miRNA cargo.

The targeting ability against the endocytotic liver ASGR1 receptor was tested with mLNPs and mEVs bioengineered with the $ASGR1_{PAB}$ (Fig. 8). For experiments involving mEVs($ASGR1_{PAB}$), six Nu/Nu male mice aged 15-17 weeks were randomly divided into two groups (n=3). For the experiments involving mLNPs($ASGR1_{PAB}$), 18 Nu/Nu female mice aged 5-8 weeks were divided into three groups (n=6). The mice then received IP injections with a total volume of 400 µl per mouse. The injections contained SFM only for the untreated mice and mEVs($ASGR1_{PAB}$, mmu-miR-298) or mLNPs($ASGR1_{PAB}$, mmu-miR-298) for treated mice.

Functionalized vesicles, mLNPs, and mEVs were bioengineered with the NPHS2 polyclonal antibody abbreviated mLNP($NPHS2_{PAB}$) and mEV($NPHS2_{PAB}$), which targets the kidney-related NPHS2 integral membrane protein (Fig. 9). Six Nu/Nu female mice, aged 5-8 weeks, were divided into two groups (n=3). These mice were IP administered 400 µl containing SFM only, mEVs($NPHS2_{PAB}$, mmu-miR-298) or mLNPs($NPHS2_{PAB}$, mmu-miR-298).

Functionalized vesicles, mEVs, and mLNPs were bioengineered with the Angiotensin-converting enzyme 2 monoclonal antibodies ($ACE2_{MAB}$) against the

membrane-associated ACE2 enzyme, which is part of the renin-angiotensin system that controls blood pressure (Fig. 10) [59]. This antibody was chosen to test the immunoreactivity of functionalized vesicles made by our method because ACE2 is expressed in many tissues ensuring direct interaction with them by these vesicles [60]. Eighteen female C57/BL6 mice, aged 5-8 weeks, were divided into three groups (n=6). These groups received 400 μl IP injections of SFM only, mEVs($ACE2_{MAB}$, mmu-miR-298), mLNPs($ACE2_{MAB}$, mmu-miR-298).

In-vivo **Quantification of miRNA**

After 72 hours, the mice were euthanized using carbon dioxide followed by a necropsy as described [61]. About 100 mg of tissue were obtained to assess the amount of miRNA delivered in the tissue. The sections were suspended in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in a 1.5 ml microcentrifuge tube. The tissues were homogenized with a 1000 µl digital pipette and a Bel-Art™ Pro Culture Cordless Homogenizer Unit (Thermo Fisher, Waltham, MA). The samples were then centrifuged at 12,000 g for five minutes at 4ºC to remove tissue debris with a Microfuge™ 22R centrifuge (Beckman Coulter, Brea, CA). The RNA was extracted using the Invitrogen PureLink™ RNA Mini Kit (Thermo Fisher, Waltham, MA) [62]. After RNA extraction, the RNA concentration was quantified using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The RNA concentration was determined using the extinction coefficient for single-stranded RNA in the spectrophotometer of 0.025 $(\mu g/ml)^{-1}$ cm^{-1}, and the RNA purity (>99%) was estimated by the 260 nm/280 nm ratio. The miRNA and U6 snRNA were amplified for quantification using the TaqMan™

microRNA reverse transcription and the TaqMan™ Universal PCR MasterMix (Thermo Fisher Scientific, Waltham, MA) as explained above.

Cytokine assay to probe immunogenicity

The LegendPlex™ 8-panel Th1/Th2 Bio-plex cytokine assay kit was used to quantitatively determine the immunogenic response of mice after 72 hours of functionalized vesicle treatment [63]. About a milliliter of blood was withdrawn immediately after euthanizing the animals in BD Microtainer® tubes containing serum separator (SST™) (Becton, Dickinson and Company, Franklin Lakes, NJ). The blood was allowed to clot at room temperature for 30 minutes. The tubes were then centrifuged at 1000 g for 15 minutes at 4°C using a Microfuge™ 22R (Beckman Coulter, Brea, CA), and the serum was then stored at -80°C. Before performing the assay, the serum was centrifuge at 10,000 g for 10 minutes at 4°C using a Microfuge™ 22R. The kit has immunoassays coupled to magnetic beads for detecting the following eight inflammatory factors, including the granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon γ (IFN-g), tumor necrosis factor α (TNF-a), and several interleukins (IL), IL-2, IL-4, IL-5, IL-10, IL-12 (p70). The plate was then read using a Luminex Magpix system (Luminex, Austin, TX).

Statistics

One-way analysis of variance (ANOVA) tests was used to determine statistical significance between groups comparing relative miRNA expression. A confidence interval of 95% with all p-values less than 0.05 was considered significant (*). Student's T-Test was also used to compare two groups, with a 95% confidence interval. Data were analyzed with Microsoft Excel (Microsoft, Redmond, WA) and GraphPad Prism 7 (GraphPad, San Diego, CA).

Results

Methods for producing functionalized vesicles

This work describes a novel method for producing functionalized vesicles involving detergent and dialysis that we called the “Functionalized Lipid Insertion Method.” This method is distinct from the “Detergent-Dialysis Method” used for decades and described in the literature since the early 1980s [17–24].

The two approaches are shown schematically in Fig. 1. In the “Detergent-Dialysis Method” (Figs. 1A-B), all functionalized vesicle components are solubilized at a relatively high detergent concentration (Step 1) [e.g., 17,24]. The detergent-solubilized mixture must be extensively dialyzed over many hours or days (Step 2). As dialysis removes the detergent, the vesicles randomly form functionalized vesicles from the solubilized components. The composition of the original components will influence the functionalized vesicle size. During the process, the functionalized lipids in the mixture will randomly orient toward the inside and outside the vesicle (red arrows near Step 3). However, even the long dialysis period is not enough to remove all the unintegrated components. Therefore, column chromatography is often needed in addition to dialysis to remove contaminants that interfere with the functionalized vesicle (Step 3) [e.g., 17].

Fig. 1B shows the production of an mEV by the “Detergent-Dialysis Method.” A natural vesicle such as an EV must be broken up into its components to be detergent-solubilized (Step 1) [19]. This process will disrupt the vesicular structure and possibly its natural functions. Next, the natural vesicle’s components, including lipids and proteins, and the functionalized lipid are dissolved at a high detergent concentration (Step 2). This detergent solubilization stage is followed by a long period of dialysis (Step 3). Because

the detergent concentration is so high, column chromatography is often used to remove any remaining contaminating detergent (Step 4). Because of the detergent solubilization, the original EV because its natural components randomly reassemble to form it. Both integral membrane proteins (green arrows) and functionalized lipids (red arrows) assume random orientations within the mEV. Some of the outward-facing proteins are now inward-facing proteins by this approach (green arrows). Some functionalized lipids face inside the vesicle cavity, where they cannot perform their intended function (red arrows).

A graphical representation of the "Functionalized Lipid Insertion Method" discussed in this work is shown in Figs. 1C and 1D. The only component that is detergent-solubilized by this approach is the functionalized lipid. In the diagram, the reactive lipid (i.e., DPSE-PEG2000-Maliemide) is solubilized by detergent and dialyzed for two hours to remove excess detergent (Step 1). The purpose of eliminating the excess detergent is to prevent it from dissolving the target preformed vesicle. Then the detergent-solubilized reactive lipid is incubated for 1 hour with an excess of a targeting component like an antibody (Ab) to allow cross-linking between them (Step 2). Afterward, the functionalized lipid is incubated for another hour with a preformed artificial vesicle or a natural vesicle (Step 3). This incubation period allows the components to slowly combine without external perturbation that might disrupt the vesicles like sonication. Gentle mixing can be performed during this stage. Finally, the detergent bound to the functionalized lipid is removed by 2 hours of dialysis (Step 4). A lot less detergent is present, so the dialysis period is significantly shortened. Also, the relatively low detergent concentration reduces the risk that excess detergent will disrupt the target vesicle. Detergent removal from the functionalized lipid exposes hydrophobic parts of the FA tail. The functionalized lipid

inserts itself into the target vesicle as an entropic process to reduce exposure to these hydrophobic surfaces. The FA tail of the functionalized lipid will insert into the target vesicle from the outside. Naturally, the functionalized part of the lipid on the other side of the molecule (red arrowhead) will be facing outward (Step 4). We have already experimentally demonstrated that we can orient integral membrane proteins in lipid bilayers using a similar principle [52,53].

Possible mechanisms for functionalized vesicle endocytosis and miRNA delivery

The general theory for delivering molecules to a cell is that a targeting molecule on the functionalized vesicle will bind to a surface receptor or excreted receptor ligand. The vesicular uptake can occur by fusion or by several potential endocytosis mechanisms [64]. The specific mechanism of vesicular uptake has not been entirely resolved but appears to be cell-dependent [64].

Fig. 2 shows the potential mechanisms that a functionalized vesicle can be endocytosed by a target cell. The top figure shows the binding of the PEG-linked Ab on the functionalized vesicle to a surface receptor on the cell (Step 1). The functionalized vesicle can bind to the receptor and induce endocytosis. Alternatively, the proximity of the functionalized vesicle to the plasma membrane may induce endocytosis through the assistance of a PEG linker (Step 2). Endocytosis of the receptor-bound vesicle leads to an invagination of the cell surface and eventually to the formation of an endosome (Step 3). The endosome eventually disintegrates intracellularly to release the miRNA or other cargo inside the target cell (Step 4).

The bottom of Fig. 2 shows the targeting of secretory receptor ligands from cells by functionalized vesicles containing miRNA. In Step 1, soluble proteins are trafficked to

the Golgi apparatus from the endoplasmic reticulum (ER) [65]. The Golgi apparatus produces secretory vesicles containing the receptor ligands that migrate to the plasma membrane [65]. At the plasma membrane, they are secreted into the extracellular (EC) space, where they are bound by a functionalized vesicle (Step 1) [65]. The functionalized vesicle bound-receptor ligands bind to cell surface receptors on the plasma membrane forming a ternary complex with the receptor. Receptor-ligand binding to the surface receptor may lead to conformational changes that lead to endocytosis (Step 2) [e.g., 66]. The functionalized vesicle is tethered to the receptor-ligand and surface receptor by a PEG linker allowing the functionalized vesicle to interact with the plasma membrane surface. Alternatively, the interaction of the functionalized vesicle through the PEG2000 linker with the plasma membrane can lead to endocytosis. In either case, the functionalized vesicle will be engulfed by the cell to form an endosome (Step 3) [64]. The endosome containing the functionalized vesicle loaded with miRNA migrates within the cell (Step 3). Eventually, the endosome and the vesicle disintegrate, releasing the miRNA or other cargo intracellularly (Step 4).

The dynamic light scattering (DLS) technique reveals how different processes affect vesicle size

The DLS technique is a well-established technique for estimating particle sizes based on their dynamic light scattering properties [67]. The technique is well suited to estimate particle size distributions [e.g., 50,68]. The DLS technique was used to determine the effects of the “Detergent-Dialysis Method” and the “Functionalized-Lipid Insertion Method” on the vesicular size distributions. Because functionalized vesicles are assembled all at once with the “Detergent-Dialysis Method,” these experiments will test

the hypothesis that the initial components of the detergent-solubilized mixture will strongly influence the vesicular size distribution.

Fig. 3 shows DLS measurements of functionalized vesicles produced by the two methods. The predominant particle sizes were determined in each solution by comparing the particle size distribution (PSD) to the particle diameter. Fig. 3A shows the predominate DDM micelle size (solid line) and a detergent-solubilized NBD-DSPE or DPSE-PEG2000-maleimide micelle size (dotted line) present in solution. The DDM micelle was 6.8 ± 1.6 nm in diameter, while the detergent-solubilized FAs were slightly smaller at 5.7 ± 1.4 nm, consistent with the sizes you would expect for these molecules.

Figs. 3B and 3C show vesicles produced using the “Detergent-Dialysis Method.” In Fig. 3B, the LNP (solid line) had an average diameter of 87.8 ± 34.7 nm, similar to previous observations [20]. However, the mLNP (dotted line) had about half the diameter of the LNP at 51.2 ± 19.5 nm. This size difference suggests that the size of LNPs may be sensitive to their initial mixtures, as we hypothesized. Fig. 3C shows the EVs (solid line) and mEVs (dotted line) produced using the “Detergent-Dialysis Method.” The EVs created using this method had an average size of 19.7 ± 5.6 nm. In contrast, the diameter of mEVs produced by this approach increased 6-fold larger or 131 ± 64.5 nm, showing another example of how the initial mixtures can influence vesicle size.

Fig. 3D and Fig. 3E show the unmodified vesicles and modified vesicles produced using the “Functionalized-Lipid Insertion Method” described in this manuscript. Fig. 3D shows the average particle size of LNPs that are unmodified (solid line). They have an average diameter of 115.6 ± 35.0 nm. LNPs modified using the “Functionalized-Lipid Insertion Method” (dotted line) increased slightly over 30% to 160.3 ± 37.4 nm. A 40 nm

diameter increase is consistent with the length of the PEG2000 linker and Ab connected to the mLNP [69,70]. The unmodified EVs shown in Fig. 3E (solid line) were 21.2 ± 5.9 nm, which is similar to the EV sizes produced with the "Detergent-Dialysis Method" (Fig. 3C, solid line). In Fig. 3E (dotted line), the EVs increased only 17% or 5 nm to 24.7 ± 6.9 nm. Thus, the size increase for mEVs was relatively modest, suggesting that their original structures were intact, but less functionalized lipid was inserted per EV than LNPs. One possibility for the size difference between mLNPs and mEVs is that the plasma membranes of EVs are crowded with many intrinsic membrane proteins that interfere with functionalized lipid insertion [e.g., 71]. The other possibility is geometrical. Because mEVs are smaller than mLNPs, their surface areas will be much larger within a given volume than mLNPs. In other words, a lot more functionalized lipids will be required to have the same functionalized lipid density on mEVs than mLNPs.

***In-vitro* delivery of miRNA by functionalized vesicles produced from the "Functionalized-Lipid Insertion Method"**

The human liver HepG2 cancer cell line has been the focus of many *in vitro* studies related to the functioning of liver cells and liver cancer [72–74]. The endocytotic asialoglycoprotein receptor 1 (ASGR1) is highly expressed on the surface of HepG2 cells [75]. The endocytotic process involves receptor-ligand binding and ASGR1 trimerization through receptor-mediated endocytosis [76]. This receptor has already been exploited to mediate small molecule uptake into HepG2 cells [77–79]. For example, galactosamine-linked albumin and nanoparticles loaded with pullulan and arabinogalactan delivered the anti-cancer drug doxorubicin to HepG2 cells [77,78]. In another study, pegylated liposomes modified with lactoferrin successfully delivered the fluorophore coumarin-6

through ASGR1 receptor-mediated endocytosis of HepG2 cells and HepG2 cells implanted in nude mice [79]. Finally, ASOs have been delivered to HepG2 cells by linking them to the ASGR1 receptor substrate *N*-acetylgalactosamine [80]. As far as we know, $ASGR1_{PAB}$ or any ASGR1 Abs have never been used to facilitate molecular uptake through the ASGR1 receptor with HepG2 cells. In this study, functionalized vesicles were made with $ASGR1_{PAB}$, which targets the EC domain of the ASGR1 receptor.

The focus of my laboratory is on drug transporters called P-glycoprotein (P-gp), which plays a major role in drug disposition, anti-cancer drug resistance, and biological barriers like the blood-brain barrier [81]. Despite being studied for decades, transporter measurements with P-gp and other transporters remain challenging [82]. The P-gp expression can vary between different cancer cell lines [83]. Even variants of the same mammalian cancer cell line can have significantly different properties [84]. Transporter transfection of mammalian cells can produce expression of other transporters in the cell [85]. Further complicating transport measurements, control mammalian cancer cells without P-gp can express different levels of endogenous transporters than the mammalian cancer cells transfected with P-gp further [83]. Additionally, culturing conditions can significantly affect P-gp expression levels [86].

We were hopeful that oligonucleotides targeting P-gp and delivered by functionalized vesicles could ameliorate some of these challenges. Our former collaborator, who is a miRNA expert, had us investigate P-gp with mouse miR-298 based on [87,88] and to be compliant with NIH funding for this project. The miR-298 in [87,88] turned out to be human miRNA-298, which has completely different targeting properties and biological functions than mouse miRNA-298 [89]. As far as we are aware, mouse

miRNA-298 is not known to affect P-gp expression in any species. That revelation was one of the primary reasons that the collaboration was terminated. Despite this setback, we felt the described functionalized vesicle technology could eventually be used to investigate P-gp, and miRNA could be used to test its feasibility since it would not have any physiological effects on the HepG2 cells.

MiRNA nomenclature can be confusing for a novice. The longer names for mouse and human miR-298 are mmu-miR-298-5p and hsa-miR-298-5p, respectively [89]. The first three letters are the species [89]. Mmu is for *Mus (M.) musculus* (house mouse) and hsa is for *Homo* (*H.*) *sapiens* [89]. The number typically reflects the order of miRNA deposition into the miRNA database called miRbase and is not related to the biological function [89]. The 5p refers to the 5' side of the precursor miR-298 stem-loop that the miRNA emanates [87]. The mouse miR-298 step loop can even have a second miRNA on the 3' side called mmu-miR-298-3p, which can be functionally distinct from the miRNA on the 5' side [90].

Fig. 4 are experiments to estimate the relative uptake of mmu-miR-298-5p in HepG2 cells. The experiments shown in Fig. 4A were used to gauge the uptake of 4 μg of mmu-miR-298-5p into the HepG2 cell line under different conditions. The relative uptake of mmu-miR-298-5p was normalized against HepG2 cells treated with DharmaFect 4 transfection reagent with miRNA (abbreviated DharmaFect(miRNA)). No mmu-miR-298-5p was detected in untreated HepG2 cells. HepG2 cells treated with EVs electroporated with mmu-miR-298-5p (abbreviated EVs(miRNA)) had miRNA uptake efficiency that was 5-fold higher than HepG2 cells treated with DharmaFect(miRNA). The miRNA uptake efficiency of HepG2 with EVs constructed with the $ASGR1_{PAB}$ and loaded

with mmu-miR-298-5p (abbreviated mEVs(ASGR1$_{PAB}$, miRNA) showed 8-fold greater improvement in miRNA uptake than DharmaFect(miRNA) control. HepG2 cells treated with exosomes genetically engineered with an Apolipoprotein A1 targeting ligand had more efficient miRNA uptake [48]. The Apolipoprotein A1 targeting ligand on the exosome may function better with the endocytotic mechanism of the ASGR1 receptor than the ASGR1$_{PAB}$ that targets different parts of the EC domains [76]. Also, internalization through the Apolipoprotein A1 targeting ligand only requires binding to the scavenger receptor, class B, type I (SR-BI) to initiate endocytosis [76,91,92]. Modified LNPs with ASGR1$_{PAB}$ were also made and loaded with 4 μg mmu-miR-298 (abbreviated mLNP(ASGR1$_{PAB}$, miRNA)). Relative miRNA uptake was almost 2-fold higher in HepG2 cells treated with mLNPs (ASGR1$_{PAB}$, miRNA) than cells treated with mEVs (ASGR1$_{PAB}$, miRNA). The higher miRNA uptake by HepG2 cells treated with mLNPs may result from more functionalized lipids per LNP or more miRNA per vesicle because of their relatively large size compared to EVs.

The miRNA uptake's temporal efficiency depends on the miRNA endocytotic mechanism, the miRNA carrier vehicle, and the miRNA type [93,94]. For example, the miRNA uptake of a human ovarian cancer cell line treated with liposomes loaded with fluorescently-labeled hsa-miR-7-5p increased linearly over 24 hours [93]. The uptake of miRNA in multiple myeloma treated with stable nucleic acid-lipid particles (SNALPs) loaded with hsa-miR-34a decreased after 48 hours [94]. In this study, the miRNA uptake in HepG2 cells after treatment with mLNP(ASGR1$_{PAB}$, miRNA) and mEV(ASGR1$_{PAB}$, mRNA) was examined over 72 hours (Fig. 4B). The uptake efficiency of HepG2 cells to mmu-miR-298-5p increased exponentially by treatment with mLNPs or mEVs. After

incubating the cells for 24 hours, the uptake efficiency approximately doubled every 12 hours for the mEVs and 8 hours for the mLNPs. The uptake at 72 hours for mLNPs and mEVs was 600 and 250-fold higher, respectively, above the background in untreated controls. The 72-hour time frame seemed like a suitable sensitivity for the *in vivo* mice experiments to determine how well the functionalized vesicles target and whether there is significant immunogenicity from the treatment.

Modified vesicles reduce HepG2 cell proliferation by enhancing hsa-miR-26a-5p uptake

The functional effects of treating HepG2 cells were tested with the mEVs(ASGR1$_{PAB}$), and mLNPs(ASGR1$_{PAB}$) loaded with miRNA hsa-miR-26a-5p (abbreviated mEV(ASGR1$_{PAB}$, miRNA) and mLNP(ASGR1$_{PAB}$, miRNA)). The miRNA hsa-miR-26a-5p was chosen to inhibit HepG2 cell proliferation and promote HepG2 cell apoptosis [48]. In other words, the miRNA will slow the closure gap of wounds made of a layer of HepG2 cells.

Wound healing assays with bright field microscopy has been effectively used to gauge cell proliferation and migration [95,96]. The approach was used to estimate the effect of mEVs and mLNPs treatment of HepG2 cells in Fig. 5. Fig. 5A shows the wound closures of HepG2 cells after various treatments at 0 and 72 hours. For untreated cells, the wound closed to about half its original width, indicating uninhibited HepG2 cell proliferation. The HepG2 cells treated with empty EVs (abbreviated EV()), unmodified extracellular vesicles electroporated with hsa-miR-26a (abbreviated EV(hsa-miR-26a)), and empty modified extracellular vesicles with the polyclonal antibody for the ASGR1 receptor (abbreviated mEV(ASGR1$_{PAB}$)) had an almost similar extent of wound closure

as the untreated cells meaning no effect on HepG2 cell proliferation. The wound area for HepG2 cells after treatment with mEVs(ASGR1$_{PAB}$, miRNA) and mLNPs(ASGR1$_{PAB}$, miRNA) almost completely inhibited wound closure, demonstrating efficient uptake of hsa-miR-26a.

This wound closure over 72 hours was assessed quantitatively in Fig. 5B. The percent wound closure by all the treatment groups was normalized to the amount of wound healing in the untreated cells. The results show that the relative amount of wound closure in the cells treated with empty EVs(), EVs(hsa-miR-26a), and mEVs(ASGR1$_{PAB}$) was virtually identical to untreated cells. For EVs(hsa-miR-26a), the lack of effect on cell proliferation may be due to inefficient uptake of the hsa-miR-26a miRNA by the HepG2 cells. In contrast, wound closure by cells treated with mEVs(ASGR1$_{PAB}$, hsa-miR-26a) or mLNPs(ASGR1$_{PAB}$, hsa-miR-26a) inhibited cell proliferation by 50%.

Along with wound closure, the relative wound area was also analyzed temporally in Fig. 5C. The wound in untreated HepG2 cells closed at 16 ± 3 % per day (open circles). This wound closure rate is close to the 19% per day observed for HepG2 treated with extracellular vesicles containing hsa-miR-26a (calculated from Fig. 5B in [48]). After three days, the wound area was reduced to 52 ± 8% of the original area, suggesting uninhibited cell proliferation. The relative wound area in the cells treated with EVs delivering miRNA (abbreviated EVs(miRNA), open squares) was reduced at the rate of 13.9 ± 1.5 % per day, thus not differing significantly from untreated cells. Treating the HepG2 cells with mEVs(ASGR1$_{PAB}$, miRNA) decreased the rate of closure to 5 ± 4.3% per day (closed circles) and with mLNPs(ASGR1$_{PAB}$, miRNA) reduced the rate of closure to 6.7 ± 1.8% per day (closed squares). HepG2 cells treated with either our mEVs or mLNPs inhibited

cell proliferation better than HepG2 cell treatment with miRNA-loaded Apo A1 targeting ligand exosomes, which only decreased HepG2 cell proliferation to 10% per day (calculated from Fig. 5B in [48]).

Process of *in vivo* targeting of modified vesicles

The uptake of miRNA in mice after treatment with mEVs and mLNPs is shown in Fig. 6. Modified vesicles are loaded with miRNA by electroporation and injected intraperitoneally (IP) into mice (Fig. 6A). Because of their small physical size, IP administration to mice was easier and more reliable than intravenous (IV) administration [97–101]. Also, vesicles administered by IP injection distribute broadly to the organs and the visceral adipose tissue because of access to the lymphatic system [97–101], which was preferred for testing our functionalized vesicles.

The treated mice are euthanized after three days using carbon dioxide followed by cervical dislocation, and their organs are harvested (Fig. 6B). The tissue was analyzed using RNA extraction protocols described in the *Materials and Methods* (Fig.6C). The relative miRNA concentration taken up in the organs is compared to the relative concentration of the U6 snRNA (Fig. 6D). The crossover threshold (C_t) is determined for the miRNA and the U6 snRNA. Finally, the relative-fold miRNA uptake in the mouse organs is determined by the $\Delta\Delta C_t$ method (Fig. 6E).

I*n vivo* studies with mmu-miR-298-5p were originally intended to modulate P-gp expression as recommended and suggested by our former collaborator, which would fit well within Specific Aim #1 of the supporting grant, but did not have that function [87,88,102–104]. As far as we are aware, no microRNAs are known to affect mouse P-gp expression or any related mouse transporters within its transporter superfamily. Our

former collaborator made miRNA sound specific, especially for P-gp transporter expression, but a single miRNA can affect several physiological functions [105]. For example, the mmu-miR-298-5p is known to target the nuclear factor (NF)-κB activator 1 (Act1) involved in cell signaling pathways and autophagy [102,106]. The miRNA is also known to regulate the expression of β-Amyloid Precursor Protein-converting Enzyme 1 [103] and regulate the expression of the insulin-like growth factor-1 β (IGF1Rβ) [104]. The miRNA also has a wide variety of theoretical targets that remain to be investigated in the TargetScanVert and miRDB databases [107,108]. Therefore, the following *in vivo* experiments with the functionalized vesicles and mice will determine the miRNA's organ specificity and immunogenicity.

Eventually, our laboratory wants to use the described functionalized vesicles to modulate *in vitro* and *in vivo* P-gp transporter expression. Luckily, siRNAs have already been shown to affect mouse P-gp expression [53,109]. Therefore, functionalized vesicles by this approach loaded with the P-gp targeting siRNA may be a practical way to investigate the P-gp transporter in the future.

***In vivo* miRNA delivery by functionalized vesicles with a non-interacting antibody**

As a negative control for *in-vivo* targeting ability of mEVs and mLNPs, mice were treated with functionalized vesicles loaded with mmu-miR-298-5p and GFP_{MAB} as the non-targeting antibody. Fig. 7 shows the uptake of mmu-miR-298 treated with modified vesicles with the GFP_{MAB} and containing mmu-miR-298 (abbreviated mEVs(GFP_{MAB}, mmu-miR-298) and mLNPs(GFP_{MAB}, mmu-miR-298-5p)). The highest miRNA uptake with the functionalized vesicles was observed in the mouse spleen, which is not surprising considering the IP administration of the functionalized vesicle. The IP injection puts the

functionalized vesicle into the lymphatic system and the spleen (a lymphoid organ) [110]. The liver had about a third of the miRNA uptake than the spleen. The kidney and the lungs had only 20% of the miRNA uptake of the liver and 5% of the miRNA uptake of the spleen. Less than 1% of miRNA uptake was observed in the brain. The miRNA uptake in mice follows a very similar pattern as biotin-labeled liposomal uptake in the organs of rats, with the exception of liposomal uptake in the lungs [111].

In vivo targeted miRNA delivery to the liver by functionalized vesicles with the ASGR1 antibody

After successfully delivering miRNAs by both mEVs and mLNPs bioengineered with ASGR1 antibody to hepatocellular carcinoma HepG2 cells (Fig. 4), we wanted to assess the capability of these vehicles in targeting the liver *in vivo* utilizing the same antibody. Mice were treated with mEVs($ASGR1_{PAB}$, mmu-miR-298) and mLNPs($ASGR1_{PAB}$, mmu-miR-298) like the *in vitro* experiments.

Fig. 8 shows the uptake of miRNA by liver, lungs, spleen, kidney, and brain in mice treated with mEVs($ASGR1_{PAB}$, mmu-miR-298) and mEVs($ASGR1_{PAB}$, mmu-miR-298) normalized to the endogenous levels of mmu-miR-298 that is present in mice. MiRNA delivery from both mEVs and mLNPs was almost entirely in the liver while relatively non-existent in other organs. The high specificity for the mEVs and mLNPs to the liver may be due to the target of $ASGR1_{PAB}$ being the ASGR1 receptor, which is naturally endocytotic.

In vivo targeted miRNA delivery to the kidney by modified vesicles with the NPHS2 antibody

NPHS2 (a.k.a. Podocin) is a non-endocytotic protein associated with the kidney [112], so we made functionalized vesicles with $NPHS2_{PAB}$ to see how well it would these

vesicles would target the kidney with mmu-miR-298. Fig. 9A shows the amount of miRNA delivered by mEVs(NPHS2$_{PAB}$, mmu-miR-298) to five major organs: kidney, liver, lungs, spleen, and brain. The amount of miRNA was normalized to the endogenous levels of mmu-miR-298 in mice only treated with SFM. The distribution of microRNA uptake was significantly different than mEVs and mLNPs engineered with the GFP$_{PAB}$ (Fig. 7), suggesting that the NPHS2$_{PAB}$ on the modified vesicles affected the organ targeting. However, these modified vesicles were less specific than mEVs and mLNPs with ASGR1$_{PAB}$ (Fig. 8), which may be because the NPHS2 protein is not naturally endocytotic [112] or because of cross-reactivity of NPHS2$_{PAB}$[113,114]. The mmu-miR-298 delivered to the kidney and spleen was the highest of all the organs and was 125-150 fold higher than control mice treated with SFM only. Since the spleen is not known to express the NPHS2 protein, the higher spleen uptake is likely due to the IP route of administration. The lungs and the liver showed significantly less uptake at ~25 fold and ~60 fold, respectively. No significant microRNA uptake was found in the brain. Modified LNPs (abbreviated mLNPs(NPHS2$_{PAB}$, mmu-miR-298) or mLNPs(NPHS2$_{PAB}$, miRNA)) also showed a very similar delivery distribution (Fig. 9B) as mEVs(NPHS2$_{PAB}$, mmu-miR-298), inferring that the NPHS2$_{PAB}$ had similar targeting effects in both of these functionalized vesicles.

Immunogenic effects of miRNA delivery by modified vesicles with the relatively non-specific ACE2 antibody

To explore the immunogenicity of mEVs and mLNPs in-vivo, a targeting vesicle was engineered with a monoclonal antibody against the membrane-associated angiotensin-converting enzyme 2 (ACE2) (ACE2$_{MAB}$). This enzyme is ubiquitously

expressed in many organs throughout the body and plays a crucial role in controlling blood pressure [60,115–118]. In rodents, ACE2 mRNA levels were highest in the ileum in both mice and rats, followed by the kidney, almost equal levels in the lungs, bladder, stomach, colon, adipose tissue, atrium, brainstem, forebrain, and ventricle [119]. The lowest ACE2 mRNA was in the spleen of the rodents [119]. A different but consistent pattern in protein levels was observed in rats with the highest in the atrium (124.5%) and ventricle (131.7%), moderate expression in the kidney (100%), lesser in the lung (19.7%), testis (28.7%), thymus (44.4%), and least amount in the spleen [119]. Therefore, targeting mEVs and mLNPs with $ACE2_{MAB}$ will be relatively non-specific but more evenly distributed among the organs than mEVs and mLNPs with GFP_{PAB}, which primarily targeted the spleen (Fig. 7).

Fig. 10 shows the uptake of mmu-miR-298 and their effects on the cytokine levels after treatment of mice with miRNA-loaded mEVs and mLNPs engineered with $ACE2_{MAB}$. In Fig. 10A, from high to low fold miRNA uptake by mEV treated mice versus mice treated with SFM only, the miRNA uptake in mice by mEVs was ~280 fold in the liver, ~250 fold in the kidneys, ~200 fold in the spleen, ~200 fold in the lungs, ~90 fold in the small intestine, ~70 fold in the heart and ~25 fold in the brain.

In Fig. 10B, the relative miRNA uptake was analyzed with mice treated with mLNPs($ACE2_{MAB}$, mmu-miR-298) versus SFM only treated mice (controls). The distribution of miRNA delivery to organs by mLNPs differed significantly from mEVs. The uptake of miRNA by treatment of mice with mLNPs($ACE2_{MAB}$, mmu-miR-298) was highest for the small intestine (~830 fold) followed by the kidney (~530 fold), spleen (~400 fold), liver (~400 fold), heart (~388 fold), and lungs (~100 fold). Virtually no miRNA uptake was

observed in the brains of mice. Except for the spleen, these relative miRNA uptake levels are consistent with the protein expression levels of the ACE2 protein in rodents and humans [60,118,119]. Since there is almost no ACE2 enzyme in the spleen [119], the relatively high miRNA uptake after treatment with functionalized vesicles is likely due to IP administration rather than specific targeting by $ACE2_{MAB}$.

The immunogenicity of the functionalized vesicles was assessed by LegendPlex™ 8-panel Th1/Th2 Bio-plex assay, which tests for 8 major anti- and pro-inflammatory cytokines from two types of CD4+ T helper lymphocytes [120]. Cytokine profiling has been used previously to determine the immunogenicity of EVs and LNPS [121,122]. Fig. 10C shows the cytokine levels in mice treated with mEVs($ACE2_{MAB}$, mmu-miR-298) and mLNPs($ACE2_{MAB}$, mmu-miR-298) in comparison to mice treated with SFM. No statistically significant change in cytokine levels was observed in mice treated with either mEVs (gray circles) or mLNPs (white circles) versus the SFM only treated mice (black circles). In addition, throughout all the *in vivo* experiments in this study (Figs. 7-10), no physical manifestations of immune response were observed, such as redness, itching, or sudden hair loss.

Discussion

In this work, we present the “Functionalized Lipid Insertion Method” to bioengineer targeting functionalized vesicles with a surface coated with PEG-linked Abs that we refer to as mEVs or mLNPs. We explain how our approach differs significantly from the “Detergent-Dialysis Method” used since the 1980s [17–24]. These functionalized vesicles are versatile and can target surface receptors. Both mEVs and mLNPs could efficiently deliver miRNA to the HEPG2 cell line. The *in-vitro* miRNA uptake efficiency was 8-fold

and 15-fold higher than the transfection reagent by mEVs and mLNPs, respectively. mEVs treatment of cells with growth-affecting miRNA caused significant effects on mRNA expression and cellular growth. Targeted delivery of miRNA to the liver (more than 80%) and kidney (more than 50%) was achieved by both mEVs and mLNPs in-vivo. The modified vesicles also were essentially non-immunogenic (Figure 10).

Even unmodified extracellular vesicles can have oligonucleotide uptake efficiencies and target cells like mEVs [123,124]. Exosomes derived from the human hepatoma Huh7 cell line targeted the human embryonic kidney line over human PBMCs or a human lymphoblast cell line [123]. The miRNA uptake efficiencies for the human embryonic kidney cell line treated with exosomes were 5-200-fold higher than the other cell lines [123]. A study with exosomes derived from MDA-MB-231 cells and H-29 colon cancer cells suggested that efficient exosomal targeting relied on complementary interactions [124]. Unmodified exosomes are the simplest to produce but predicting the exosomal target requires analyzing the oligonucleotide uptake in various tissues and cell lines to determine specificity.

Several methods have been developed to modify the surfaces of natural vesicles to control targeting to cells and improve uptake of oligonucleotides [15,53,124]. Targeting and miRNA uptake of CD8+ T-cell-derived exosomes to effector T-cells was enhanced by having a constitutive exosomal protein bound with antibodies [15]. Unfortunately, this simple approach does not have broader applicability to exosomal targeting beyond T-cells. Another strategy to exploit constitutive exosomal proteins is to fuse them with targeting proteins by genetic engineering [125]. Exosomes were genetically engineered with a lysosomal protein (Lamp2b) and the rabies viral glycoprotein (RVG) [72]. They

were able to enhance siRNA uptake efficiency in cells and overcome the blood-brain barrier (BBB) in mice [53]. Exosomes have been genetically engineered using a similar technique with fusion proteins of the lysosomal protein (Lamp2) with the green fluorescent protein (GFP) for fluorescent tracking and with the HER2 affibody for targeting [126]. Exosomes have also been genetically engineered with a construct made from the vesicular stomatitis virus glycoprotein with improved loading, delivery, and tracking [127]. The development of modified exosomes by genetic engineering is limited by the complexity of engineering the fusion protein construct and getting the construct to express in budding exosomes [125].

A highly PEGylated exosome with a relatively low surface density of Abs than Abi-exosomes was developed using a two-stage process to deliver anticancer drugs cells [16]. The modified exosomes provided only a ~30% increase in anticancer drug uptake by a human pancreatic cancer cell line [16].

Similarly, modified and unmodified LNPs have been used to deliver miRNA previously. Cationic liposomes have been used as transfection agents to deliver miRNA such as miR-7 to ovarian cancer cells, miR-143 and -145 in human colorectal tumors, and miR-122 in liver cells achieving up to 80% efficiency [93,128,129]. Cationic liposomes typically consist of a cationic lipid, neutral lipid and/or cholesterol, and a PEG-lipid. Cationic liposomes are known to form ion pairs with anionic phospholipids of the endosomal membrane leading to the release of cargo efficiently, which makes them one of the promising drug delivery vehicles. Cationic liposomes can form complexes with oligonucleotides such as miRNA and are known as lipoplexes. For instance, a cationic-based lipoplex delivery system has been used to deliver miR-29b and miR-133b to non-

small cell lung cancer cells. The lipoplexes achieved over 2-fold delivery efficiency in comparison to the transfection reagent *in vitro*. In addition to low delivery efficiency, when injected intravenously, only 30% accumulation was achieved in the target organ, lungs, with the highest amount found in the liver, followed by kidney and spleen [130,131]. The process of preparing lipoplexes, unfortunately, renders them unoptimizable down the process and increases the risk of interaction of cationic lipids with other tissues making them non-specific, limiting their potential as targeted drug delivery vehicles.

In addition to cationic liposomes, liposomes can also be conjugated with target-specific vectors to design cell-specific vehicles. Targeted liposomes have been prepared by conjugating Aptamer (AS1411), a target-specific single-stranded oligonucleotide, using thioether linkage between DSPE-PEG2000 maleimide on the liposomal surface. These Aptamer functionalized liposomes were used to deliver miR-29b to A2780 cells, ovarian cancer cells [132]. Another study prepared a liposome-polycation-hyaluronic acid (LPH) modified with GC4 single-chain variable fragment, a tumor-targeting monoclonal antibody to deliver siRNA and miRNA to B16F10, metastatic lung cancer cells [133]. The GC4 targeted liposomes achieved 40% delivery of siRNA in the tumor, and the highest accumulation was achieved in the liver (>50%) when dosed intravenously to the tumor-bearing mice [133]. An antibody against the CD59 receptor, a receptor overexpressed in cervical cancers, was conjugated to Cisplatin (CDDP)/miR-1284-loaded liposomes. CD59 conjugated vehicles showed a significant increase in miRNA-induced cytotoxicity in cervical cancer cells and prolonged blood circulation of cisplatin [134]. All these studies have used the post-conjugation method to modify liposomes, where they attach derivatized PEGylated lipids to the liposomes while preparing liposomes followed by

conjugation of antibodies. Although successful, this method is more complicated as you functionalize lipid derivatives even before preparing liposomes. However, the post-insertion method, also known as the detergent dialysis method, attachment of antibodies to derivatized lipids first, and then inserting antibody derivatized lipids in PEGylated liposomes, gives more room to play with designing or modifying liposomes as desired. The Detergent-dialysis method has been shown to be as effective as if not more than the post conjugation method [135]. This method has been used to attach target-specific antibodies such as CD44 and CD133 to deliver cargoes like small molecules like doxorubicin or antibodies like anti-IL6R antibodies [136,137]. However, as mentioned earlier, this method requires extensive dialysis over a long time, increasing the risk of degradation (in the case of natural vesicles), aggregation, and attachment of functionalizing ligands both internally and externally.

Considering the versatility of the “Functionalized Lipid Insertion Method,” the approach seems to be the simplest and most efficient way to modify therapeutic delivery vehicles. Fluorescence tracking, PEGylation, and Ab targeting are integrated into a single dialysis step followed by electroporation for oligonucleotide loading. Many of the pieces for industrial-scale production of mEVs are already available. Industrial methods for dialyzing and electroporation have already been around for decades [138,139]. Synthetic methods are available to produce large amounts of miRNA [140]. Innovative large-scale production of monoclonal Abs is being developed from plants [141]. Currently, getting EVs is rate-limiting, but methods are on the horizon for getting from abundant sources such as milk [138,139]. Liposomes have been around for more than a few decades, with different methods available to upscale their preparation, including microfluidics [142,143].

Several challenges exist for implementing mEVs and mLNPs therapeutically. For long-term viability, EVs and, by extension, mEVs are most often stored at -80ºC. However, month-long room temperature storage has been shown after lyophilization and in the presence of trehalose [41,144]. In contrast, lyophilized LNPs can last up to a year at 4ºC or room temperature in an oxygen-free environment [145–148]. MicroRNAs also have their challenges and must often be stored at -80ºC [149], but methods are available to increase temperature stability through chemical modification [150]. MiRNA also has the potential for slowly leaking from functionalized vesicles [151].

Authors contributions

Authorship was determined using guidelines recommended by *The International Committee of Medical Journal Editors* [152–155], which requires significant and novel contributions to scholarship and significant contributions to the organization, writing, and intellectual ideas within this manuscript. The original development of the "Functionalized Lipid Insertion Method" was based on membrane protein reconstitution in liposomes previously done in Dr. Arthur G. Roberts laboratory [52,53]. Ms. Pragati Jain made significant improvements to the functionalized vesicle experimental protocol that was part of Dr. Sudeepti Kuppa's Ph.D. thesis. Ms. Pragati Jain and Dr. Arthur G. Roberts played the largest roles in designing the experiments within the manuscript, as evidenced by their many individual meetings. They both played major roles in the writing and the organization of the manuscript. Ms. Pragati Jain selected the specific antibodies and most of the other materials that were used within the project. Dr. Arthur G. Roberts selected the fluorescent and reactive lipids used in this project and designed the original protocol for functionalized vesicles, as well as contributed to modified versions by Dr. Sudeepti Kuppa and Ms. Pragati Jain. Ms. Pragati Jain did all of the data collection and almost all of the data analysis.

Acknowledgments

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Figure Legends.

Figure 1. Bioengineering functionalized vesicles by the "Detergent-Dialysis Method" and the "Functionalized-Lipid Method." A) Shows the "Detergent-Dialysis Method" that has been used since the 1980s to produce functionalized vesicles from LNPs. In Step 1, functionalized lipids containing a targeting molecule such as an Ab are mixed with lipids and high concentrations of detergent, leading to complete solubilization of all the components. In Step 2, the high concentrations of detergent are dialyzed over a long time period to remove the detergent. When enough detergent is removed, the mLNPs will spontaneously form, but the orientations of the functionalized lipids become randomized (red arrows) within the lipid bilayer. Even after this long dialysis period, column chromatography may be required to remove detergent-solubilized components in Step 3. B) Shows the process of using the method to produce functionalized vesicles from natural vesicles like EVs. A key difference with producing mEVs over producing mLNPs is that they must be lysed into their individual components in Step 1 prior to detergent solubilization and dialysis in Steps 2 and 3. In Step 3, Because proteins are likely to be embedded in the natural vesicle lipid bilayer, their orientations will be randomly oriented (green arrows) like the functionalized lipids (red arrows). Column chromatography might be required afterward in Step 4 to remove detergent-solubilized contaminants. C) Shows the process of producing mLNPs using the "Functionalized Lipid Insertion Method." In Step 1, detergent-solubilized lipids with reactive functional groups are dialyzed for two hours to remove excess detergent. These solubilized lipids are mixed with targeting molecules such as Abs for 1 hour to form functionalized lipids in Step 2. In Step 3, the functionalized lipids are then incubated (or mixed gently) with preformed LNPs for another

hour to ensure the integrity of the preformed vesicle. In Step 4, because the detergent concentration is relatively small compared with the detergent concentrations used in the "Detergent-Dialysis Method," a lot shorter time period is required for their removal. Removal of detergent from the functionalized lipids causes them to directionally insert into the preformed liposomes, becoming mLNPs. D) Shows natural vesicles like EVs being produced by the "Functionalized Lipid Insertion Method." The process of producing mEVs is virtually identical to producing mLNPs. In Steps 1 and 2, a detergent-solubilized functionalized lipid is produced from dialysis and incubation with targeting molecules such as Abs. Then in Step 3, they are incubated (or gently mixed) with the EV to maintain vesicle integrity. Detergent removal through dialysis inserts the functionalized lipid directionally into the EV forming an mEV.

Figure 2. Potential mechanisms for cellular endocytosis of functionalized vesicles. A) A functionalized vesicle binds to a cell surface endocytotic receptor in Step 1 and then undergoes endocytosis in Step 2. The receptor-bound functionalized vesicle forms an endosome in Step 3 and eventually disintegrates in Step 4 to release its contents like R. B) A soluble receptor-ligand migrates to the plasma membrane surface from the Golgi apparatus, where it binds to a functionalized vesicle in Step 1. In order for the soluble protein-bound functionalized vesicle to be internalized by a cell, the complex binds to a cell surface receptor forming a ternary complex in step 2. If the surface receptor is endocytotic, the functionalized vesicle-bound receptor ligand is endocytosed to form an endosome in Step 3. Alternatively, if the functionalized vesicle is connected to the receptor-ligand through a long linker like PEG2000, the functionalized vesicle might

contact the plasma surface leading to endocytosis. The resulting endosome disintegrates and releases its contents, such as R in Step 4. Abbreviations: R, miRNA.

Figure 3. Size distribution of functionalized vesicles determined by dynamic light scattering (DLS). A) Detergent DDM micelles (solid line) and DDM-solubilized FAs (dashed line). The "Detergent-Dialysis Method" was used B) to produce the size distributions of LNPs (solid line) and mLNPs (dashed line) and C) to produce the size distributions of EVs (solid line) and mEVs (dashed line). The solid lines in panels D) and E) show the size distributions produced for LNPs using the extrusion approach [44] and EVs through isolation and purification protocols described in the Materials and Methods. The dashed lines in panels D) and E) show the size distributions produced for mLNPs and mEVs using the "Functionalized Lipid Insertion Method" described in the text. The FAs shown in panel A are an equal mixture of NBD-DSPE and DSPE-PEG2000-maleimide.

Figure 4. Relative miRNA uptake efficiency into HEPG2 cells with different treatments. A) The relative miRNA (i.e., mmu-miR-298-5p) uptake into HepG2 cells under the various treatments: Untreated HepG2 cells (Cells), HepG2 cells treated with DharmaFect 4 transfection reagent and miRNA (Cells + DharmaFect(miRNA)), HepG2 cells treated with EVs and miRNA (Cells + EVs(miRNA)), HepG2 cells treated with mEVs with an $ASGR1_{PAB}$ and containing miRNA (Cells + mEVs($ASGR1_{PAB}$, miRNA), and HepG2 cells treated with mLNPs with an $ASGR1_{PAB}$ and containing miRNA (Cells + mLNPs($ASGR1_{PAB}$, miRNA). The relative miRNA uptake was normalized against the miRNA uptake by DharmaFect(miRNA). The values in the panel represent the mean ± SD of at least three independent experiments. B) Temporal changes in miRNA uptake

efficiency for mLNPs(ASGR1$_{PAB}$, miRNA) (solid squares) and mEVs(ASGR1$_{PAB}$, miRNA) (solid circles) over 72 hours with respect to U6 snRNA. The points and bars represent the average and SD, respectively. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$. Abbreviations: SD, standard deviation.

Figure 5. Anti-proliferative effects miRNA hsa-miR-26a-5p with HepG2 cells under various treatments. A) Microscopic images of a HepG2 layer during the migration assay with the following treatments after 0 (top) and 72 hours (bottom): Untreated HepG2 cells (Cells), HepG2 cells treated with empty EVs (Cells + EVs()), HepG2 cells treated with EVs containing miRNA (EVs(miRNA)), HepG2 cells treated with empty mEVs engineered with an ASGR1$_{PAB}$ (Cells + mEVs(ASGR1$_{PAB}$)), HepG2 cells treated with mEVs with the ASGR1$_{PAB}$ and miRNA (Cells + mEVs(ASGR1$_{PAB}$, miRNA)), and HepG2 cells treated mLNPs with the ASGR1$_{PAB}$ and miRNA (Cells + mLNPs(ASGR1$_{PAB}$, miRNA)). The black bar in the lower left-hand figure represents a distance of 200 μm. B) Shows quantitative measurements of HepG2 cell migration using the data and same abbreviations from panel A. The values in the bar graphs represent the mean ± SD of at least three independent experiments. C) Temporal changes in HepG2 cell migration in untreated cells (open circles) and cells treated with EVs(miRNA) (open squares), mEVs(ASGR1$_{PAB}$, miRNA) (closed circles), and mLNPs(ASGR1$_{PAB}$, miRNA) (closed squares) over 72 hours. To emphasize the differences, the time course for HepG2 cells treated with mEVs(ASGR1$_{PAB}$, miRNA) and mLNPs(ASGR1$_{PAB}$, miRNA) is shown as thick lines. The error bars represent the mean of three independent experiments ± SD. The dose miRNA in each of the experiments was 0.35 μg. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$. Abbreviations: SD, standard deviation.

Figure 6. Process of administering functionalized vesicles into mice. A) On the first day, functionalized vesicles, mEVs or mLNPs, with miRNA (R) and targeting ligands such as Abs are intraperitoneally (IP) injected into mice. B) Three days later, the mice are euthanized, and the organs are harvested. C) RNA is harvested and purified from the mouse organs. D) The amount of purified RNA from each of the organs is analyzed against U6 snRNA by qRT-PCR. E) The relative level of miRNA uptake by the mouse organs from treatment with the functionalized vesicles is determined using the $\Delta\Delta C_t$ method. Abbreviations: R, miRNA.

Figure 7. Uptake of miRNA in organs of mice treated with functionalized vesicles containing the non-targeting polyclonal green fluorescent GFP (GFP_{PAB}) antibodies. The figure shows the relative-fold uptake of miRNA in the organs of mice treated with (A) mEVs(GFP_{PAB}, mmu-miR-298) (gray) and (B) mLNPs(GFP_{PAB}, mmu-miR-298) (white) versus mice that were treated with SFM only (black and barely visible in the figure). The data were normalized against the U6 snRNA and expressed as mean ± SEM. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$.

Abbreviations: SEM, standard error of the mean.

Figure 8. Targeting of mmu-miR-298 with the ASGR1 antibody in mice. The following figure shows the fold-difference of mmu-miR-298 uptake in various organs of mice after being treated with (A) mEVs (n=3) (gray) and (B) mLNPs (n=6) (white) versus the SFM only treated mouse controls. The data were normalized to U6 and expressed as mean± SEM of at least three independent samples. The injection volume in the mice varied between 250 and 300 μl. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$. Abbreviations: SEM, standard error of the mean.

Figure 9. Relative mmu-miR-298 uptake by organs with the treatment of mEVs and mLNPs bioengineered with NPHS2 antibody. (A) mEVs (n=3) (gray) and (B) mLNPs (n=6) (white) bioengineered with an NPHS2 antibody in mice. The relative mmu-miR-298 uptake of mice treated with serum-free media (black) and mEVs/mLNPs treated (white). All the data were normalized to the constitutive level of U6 snRNA and represent the mean± SEM. The dosage for each of the mice was approximately 114 μg with an injection volume of 250-300 μl. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$.

Figure 10. ACE2 targeting and immune reactivity of mEV(ACE2) and mLNP(ACE2) in-vivo. The relative ratio of mmu-miR-298 uptake in various organs of mice after being treated with (A) mEVs($ACE2_{PAB}$, miRNA) (gray) and (B) mLNP($ACE2_{PAB}$, miRNA) (white) versus SFM only treated mice (black) (n=6). The data were normalized to U6 and expressed as mean± SEM of at least three independent experiments. C) Levels of 8-major cytokine factors as obtained after conducting cytokine assay on the blood samples withdrawn from mice treated with mEVs($ACE2_{PAB}$, miRNA) (gray circles), mLNP($ACE2_{PAB}$, miRNA) (white circles), and SFM only (black circles) (n=6) 72 hours after the treatment. The dosage for the mice was 110 μg and the injection volume was 250-300 μl. The p-values are * $p \leqslant 0.05$, ** $p \leqslant 0.01$, *** $p \leqslant 0.001$, **** $p<0.0001$.

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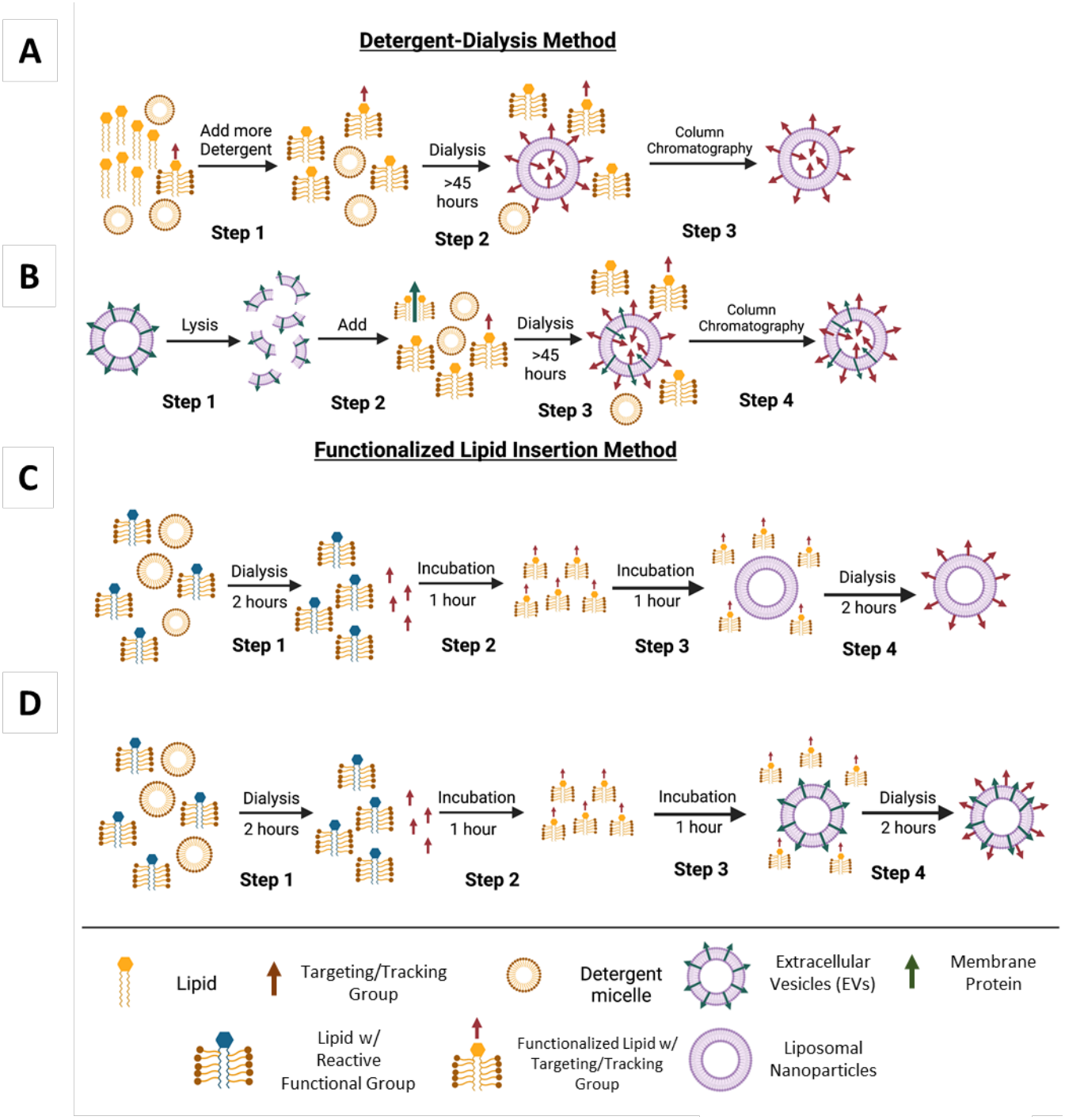

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2 **Figure 2.**

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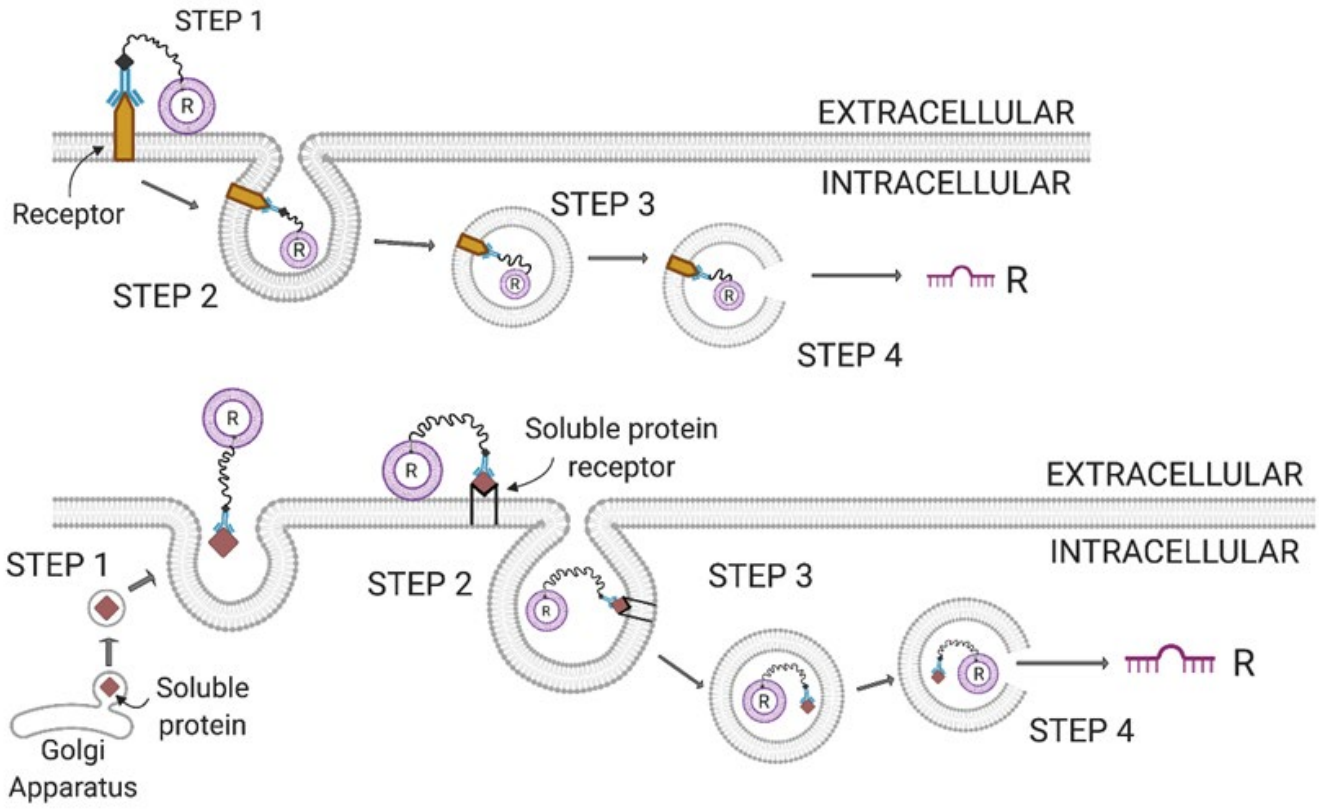
STEP 1
EXTRACELLULAR
INTRACELLULAR
Receptor
STEP 2
STEP 3
STEP 4
R
Soluble protein
receptor
EXTRACELLULAR
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STEP 3
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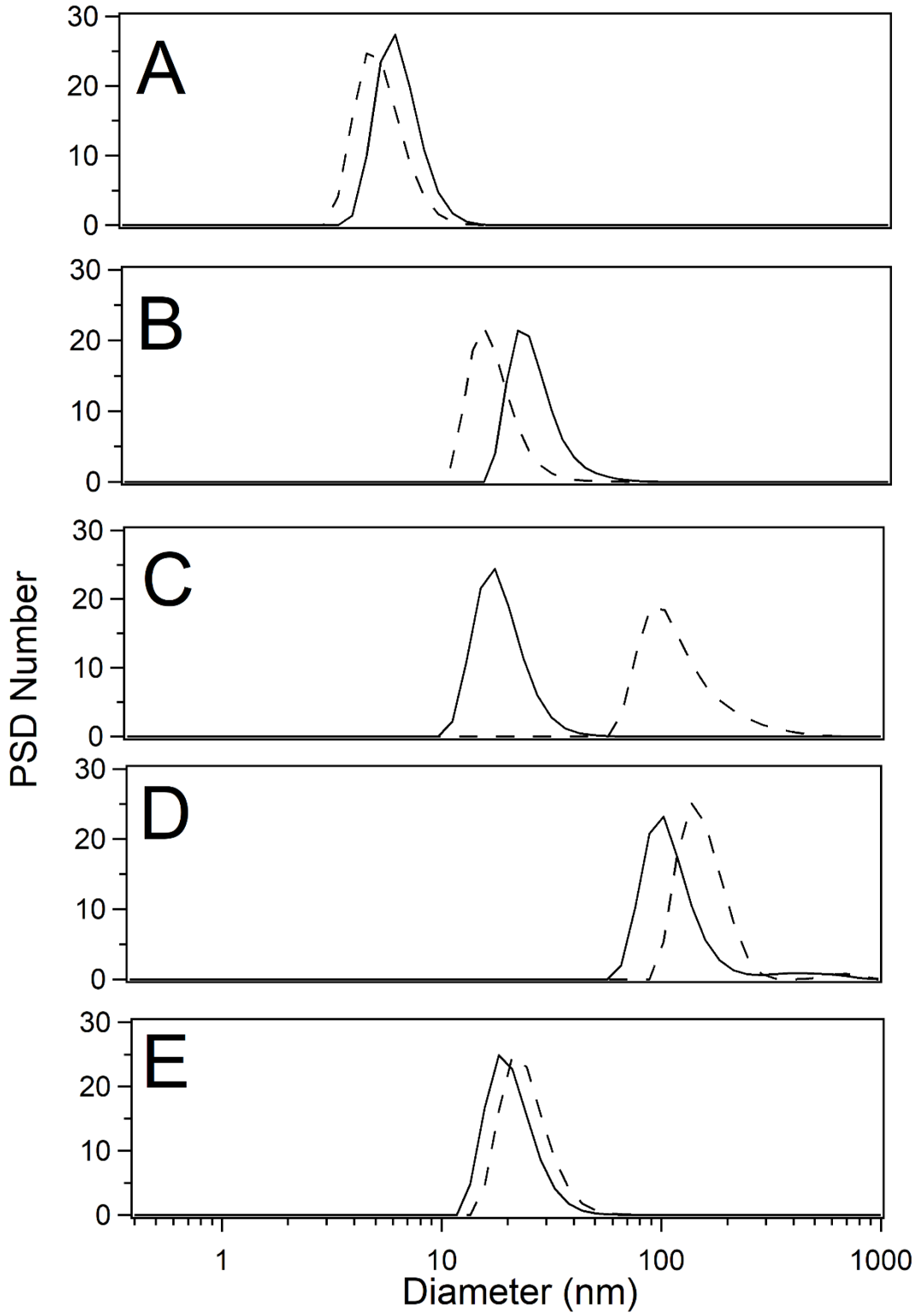

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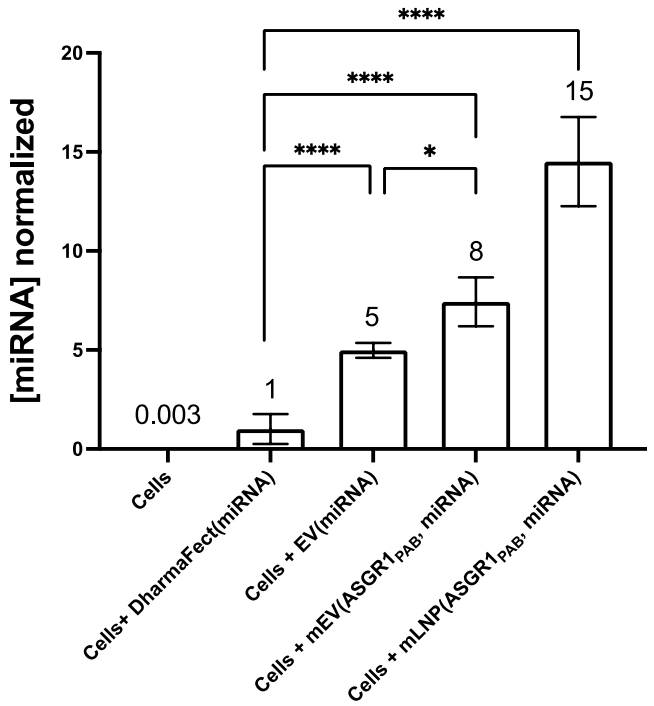
Figure 4.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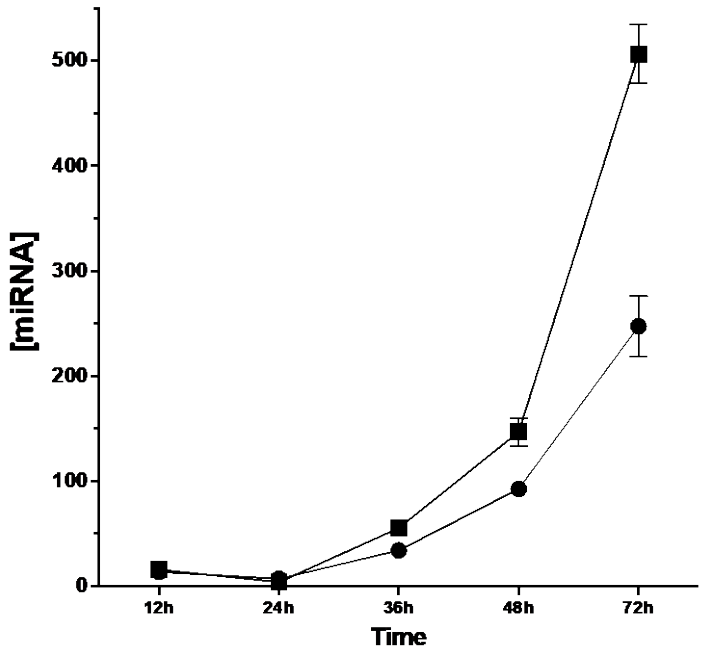
Cells + mLNP(ASGR1PAB, miRNA)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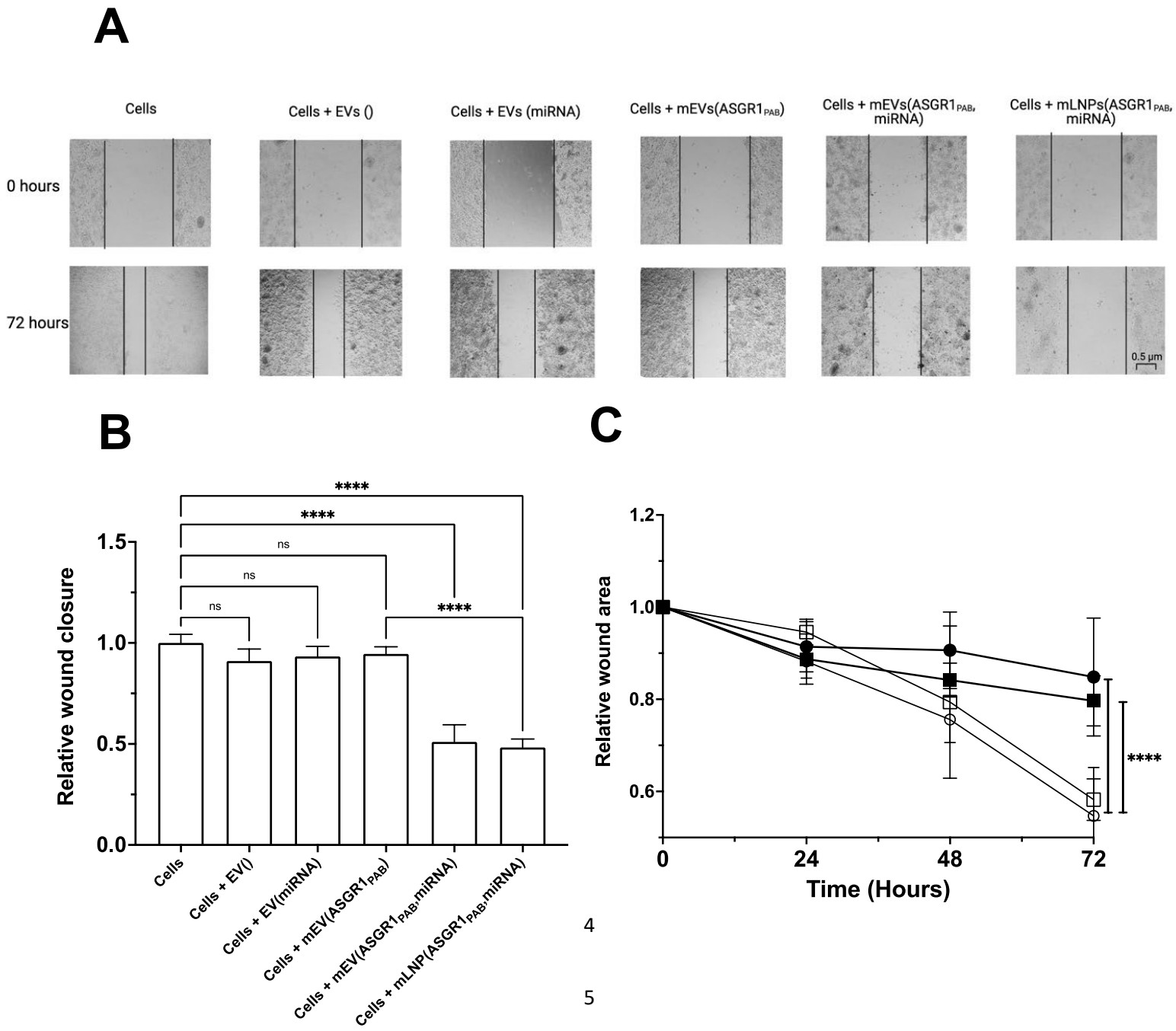

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6 S\

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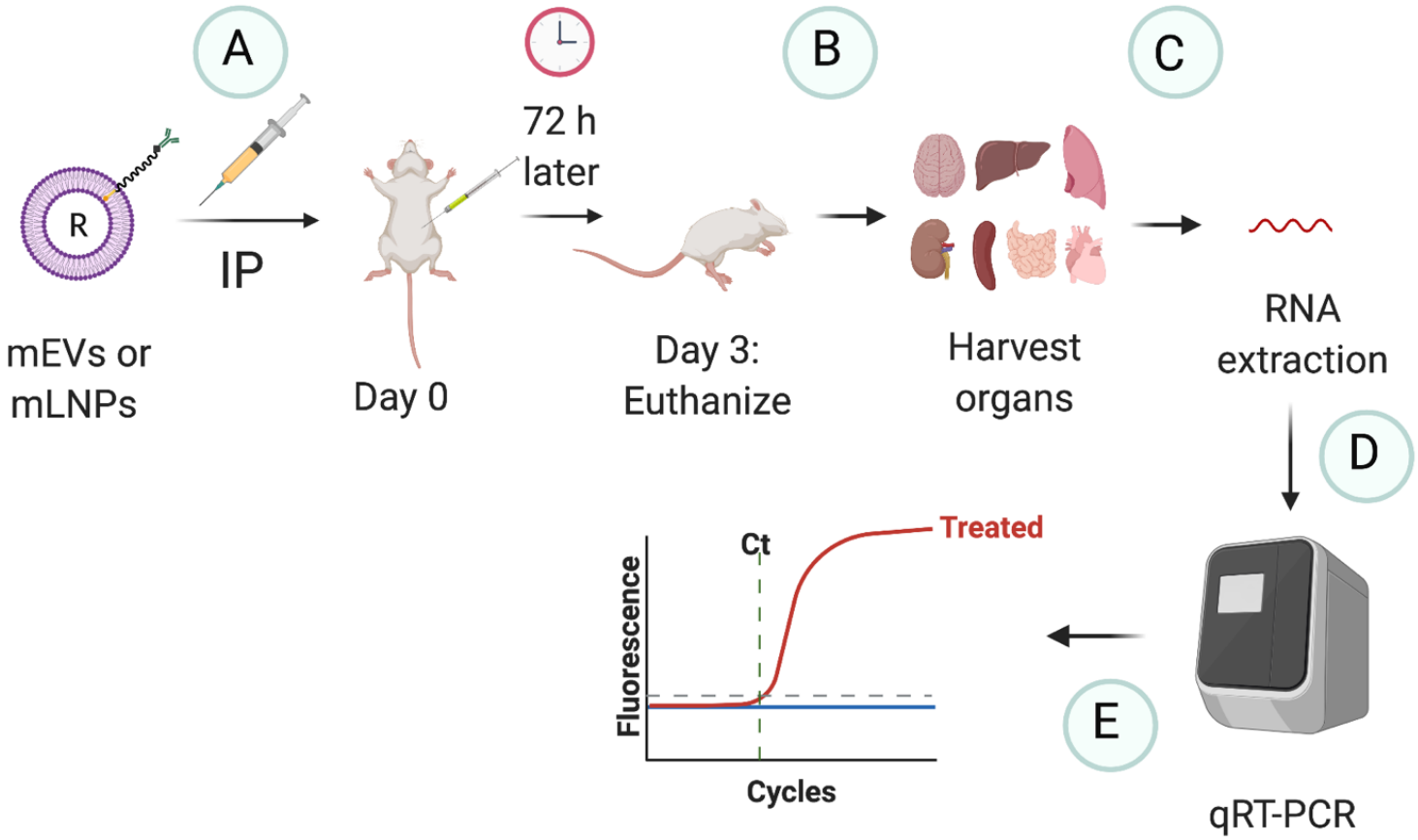
A
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E
R
IP
mEVs or
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Day 0
72 h
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Fluorescence
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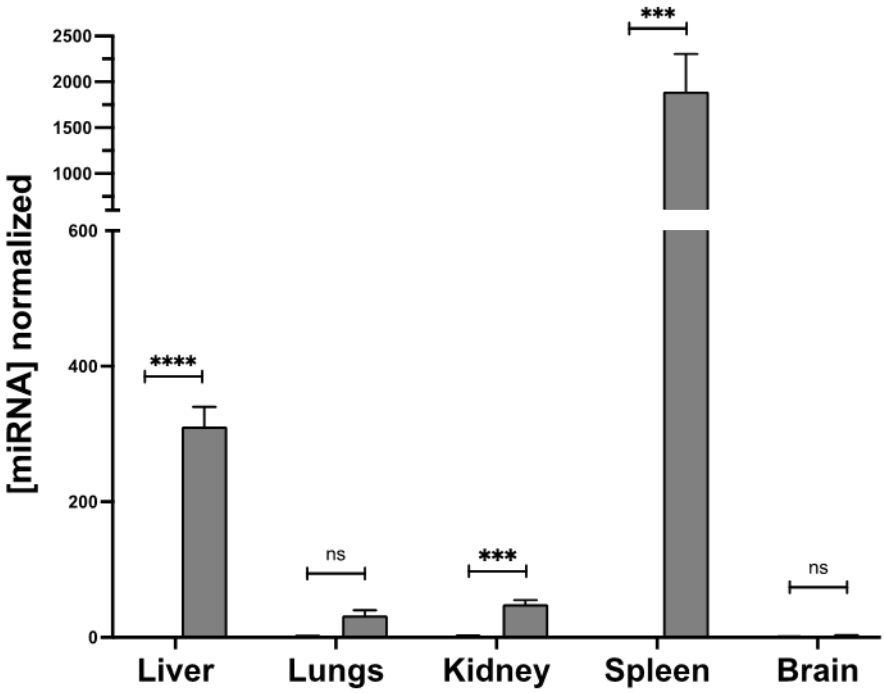
Figure 7.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Spleen
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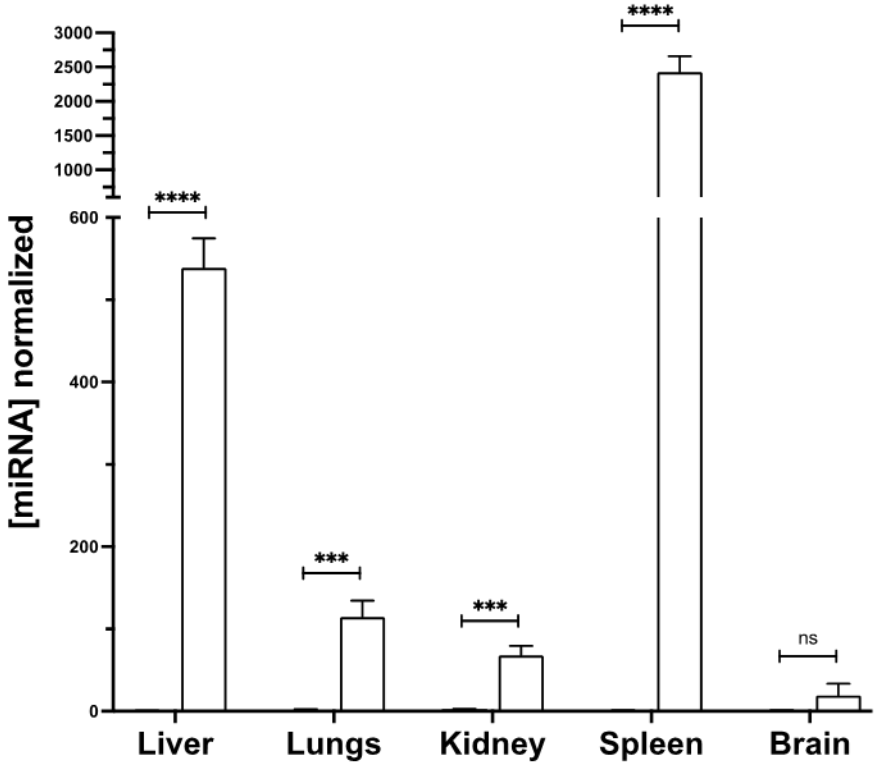
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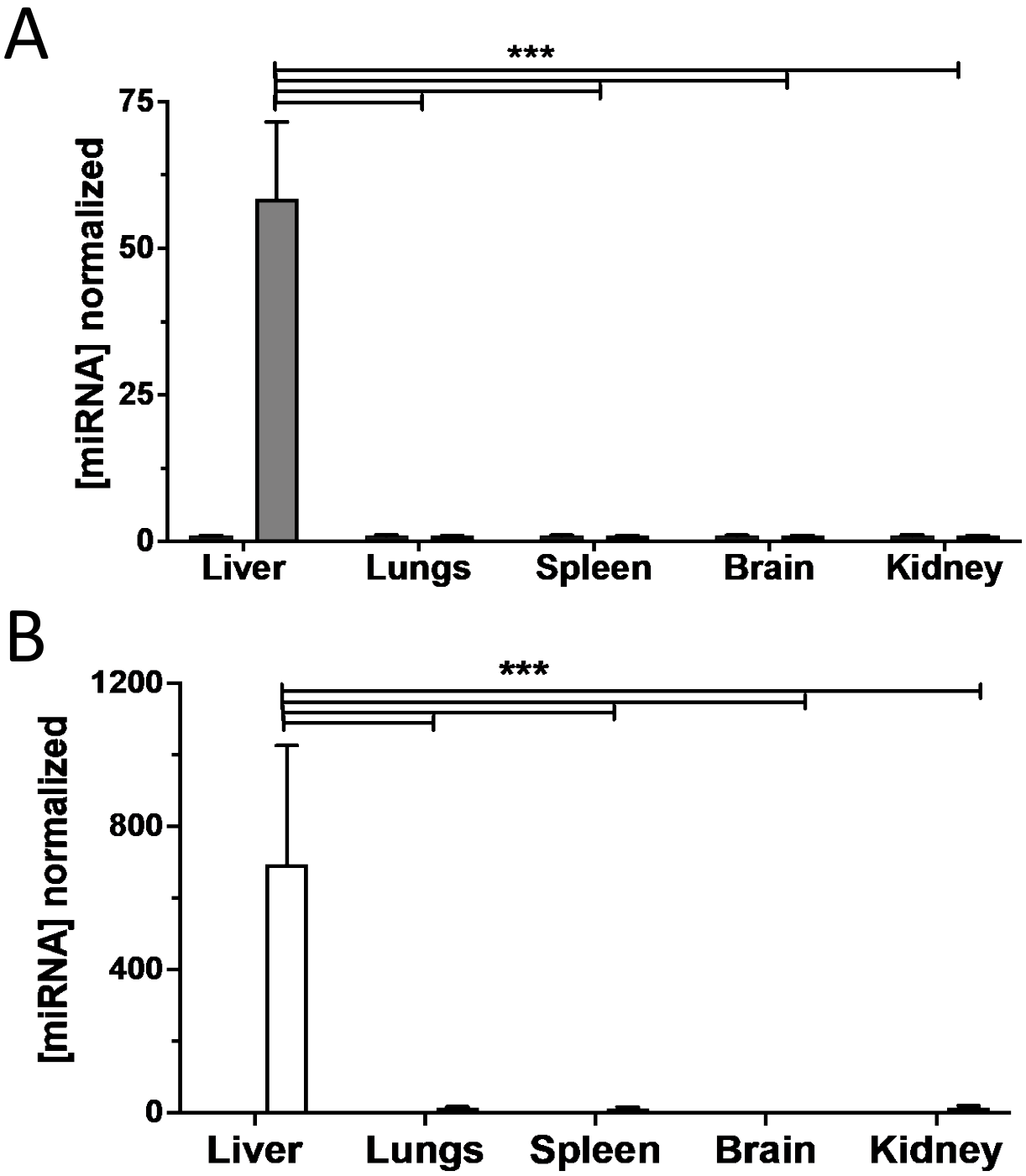
1 **Figure 8.**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Lungs
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Brain
Kidney

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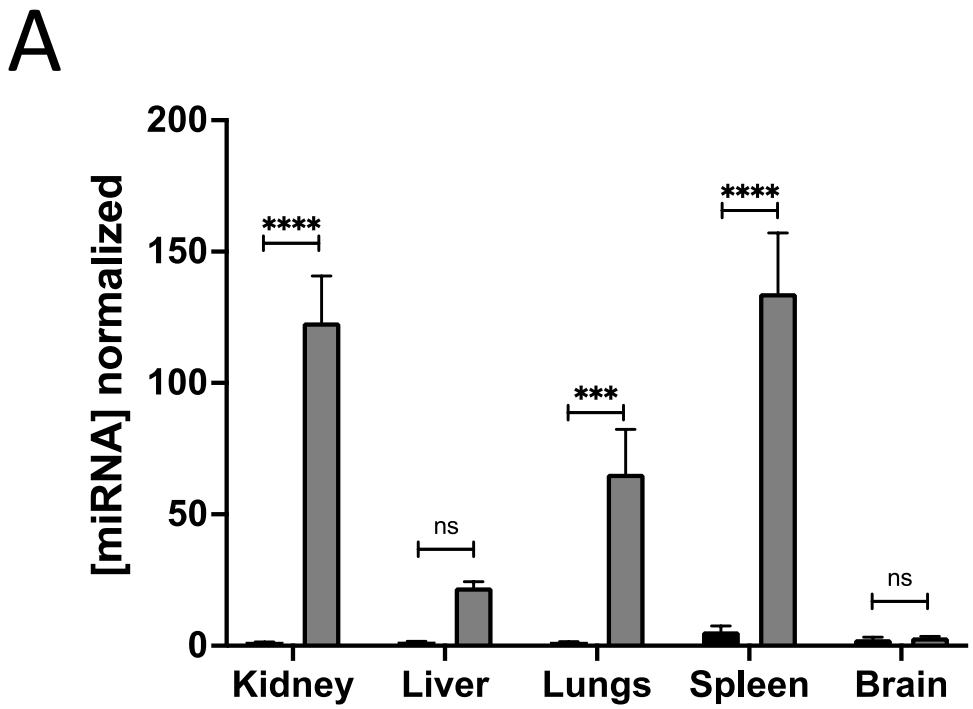
Figure 9.


A
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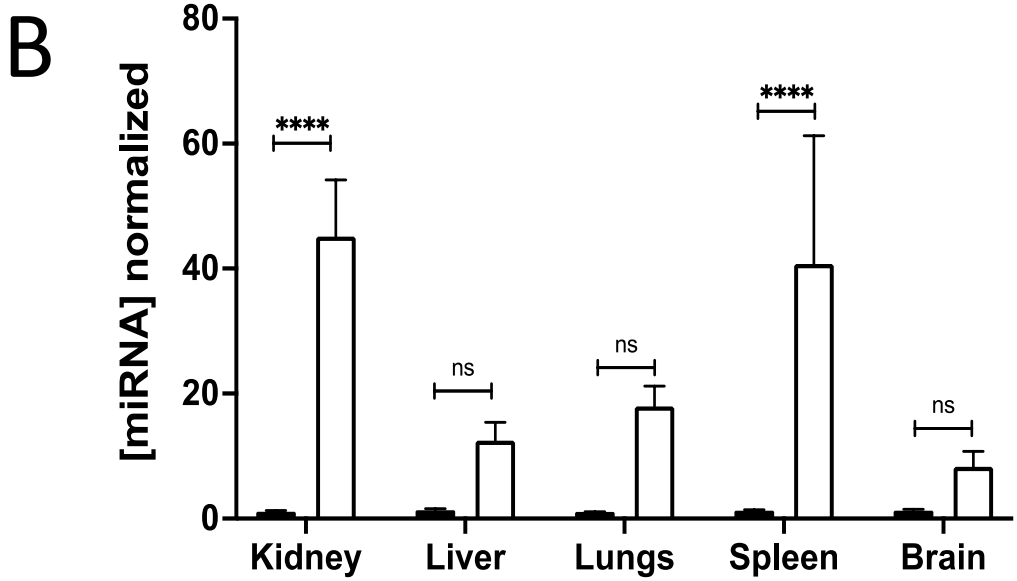
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1 **Figure 10.**

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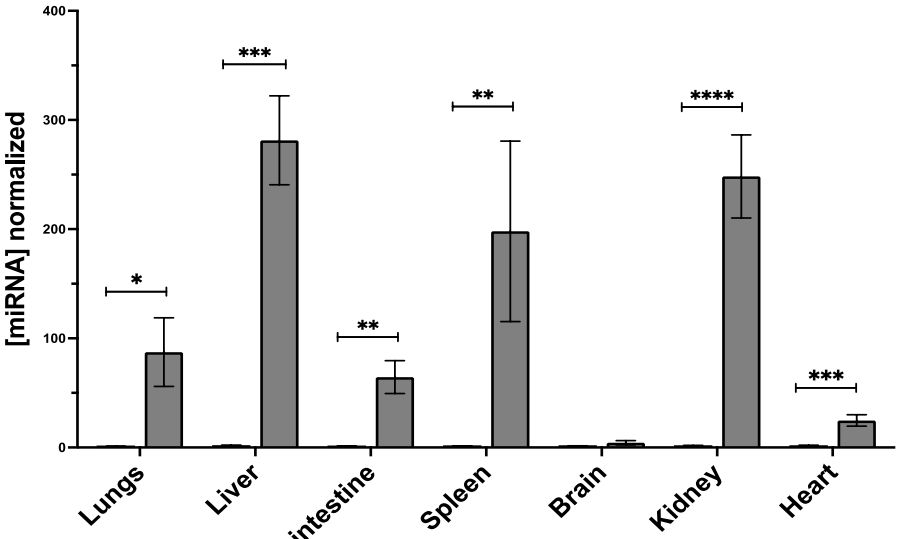

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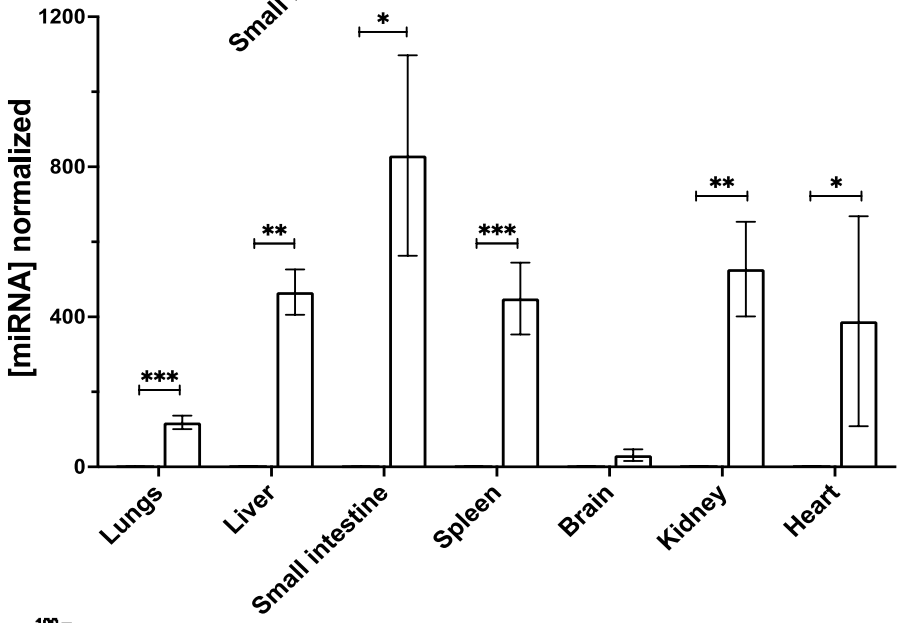
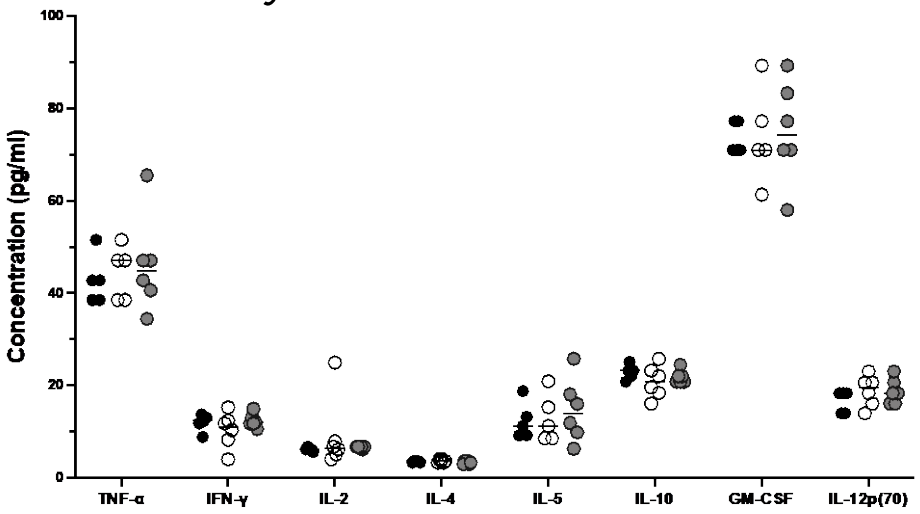


C



Bibliography

[1] J.K. Patra, G. Das, L.F. Fraceto, E.V.R. Campos, M. del P. Rodriguez-Torres, L.S. Acosta-Torres, L.A. Diaz-Torres, R. Grillo, M.K. Swamy, S. Sharma, S. Habtemariam, H.-S. Shin, Nano based drug delivery systems: recent developments and future prospects, Journal of Nanobiotechnology. 16 (2018) 71. https://doi.org/10.1186/s12951-018-0392-8.

[2] D. Das, N. Maity, A.H. V, Nanotechnology: a revolution in targeted drug delivery, International Journal of Basic & Clinical Pharmacology. 6 (2017) 2766–2773. https://doi.org/10.18203/2319-2003.ijbcp20175200.

[3] B.S. Zolnik, Á. González-Fernández, N. Sadrieh, M.A. Dobrovolskaia, Nanoparticles and the Immune System, Endocrinology. 151 (2010) 458–465. https://doi.org/10.1210/en.2009-1082.

[4] S. Su, P.M. Kang, Systemic Review of Biodegradable Nanomaterials in Nanomedicine, Nanomaterials (Basel). 10 (2020). https://doi.org/10.3390/nano10040656.

[5] X. Li, L. Wang, Y. Fan, Q. Feng, F. Cui, Biocompatibility and Toxicity of Nanoparticles and Nanotubes, Journal of Nanomaterials. 2012 (2012) e548389. https://doi.org/10.1155/2012/548389.

[6] F. Alexis, E. Pridgen, L.K. Molnar, O.C. Farokhzad, Factors Affecting the Clearance and Biodistribution of Polymeric Nanoparticles, Mol Pharm. 5 (2008) 505–515. https://doi.org/10.1021/mp800051m.

[7] S.T. Jahan, S.M.A. Sadat, M. Walliser, A. Haddadi, Targeted Therapeutic Nanoparticles: An Immense Promise to Fight against Cancer, Journal of Drug Delivery. 2017 (2017) e9090325. https://doi.org/10.1155/2017/9090325.

[8] X. Luan, K. Sansanaphongpricha, I. Myers, H. Chen, H. Yuan, D. Sun, Engineering exosomes as refined biological nanoplatforms for drug delivery, Acta Pharmacologica Sinica. 38 (2017) 754–763. https://doi.org/10.1038/aps.2017.12.

[9] A. Akbarzadeh, R. Rezaei-Sadabady, S. Davaran, S.W. Joo, N. Zarghami, Y. Hanifehpour, M. Samiei, M. Kouhi, K. Nejati-Koshki, Liposome: classification, preparation, and applications, Nanoscale Res Lett. 8 (2013) 102. https://doi.org/10.1186/1556-276X-8-102.

[10] E.M. Veziroglu, G.I. Mias, Characterizing Extracellular Vesicles and Their Diverse RNA Contents, Front Genet. 11 (2020) 700. https://doi.org/10.3389/fgene.2020.00700.

[11] R. Kalluri, V.S. LeBleu, The biology, function, and biomedical applications of exosomes, Science. 367 (2020). https://doi.org/10.1126/science.aau6977.

[12] J. Qin, Q. Xu, Functions and application of exosomes, Acta Pol Pharm. 71 (2014) 537–43.

[13] R.R. Sawant, V.P. Torchilin, Challenges in Development of Targeted Liposomal Therapeutics, AAPS J. 14 (2012) 303–315. https://doi.org/10.1208/s12248-012-9330-0.

[14] A. Samad, Y. Sultana, M. Aqil, Liposomal drug delivery systems: an update review, Curr Drug Deliv. 4 (2007) 297–305. https://doi.org/10.2174/156720107782151269.

[15] K. Bryniarski, W. Ptak, A. Jayakumar, K. Püllmann, M.J. Caplan, A. Chairoungdua, J. Lu, B.D. Adams, E. Sikora, K. Nazimek, S. Marquez, S.H. Kleinstein, P. Sangwung, Y. Iwakiri, E. Delgato, F. Redegeld, B.R. Blokhuis, J. Wojcikowski, A.W. Daniel, T.G. Kormelink, P.W. Askenase, Antigen-specific, antibody-coated, exosome-like nanovesicles deliver suppressor T-cell microRNA-150 to effector T cells to inhibit contact sensitivity, Journal of Allergy and Clinical Immunology. 132 (2013) 170-181.e9. https://doi.org/10.1016/j.jaci.2013.04.048.

[16] Y. Si, S. Kim, E. Zhang, Y. Tang, R. Jaskula-Sztul, J.M. Markert, H. Chen, L. Zhou, X. (Margaret) Liu, Targeted Exosomes for Drug Delivery: Biomanufacturing, Surface Tagging, and Validation, Biotechnology Journal. 15 (2020) 1900163. https://doi.org/10.1002/biot.201900163.

[17] A. Huang, L. Huang, S.J. Kennel, Monoclonal antibody covalently coupled with fatty acid. A reagent for in vitro liposome targeting., Journal of Biological Chemistry. 255 (1980) 8015–8018. https://doi.org/10.1016/S0021-9258(19)70595-X.

[18] J.L. Rigaud, D. Levy, Reconstitution of membrane proteins into liposomes, Methods in Enzymology. 372 (2003) 65–86. https://doi.org/10.1016/S0076-6879(03)72004-7.

[19] L.E. Westerman, P.E. Jensen, Liposomes Composed of Reconstituted Membranes for Induction of Tumor-Specific Immunity, in: Methods in Enzymology, Academic Press, 2003: pp. 118–127. https://doi.org/10.1016/S0076-6879(03)73008-0.

[20] A. Huang, S.J. Kennel, L. Huang, Immunoliposome labeling: A sensitive and specific method for cell surface labeling, Journal of Immunological Methods. 46 (1981) 141–151. https://doi.org/10.1016/0022-1759(81)90131-9.

[21] H. Alpes, K. Allmann, H. Plattner, J. Reichert, R. Rick, S. Schulz, Formation of large unilamellar vesicles using alkyl maltoside detergents, Biochimica et Biophysica Acta (BBA) - Biomembranes. 862 (1986) 294–302. https://doi.org/10.1016/0005-2736(86)90231-2.

[22] L. Huang, United States Patent: 4957735 - Target-sensitive immunoliposomes-preparation and characterization, 4957735, 1990. https://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&d=PALL&p=1&u=%2Fnetahtml%2FPTO%2Fsrchnum.htm&r=1&f=G&l=50&s1=4,957,735.PN.&OS=PN/4,957,735&RS=PN/4,957,735 (accessed August 31, 2021).

[23] R.J.Y. Ho, B.T. Rouse, L. Huang, Target-sensitive immunoliposomes: preparation and characterization, Biochemistry. 25 (1986) 5500–5506. https://doi.org/10.1021/bi00367a023.

[24] M. Micklus, N. Greig, S. Rapoport, Targeting of liposomes to the blood-brain barrier, US20020025313A1, 2002. https://patents.google.com/patent/US20020025313A1/en (accessed August 21, 2021).

[25] Y. Cheng, Q. Zeng, Q. Han, W. Xia, Effect of pH, temperature and freezing-thawing on quantity changes and cellular uptake of exosomes, Protein Cell. 10 (2019) 295–299. https://doi.org/10.1007/s13238-018-0529-4.

[26] H. Ma, C. Ó'Fágáin, R. O'Kennedy, Antibody stability: A key to performance - Analysis, influences and improvement, Biochimie. 177 (2020) 213–225. https://doi.org/10.1016/j.biochi.2020.08.019.

[27] C.I.E. Smith, R. Zain, Therapeutic Oligonucleotides: State of the Art, Annual Review of Pharmacology and Toxicology. 59 (2019) 605–630. https://doi.org/10.1146/annurev-pharmtox-010818-021050.

[28] T.C. Roberts, R. Langer, M.J.A. Wood, Advances in oligonucleotide drug delivery, Nat Rev Drug Discov. 19 (2020) 673–694. https://doi.org/10.1038/s41573-020-0075-7.

[29] K.E. Lundin, O. Gissberg, C.I.E. Smith, R. Zain, Chemical Development of Therapeutic Oligonucleotides, in: O. Gissberg, R. Zain, K.E. Lundin (Eds.), Oligonucleotide-Based Therapies: Methods and Protocols, Springer, New York, NY, 2019: pp. 3–16. https://doi.org/10.1007/978-1-4939-9670-4_1.

[30] K. Tatiparti, S. Sau, S.K. Kashaw, A.K. Iyer, siRNA Delivery Strategies: A Comprehensive Review of Recent Developments, Nanomaterials. 7 (2017) 77. https://doi.org/10.3390/nano7040077.

[31] K. Gavrilov, W.M. Saltzman, Therapeutic siRNA: Principles, Challenges, and Strategies, Yale J Biol Med. 85 (2012) 187–200.

[32] K. Dhuri, C. Bechtold, E. Quijano, H. Pham, A. Gupta, A. Vikram, R. Bahal, Antisense Oligonucleotides: An Emerging Area in Drug Discovery and Development, Journal of Clinical Medicine. 9 (2020) 2004. https://doi.org/10.3390/jcm9062004.

[33] M. Gagliardi, A.T. Ashizawa, The Challenges and Strategies of Antisense Oligonucleotide Drug Delivery, Biomedicines. 9 (2021) 433. https://doi.org/10.3390/biomedicines9040433.

[34] R. Rupaimoole, F.J. Slack, MicroRNA therapeutics: towards a new era for the management of cancer and other diseases, Nat Rev Drug Discov. 16 (2017) 203–222. https://doi.org/10.1038/nrd.2016.246.

[35] R.C. Wilson, J.A. Doudna, Molecular Mechanisms of RNA Interference, Annual Review of Biophysics. 42 (2013) 217–239. https://doi.org/10.1146/annurev-biophys-083012-130404.

[36] M.M. Zhang, R. Bahal, T.P. Rasmussen, J.E. Manautou, X. Zhong, The growth of siRNA-based therapeutics: Updated clinical studies, Biochemical Pharmacology. 189 (2021) 114432. https://doi.org/10.1016/j.bcp.2021.114432.

[37] S. Akhtar, M.D. Hughes, A. Khan, M. Bibby, M. Hussain, Q. Nawaz, J. Double, P. Sayyed, The delivery of antisense therapeutics, Advanced Drug Delivery Reviews. 44 (2000) 3–21. https://doi.org/10.1016/S0169-409X(00)00080-6.

[38] J. O'Brien, H. Hayder, Y. Zayed, C. Peng, Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation, Front. Endocrinol. 9 (2018). https://doi.org/10.3389/fendo.2018.00402.

[39] C.E. Condrat, D.C. Thompson, M.G. Barbu, O.L. Bugnar, A. Boboc, D. Cretoiu, N. Suciu, S.M. Cretoiu, S.C. Voinea, miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis, Cells. 9 (2020). https://doi.org/10.3390/cells9020276.

[40] W. Si, J. Shen, H. Zheng, W. Fan, The role and mechanisms of action of microRNAs in cancer drug resistance, Clinical Epigenetics. 11 (2019) 25. https://doi.org/10.1186/s13148-018-0587-8.

[41] A. Jeyaram, S.M. Jay, Preservation and Storage Stability of Extracellular Vesicles for Therapeutic Applications, AAPS J. 20 (2017) 1. https://doi.org/10.1208/s12248-017-0160-y.

[42] S.K. Panda, B. Ravindran, F. Histopaque, Isolation of human PBMCs, Bio-Protocol. 3 (2013) e323.

[43] G.E. Moore, R.E. Gerner, H.A. Franklin, Culture of Normal Human Leukocytes, JAMA. 199 (1967) 519–524. https://doi.org/10.1001/jama.1967.03120080053007.

[44] B. Mui, L. Chow, M.J. Hope, Extrusion technique to generate liposomes of defined size, Methods in Enzymology. 367 (2003) 3–14. https://doi.org/10.1016/S0076-6879(03)67001-1.

[45] K.V. Ledwitch, R.W. Barnes, A.G. Roberts, Unravelling the complex drug-drug interactions of the cardiovascular drugs, verapamil and digoxin, with P-glycoprotein, Biosci. Rep. 36 (2016) 1–14. https://doi.org/10.1042/BSR20150317.

[46] J.P. Kampf, D. Cupp, A.M. Kleinfeld, Different Mechanisms of Free Fatty Acid Flip-Flop and Dissociation Revealed by Temperature and Molecular Species Dependence of Transport across Lipid Vesicles*, Journal of Biological Chemistry. 281 (2006) 21566–21574. https://doi.org/10.1074/jbc.M602067200.

[47] S. Hupfeld, A.M. Holsæter, M. Skar, C.B. Frantzen, M. Brandl, Liposome Size Analysis by Dynamic/Static Light Scattering upon Size Exclusion-/Field Flow-Fractionation, Journal of Nanoscience and Nanotechnology. 6 (2006) 3025–3031. https://doi.org/10.1166/jnn.2006.454.

[48] G. Liang, S. Kan, Y. Zhu, S. Feng, W. Feng, S. Gao, Engineered exosome-mediated delivery of functionally active miR-26a and its enhanced suppression

effect in HepG2 cells, Int J Nanomedicine. 13 (2018) 585–599. https://doi.org/10.2147/IJN.S154458.

[49] O. López, A. de la Maza, L. Coderch, C. López-Iglesias, E. Wehrli, J.L. Parra, Direct formation of mixed micelles in the solubilization of phospholipid liposomes by Triton X-100, FEBS Letters. 426 (1998) 314–318. https://doi.org/10.1016/S0014-5793(98)00363-9.

[50] V. Palmieri, D. Lucchetti, I. Gatto, A. Maiorana, M. Marcantoni, G. Maulucci, M. Papi, R. Pola, M. De Spirito, A. Sgambato, Dynamic light scattering for the characterization and counting of extracellular vesicles: a powerful noninvasive tool, J Nanopart Res. 16 (2014) 2583. https://doi.org/10.1007/s11051-014-2583-z.

[51] R. Szatanek, M. Baj-Krzyworzeka, J. Zimoch, M. Lekka, M. Siedlar, J. Baran, The Methods of Choice for Extracellular Vesicles (EVs) Characterization, Int J Mol Sci. 18 (2017). https://doi.org/10.3390/ijms18061153.

[52] I. Makra, P. Terejánszky, R.E. Gyurcsányi, A method based on light scattering to estimate the concentration of virus particles without the need for virus particle standards, MethodsX. 2 (2015) 91–99. https://doi.org/10.1016/j.mex.2015.02.003.

[53] L. Alvarez-Erviti, Y. Seow, H. Yin, C. Betts, S. Lakhal, M.J.A. Wood, Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes, Nature Biotechnology. 29 (2011) 341–345. https://doi.org/10.1038/nbt.1807.

[54] S. Pirkmajer, A.V. Chibalin, Serum starvation: caveat emptor, American Journal of Physiology-Cell Physiology. 301 (2011) C272–C279. https://doi.org/10.1152/ajpcell.00091.2011.

[55] M.W. Pfaffl, Relative quantitation, in: M.T. Dorak (Ed.), Real-Time PCR, 1st edition, Taylor & Francis, 2007.

[56] A.L. Didychuk, S.E. Butcher, D.A. Brow, The life of U6 small nuclear RNA, from cradle to grave, RNA. 24 (2018) 437–460. https://doi.org/10.1261/rna.065136.117.

[57] R.L. Causin, D. Pessôa-Pereira, K.C.B. Souza, A.F. Evangelista, R.M.V. Reis, J.H.T.G. Fregnani, M.M.C. Marques, Identification and performance evaluation of housekeeping genes for microRNA expression normalization by reverse transcription-quantitative PCR using liquid-based cervical cytology samples, Oncol Lett. 18 (2019) 4753–4761. https://doi.org/10.3892/ol.2019.10824.

[58] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C T method, Nature Protocols. 3 (2008) 1101–1108. https://doi.org/10.1038/nprot.2008.73.

[59] I. Hamming, M. Cooper, B. Haagmans, N. Hooper, R. Korstanje, A. Osterhaus, W. Timens, A. Turner, G. Navis, H. van Goor, The emerging role of ACE2 in physiology and disease, J Pathol. 212 (2007) 1–11. https://doi.org/10.1002/path.2162.

[60] I. Hamming, W. Timens, M. Bulthuis, A. Lely, G. Navis, H. van Goor, Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis, J Pathol. 203 (2004) 631–637. https://doi.org/10.1002/path.1570.

[61] C. Km, S. Ml, R. An, K. La, Carbon Dioxide for Euthanasia: Concerns Regarding Pain and Distress, With Special Reference to Mice and Rats, Laboratory Animals. 39 (2005). https://doi.org/10.1258/0023677053739747.

[62] TRIzol Products - US, (n.d.). https://www.thermofisher.com/us/en/home/brands/product-brand/trizol.html (accessed May 16, 2020).

[63] Bio-Plex Pro™ Mouse Cytokine, Chemokine, and Growth Factor Assays | Life Science Research | Bio-Rad, (n.d.). https://www.bio-rad.com/en-us/category/bio-plex-pro-mouse-cytokine-chemokine-growth-factor-assays?ID=37dd4c58-2aa7-4991-a7cb-5c51024959c6 (accessed December 11, 2020).

[64] L.A. Mulcahy, R.C. Pink, D.R.F. Carter, Routes and mechanisms of extracellular vesicle uptake, J Extracell Vesicles. 3 (2014). https://doi.org/10.3402/jev.v3.24641.

[65] G. Costaguta, G.S. Payne, Overview of Protein Trafficking Mechanisms, in: N. Segev (Ed.), Trafficking Inside Cells: Pathways, Mechanisms and Regulation, Springer, New York, NY, 2009: pp. 105–118. https://doi.org/10.1007/978-0-387-93877-6_6.

[66] A. Perrakis, W.H. Moolenaar, Autotaxin: structure-function and signaling, J. Lipid Res. 55 (2014) 1010–1018. https://doi.org/10.1194/jlr.R046391.

[67] J. Stetefeld, S.A. McKenna, T.R. Patel, Dynamic light scattering: a practical guide and applications in biomedical sciences, Biophys Rev. 8 (2016) 409–427. https://doi.org/10.1007/s12551-016-0218-6.

[68] P.M. Carvalho, M.R. Felício, N.C. Santos, S. Gonçalves, M.M. Domingues, Application of Light Scattering Techniques to Nanoparticle Characterization and Development, Frontiers in Chemistry. 6 (2018) 237. https://doi.org/10.3389/fchem.2018.00237.

[69] K.L. Linegar, A.E. Adeniran, A.F. Kostko, M.A. Anisimov, Hydrodynamic radius of polyethylene glycol in solution obtained by dynamic light scattering, Colloid J. 72 (2010) 279–281. https://doi.org/10.1134/S1061933X10020195.

[70] S. Niimi, [Determination of the particle size and relative light scattering intensity of aggregates of human IgG and humanized monoclonal antibody product induced by various stress using dynamic light scattering], Kokuritsu Iyakuhin Shokuhin Eisei Kenkyusho Hokoku. (2011) 55–60.

[71] S. Hu, L. Musante, D. Tataruch, X. Xu, O. Kretz, M. Henry, P. Meleady, H. Luo, H. Zou, Y. Jiang, H. Holthofer, Purification and Identification of Membrane Proteins from Urinary Extracellular Vesicles using Triton X-114 Phase Partitioning, J. Proteome Res. 17 (2018) 86–96. https://doi.org/10.1021/acs.jproteome.7b00386.

[72] M.T. Donato, L. Tolosa, M.J. Gómez-Lechón, Culture and Functional Characterization of Human Hepatoma HepG2 Cells, Methods Mol. Biol. 1250 (2015) 77–93. https://doi.org/10.1007/978-1-4939-2074-7_5.

[73] S. Wilkening, F. Stahl, A. Bader, Comparison of Primary Human Hepatocytes and Hepatoma Cell Line Hepg2 with Regard to Their Biotransformation Properties, Drug Metab Dispos. 31 (2003) 1035–1042. https://doi.org/10.1124/dmd.31.8.1035.

[74] G.-H. Qiu, X. Xie, F. Xu, X. Shi, Y. Wang, L. Deng, Distinctive pharmacological differences between liver cancer cell lines HepG2 and Hep3B, Cytotechnology. 67 (2015) 1–12. https://doi.org/10.1007/s10616-014-9761-9.

[75] D. Witzigmann, L. Quagliata, S.H. Schenk, C. Quintavalle, L.M. Terracciano, J. Huwyler, Variable asialoglycoprotein receptor 1 expression in liver disease:

Implications for therapeutic intervention, Hepatology Research. 46 (2016) 686–696. https://doi.org/10.1111/hepr.12599.

[76] S. Das, P. Kudale, P. Dandekar, P.V. Devarajan, Asialoglycoprotein Receptor and Targeting Strategies, in: P.V. Devarajan, P. Dandekar, A.A. D'Souza (Eds.), Targeted Intracellular Drug Delivery by Receptor Mediated Endocytosis, Springer International Publishing, Cham, 2019: pp. 353–381. https://doi.org/10.1007/978-3-030-29168-6_12.

[77] Z. Shen, W. Wei, H. Tanaka, K. Kohama, G. Ma, T. Dobashi, Y. Maki, H. Wang, J. Bi, S. Dai, A galactosamine-mediated drug delivery carrier for targeted liver cancer therapy, Pharmacological Research. 64 (2011) 410–419. https://doi.org/10.1016/j.phrs.2011.06.015.

[78] S. Pranatharthiharan, M.D. Patel, V.C. Malshe, V. Pujari, A. Gorakshakar, M. Madkaikar, K. Ghosh, P.V. Devarajan, Asialoglycoprotein receptor targeted delivery of doxorubicin nanoparticles for hepatocellular carcinoma, Drug Delivery. 24 (2017) 20–29. https://doi.org/10.1080/10717544.2016.1225856.

[79] M. Wei, Y. Xu, Q. Zou, L. Tu, C. Tang, T. Xu, L. Deng, C. Wu, Hepatocellular carcinoma targeting effect of PEGylated liposomes modified with lactoferrin, European Journal of Pharmaceutical Sciences. 46 (2012) 131–141. https://doi.org/10.1016/j.ejps.2012.02.007.

[80] A.A. D'Souza, P.V. Devarajan, Asialoglycoprotein receptor mediated hepatocyte targeting — Strategies and applications, Journal of Controlled Release. 203 (2015) 126–139. https://doi.org/10.1016/j.jconrel.2015.02.022.

[81] A.G. Roberts, The Structure and Mechanism of Drug Transporters, in: S. Nagar, U.A. Argikar, D. Tweedie (Eds.), Enzyme Kinetics in Drug Metabolism: Fundamentals and Applications, Springer US, New York, NY, 2021: pp. 193–234. https://doi.org/10.1007/978-1-0716-1554-6_8.

[82] J. Bentz, M.P. O'Connor, D. Bednarczyk, J. Coleman, C. Lee, J. Palm, Y.A. Pak, E.S. Perloff, E. Reyner, P. Balimane, M. Brännström, X. Chu, C. Funk, A. Guo, I. Hanna, K. Herédi-Szabó, K. Hillgren, L. Li, E. Hollnack-Pusch, M. Jamei, X. Lin, A.K. Mason, S. Neuhoff, A. Patel, L. Podila, E. Plise, G. Rajaraman, L. Salphati, E. Sands, M.E. Taub, J.-S. Taur, D. Weitz, H.M. Wortelboer, C.Q. Xia, G. Xiao, J. Yabut, T. Yamagata, L. Zhang, H. Ellens, Variability in P-Glycoprotein Inhibitory Potency (IC50) Using Various in Vitro Experimental Systems: Implications for Universal Digoxin Drug-Drug Interaction Risk Assessment Decision Criteria, Drug Metab Dispos. 41 (2013) 1347–1366. https://doi.org/10.1124/dmd.112.050500.

[83] L.-B. Goh, K.J. Spears, D. Yao, A. Ayrton, P. Morgan, C. Roland Wolf, T. Friedberg, Endogenous drug transporters in in vitro and in vivo models for the prediction of drug disposition in man, Biochemical Pharmacology. 64 (2002) 1569–1578. https://doi.org/10.1016/S0006-2952(02)01355-2.

[84] J.D. Dukes, P. Whitley, A.D. Chalmers, The MDCK variety pack: choosing the right strain, BMC Cell Biol. 12 (2011) 43. https://doi.org/10.1186/1471-2121-12-43.

[85] J. Li, Y. Wang, I.J. Hidalgo, Kinetic analysis of human and canine P-glycoprotein-mediated drug transport in MDR1–MDCK cell model: Approaches to reduce false-negative substrate classification, Journal of Pharmaceutical Sciences. 102 (2013) 3436–3446. https://doi.org/10.1002/jps.23523.

[86] P. Anderle, E. Niederer, W. Rubas, C. Hilgendorf, H. Spahn-Langguth, H. Wunderli-Allenspach, H.P. Merkle, P. Langguth, P-Glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: the influence of culturing conditions and drug exposure on P-gp expression levels, J Pharm Sci. 87 (1998) 757–762. https://doi.org/10.1021/js970372e.

[87] L. Bao, S. Hazari, S. Mehra, D. Kaushal, K. Moroz, S. Dash, Increased expression of P-glycoprotein and doxorubicin chemoresistance of metastatic breast cancer is regulated by miR-298, Am. J. Pathol. 180 (2012) 2490–2503. https://doi.org/10.1016/j.ajpath.2012.02.024.

[88] Y. Xie, Y. Shao, X. Deng, M. Wang, Y. Chen, MicroRNA-298 Reverses Multidrug Resistance to Antiepileptic Drugs by Suppressing MDR1/P-gp Expression in vitro, Front Neurosci. 12 (2018). https://doi.org/10.3389/fnins.2018.00602.

[89] A. Kozomara, M. Birgaoanu, S. Griffiths-Jones, miRBase: from microRNA sequences to function, Nucleic Acids Res. 47 (2019) D155–D162. https://doi.org/10.1093/nar/gky1141.

[90] A. Marco, J.I. MacPherson, M. Ronshaugen, S. Griffiths-Jones, MicroRNAs from the same precursor have different targeting properties, Silence. 3 (2012) 8. https://doi.org/10.1186/1758-907X-3-8.

[91] P.E. Marques, S. Nyegaard, R.F. Collins, F. Troise, S.A. Freeman, W.S. Trimble, S. Grinstein, Multimerization and Retention of the Scavenger Receptor SR-B1 in the Plasma Membrane, Developmental Cell. 50 (2019) 283-295.e5. https://doi.org/10.1016/j.devcel.2019.05.026.

[92] D. Sahoo, Y.F. Darlington, D. Pop, D.L. Williams, M.A. Connelly, Scavenger receptor class B Type I (SR-BI) assembles into detergent-sensitive dimers and tetramers, Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 1771 (2007) 807–817. https://doi.org/10.1016/j.bbalip.2006.03.003.

[93] X. Cui, K. Song, X. Lu, W. Feng, W. Di, Liposomal Delivery of MicroRNA-7 Targeting EGFR to Inhibit the Growth, Invasion, and Migration of Ovarian Cancer, ACS Omega. (2021). https://doi.org/10.1021/acsomega.1c00992.

[94] M.T.D. Martino, V. Campani, G. Misso, M.E.G. Cantafio, A. Gullà, U. Foresta, P.H. Guzzi, M. Castellano, A. Grimaldi, V. Gigantino, R. Franco, S. Lusa, M. Cannataro, P. Tagliaferri, G.D. Rosa, P. Tassone, M. Caraglia, In Vivo Activity of MiR-34a Mimics Delivered by Stable Nucleic Acid Lipid Particles (SNALPs) against Multiple Myeloma, PLOS ONE. 9 (2014) e90005. https://doi.org/10.1371/journal.pone.0090005.

[95] A. Stamm, K. Reimers, S. Strauß, P. Vogt, T. Scheper, I. Pepelanova, In vitro wound healing assays – state of the art, BioNanoMaterials. 17 (2016) 79–87. https://doi.org/10.1515/bnm-2016-0002.

[96] J.E.N. Jonkman, J.A. Cathcart, F. Xu, M.E. Bartolini, J.E. Amon, K.M. Stevens, P. Colarusso, An introduction to the wound healing assay using live-cell microscopy, Cell Adhesion & Migration. 8 (2014) 440–451. https://doi.org/10.4161/cam.36224.

[97] M.J. Haney, Y. Zhao, J. Fay, H. Duhyeong, M. Wang, H. Wang, Z. Li, Y.Z. Lee, M.K. Karuppan, N. El-Hage, A.V. Kabanov, E.V. Batrakova, Genetically modified macrophages accomplish targeted gene delivery to the inflamed brain in transgenic

Parkin Q311X(A) mice: importance of administration routes, Scientific Reports. 10 (2020) 11818. https://doi.org/10.1038/s41598-020-68874-7.

[98] O.P.B. Wiklander, J.Z. Nordin, A. O'Loughlin, Y. Gustafsson, G. Corso, I. Mäger, P. Vader, Y. Lee, H. Sork, Y. Seow, N. Heldring, L. Alvarez-Erviti, C.E. Smith, K. Le Blanc, P. Macchiarini, P. Jungebluth, M.J.A. Wood, S.E. Andaloussi, Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting, J Extracell Vesicles. 4 (2015) 10.3402/jev.v4.26316. https://doi.org/10.3402/jev.v4.26316.

[99] P. Rosa, F. Clementi, Absorption and Tissue Distribution of Doxorubicin Entrapped in Liposomes following Intravenous or Intraperitoneal Administration, PHA. 26 (1983) 221–229. https://doi.org/10.1159/000137805.

[100] G. Lee, S. Han, I. Inocencio, E. Cao, J. Hong, A.R.J. Phillips, J.A. Windsor, C.J.H. Porter, N.L. Trevaskis, Lymphatic Uptake of Liposomes after Intraperitoneal Administration Primarily Occurs via the Diaphragmatic Lymphatics and is Dependent on Liposome Surface Properties, Mol. Pharmaceutics. 16 (2019) 4987–4999. https://doi.org/10.1021/acs.molpharmaceut.9b00855.

[101] Z. Xueying, L. Zhelong, S. Wenqi, Y. Guodong, X. Changyang, Y. Lijun, Delivery Efficacy Differences of Intravenous and Intraperitoneal Injection of Exosomes: Perspectives from Tracking Dye Labeled and MiRNA Encapsulated Exosomes, Current Drug Delivery. 17 (2020) 186–194.

[102] H. Sun, D. Zhong, C. Wang, Y. Sun, J. Zhao, G. Li, MiR-298 Exacerbates Ischemia/Reperfusion Injury Following Ischemic Stroke by Targeting Act1, CPB. 48 (2018) 528–539. https://doi.org/10.1159/000491810.

[103] V. Boissonneault, I. Plante, S. Rivest, P. Provost, MicroRNA-298 and MicroRNA-328 Regulate Expression of Mouse β-Amyloid Precursor Protein-converting Enzyme 1 *, Journal of Biological Chemistry. 284 (2009) 1971–1981. https://doi.org/10.1074/jbc.M807530200.

[104] D. Barbagallo, S. Piro, A.G. Condorelli, L.G. Mascali, F. Urbano, N. Parrinello, A. Monello, L. Statello, M. Ragusa, A.M. Rabuazzo, C. Di Pietro, F. Purrello, M. Purrello, miR-296-3p, miR-298-5p and their downstream networks are causally involved in the higher resistance of mammalian pancreatic α cells to cytokine-induced apoptosis as compared to β cells, BMC Genomics. 14 (2013) 62. https://doi.org/10.1186/1471-2164-14-62.

[105] N. Bushati, S.M. Cohen, microRNA Functions, Annual Review of Cell and Developmental Biology. 23 (2007) 175–205. https://doi.org/10.1146/annurev.cellbio.23.090506.123406.

[106] X. Li, M. Commane, H. Nie, X. Hua, M. Chatterjee-Kishore, D. Wald, M. Haag, G.R. Stark, Act1, an NF-κB-activating protein, PNAS. 97 (2000) 10489–10493. https://doi.org/10.1073/pnas.160265197.

[107] V. Agarwal, G.W. Bell, J.-W. Nam, D.P. Bartel, Predicting effective microRNA target sites in mammalian mRNAs, ELife. 4 (2015) e05005. https://doi.org/10.7554/eLife.05005.

[108] Y. Chen, X. Wang, miRDB: an online database for prediction of functional microRNA targets, Nucleic Acids Research. 48 (2020) D127–D131. https://doi.org/10.1093/nar/gkz757.

[109] L.-L. Kong, X.-M. Zhuang, H.-Y. Yang, M. Yuan, L. Xu, H. Li, Inhibition of P-glycoprotein Gene Expression and Function Enhances Triptolide-induced Hepatotoxicity in Mice, Sci Rep. 5 (2015) 11747. https://doi.org/10.1038/srep11747.

[110] R.E. Mebius, G. Kraal, Structure and function of the spleen, Nat Rev Immunol. 5 (2005) 606–616. https://doi.org/10.1038/nri1669.

[111] L.A. Medina, R. Klipper, W.T. Phillips, B. Goins, Pharmacokinetics and biodistribution of [111In]-avidin and [99mTc]-biotin-liposomes injected in the pleural space for the targeting of mediastinal nodes, Nuclear Medicine and Biology. 31 (2004) 41–51. https://doi.org/10.1016/S0969-8051(03)00122-7.

[112] K. Schwarz, M. Simons, J. Reiser, M.A. Saleem, C. Faul, W. Kriz, A.S. Shaw, L.B. Holzman, P. Mundel, Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin, J Clin Invest. 108 (2001) 1621–1629. https://doi.org/10.1172/JCI12849.

[113] Polyclonal vs. monoclonal antibodies, (n.d.). https://www.ptglab.com/news/blog/polyclonal-vs-monoclonal-antibodies/ (accessed July 30, 2021).

[114] What is a polyclonal antibody? - Ximbio FAQ, (n.d.). https://ximbio.com/faq/57/what-is-a-polyclonal-antibody (accessed July 30, 2021).

[115] P. Towler, B. Staker, S.G. Prasad, S. Menon, J. Tang, T. Parsons, D. Ryan, M. Fisher, D. Williams, N.A. Dales, M.A. Patane, M.W. Pantoliano, ACE2 X-Ray Structures Reveal a Large Hinge-bending Motion Important for Inhibitor Binding

and Catalysis*, Journal of Biological Chemistry. 279 (2004) 17996–18007. https://doi.org/10.1074/jbc.M311191200.

[116] A.J. Turner, Chapter 25 - ACE2 Cell Biology, Regulation, and Physiological Functions, in: T. Unger, U.M. Steckelings, R.A.S. dos Santos (Eds.), The Protective Arm of the Renin Angiotensin System (RAS), Academic Press, Boston, 2015: pp. 185–189. https://doi.org/10.1016/B978-0-12-801364-9.00025-0.

[117] M. Donoghue, F. Hsieh, E. Baronas, K. Godbout, M. Gosselin, N. Stagliano, M. Donovan, B. Woolf, K. Robison, R. Jeyaseelan, R.E. Breitbart, S. Acton, A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9, Circ Res. 87 (2000) E1-9. https://doi.org/10.1161/01.res.87.5.e1.

[118] D. Harmer, M. Gilbert, R. Borman, K.L. Clark, Quantitative mRNA expression profiling of ACE 2, a novel homologue of angiotensin converting enzyme, FEBS Letters. 532 (2002) 107–110. https://doi.org/10.1016/S0014-5793(02)03640-2.

[119] F. Gembardt, A. Sterner-Kock, H. Imboden, M. Spalteholz, F. Reibitz, H.-P. Schultheiss, W.-E. Siems, T. Walther, Organ-specific distribution of ACE2 mRNA and correlating peptidase activity in rodents, Peptides. 26 (2005) 1270–1277. https://doi.org/10.1016/j.peptides.2005.01.009.

[120] S. Romagnani, T-cell subsets (Th1 versus Th2), Annals of Allergy, Asthma & Immunology. 85 (2000) 21. https://doi.org/10.1016/S1081-1206(10)62426-X.

[121] S. Kodidela, S. Ranjit, N. Sinha, C. McArthur, A. Kumar, S. Kumar, Cytokine profiling of exosomes derived from the plasma of HIV-infected alcohol drinkers and

cigarette smokers, PLOS ONE. 13 (2018) e0201144. https://doi.org/10.1371/journal.pone.0201144.

[122] M.T. Abrams, M.L. Koser, J. Seitzer, S.C. Williams, M.A. DiPietro, W. Wang, A.W. Shaw, X. Mao, V. Jadhav, J.P. Davide, P.A. Burke, A.B. Sachs, S.M. Stirdivant, L. Sepp-Lorenzino, Evaluation of Efficacy, Biodistribution, and Inflammation for a Potent siRNA Nanoparticle: Effect of Dexamethasone Co-treatment, Molecular Therapy. 18 (2010) 171–180. https://doi.org/10.1038/mt.2009.208.

[123] Q. Pan, V. Ramakrishnaiah, S. Henry, S. Fouraschen, P.E. de Ruiter, J. Kwekkeboom, H.W. Tilanus, H.L.A. Janssen, L.J.W. van der Laan, Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNA interference (RNAi), Gut. 61 (2012) 1330–1339. https://doi.org/10.1136/gutjnl-2011-300449.

[124] H. Nie, X. Xie, D. Zhang, Y. Zhou, B. Li, F. Li, F. Li, Y. Cheng, H. Mei, H. Meng, L. Jia, Use of lung-specific exosomes for miRNA-126 delivery in non-small cell lung cancer, Nanoscale. 12 (2020) 877–887. https://doi.org/10.1039/C9NR09011H.

[125] C. Liu, C. Su, Design strategies and application progress of therapeutic exosomes, Theranostics. 9 (2019) 1015–1028. https://doi.org/10.7150/thno.30853.

[126] G. Liang, Y. Zhu, D.J. Ali, T. Tian, H. Xu, K. Si, B. Sun, B. Chen, Z. Xiao, Engineered exosomes for targeted co-delivery of miR-21 inhibitor and chemotherapeutics to reverse drug resistance in colon cancer, Journal of Nanobiotechnology. 18 (2020) 10. https://doi.org/10.1186/s12951-019-0563-2.

[127] C. Meyer, J. Losacco, Z. Stickney, L. Li, G. Marriott, B. Lu, Pseudotyping exosomes for enhanced protein delivery in mammalian cells, IJN. 12 (2017) 3153–3170. https://doi.org/10.2147/IJN.S133430.

[128] S. Hsu, B. Yu, X. Wang, Y. Lu, C.R. Schmidt, R.J. Lee, L.J. Lee, S.T. Jacob, K. Ghoshal, Cationic lipid nanoparticles for therapeutic delivery of siRNA and miRNA to murine liver tumor, Nanomedicine: Nanotechnology, Biology and Medicine. 9 (2013) 1169–1180. https://doi.org/10.1016/j.nano.2013.05.007.

[129] Y. Akao, Y. Nakagawa, I. Hirata, A. Iio, T. Itoh, K. Kojima, R. Nakashima, Y. Kitade, T. Naoe, Role of anti-oncomirs miR-143 and -145 in human colorectal tumors, Cancer Gene Ther. 17 (2010) 398–408. https://doi.org/10.1038/cgt.2009.88.

[130] Y. Wu, M. Crawford, B. Yu, Y. Mao, S.P. Nana-Sinkam, L.J. Lee, MicroRNA Delivery by Cationic Lipoplexes for Lung Cancer Therapy, Mol. Pharmaceutics. 8 (2011) 1381–1389. https://doi.org/10.1021/mp2002076.

[131] Y. Wu, M. Crawford, Y. Mao, R.J. Lee, I.C. Davis, T.S. Elton, L.J. Lee, S.P. Nana-Sinkam, Therapeutic Delivery of MicroRNA-29b by Cationic Lipoplexes for Lung Cancer, Molecular Therapy - Nucleic Acids. 2 (2013) e84. https://doi.org/10.1038/mtna.2013.14.

[132] L. Jiang, H. Wang, S. Chen, Aptamer (AS1411)-Conjugated Liposome for Enhanced Therapeutic Efficacy of miRNA-29b in Ovarian Cancer, Journal of Nanoscience and Nanotechnology. 20 (2020) 2025–2031.

[133] Y. Chen, X. Zhu, X. Zhang, B. Liu, L. Huang, Nanoparticles Modified With Tumor-targeting scFv Deliver siRNA and miRNA for Cancer Therapy, Molecular Therapy. 18 (2010) 1650–1656. https://doi.org/10.1038/mt.2010.136.

[134] L. Wang, T.-T. Liang, CD59 receptor targeted delivery of miRNA-1284 and cisplatin-loaded liposomes for effective therapeutic efficacy against cervical cancer cells, AMB Express. 10 (2020) 1–11.

[135] D.L. Iden, T.M. Allen, In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach, Biochimica et Biophysica Acta (BBA) - Biomembranes. 1513 (2001) 207–216. https://doi.org/10.1016/S0005-2736(01)00357-1.

[136] C. Guo, Y. Chen, W. Gao, A. Chang, Y. Ye, W. Shen, Y. Luo, S. Yang, P. Sun, R. Xiang, N. Li, Liposomal Nanoparticles Carrying anti-IL6R Antibody to the Tumour Microenvironment Inhibit Metastasis in Two Molecular Subtypes of Breast Cancer Mouse Models, Theranostics. 7 (2017) 775–788. https://doi.org/10.7150/thno.17237.

[137] Optimization of post-insertion method to conjugate Doxil with anti-CD133 monoclonal antibodies: Investigating the specific binding and cytotoxicity to colorectal cancer cells in vitro - ScienceDirect, (n.d.). https://www.sciencedirect.com/science/article/pii/S1319016420302097 (accessed September 14, 2021).

[138] R.W. Baker, B.T. Low, Membrane Separation☆, in: Reference Module in Chemistry, Molecular Sciences and Chemical Engineering, Elsevier, 2015. https://doi.org/10.1016/B978-0-12-409547-2.11674-9.

[139] M. Sack, J. Sigler, S. Frenzel, Chr. Eing, J. Arnold, Th. Michelberger, W. Frey, F. Attmann, L. Stukenbrock, G. Müller, Research on Industrial-Scale Electroporation Devices Fostering the Extraction of Substances from Biological Tissue, Food Eng. Rev. 2 (2010) 147–156. https://doi.org/10.1007/s12393-010-9017-1.

[140] S. Kosuri, G.M. Church, Large-scale de novo DNA synthesis: technologies and applications, Nature Methods. 11 (2014) 499–507. https://doi.org/10.1038/nmeth.2918.

[141] J.F. Buyel, R.M. Twyman, R. Fischer, Very-Large-Scale Production of Monoclonal Antibodies in Plants, in: Process Scale Purification of Antibodies, John Wiley & Sons, Ltd, 2017: pp. 655–672. https://doi.org/10.1002/9781119126942.ch30.

[142] B. Yu, R.J. Lee, L.J. Lee, Chapter 7 - Microfluidic Methods for Production of Liposomes, in: Methods in Enzymology, Academic Press, 2009: pp. 129–141. https://doi.org/10.1016/S0076-6879(09)65007-2.

[143] C. Walsh, K. Ou, N.M. Belliveau, T.J. Leaver, A.W. Wild, J. Huft, P.J. Lin, S. Chen, A.K. Leung, J.B. Lee, C.L. Hansen, R.J. Taylor, E.C. Ramsay, P.R. Cullis, Microfluidic-based manufacture of siRNA-lipid nanoparticles for therapeutic applications, Methods Mol Biol. 1141 (2014) 109–120. https://doi.org/10.1007/978-1-4939-0363-4_6.

[144] C. Charoenviriyakul, Y. Takahashi, M. Nishikawa, Y. Takakura, Preservation of exosomes at room temperature using lyophilization, International Journal of Pharmaceutics. 553 (2018) 1–7. https://doi.org/10.1016/j.ijpharm.2018.10.032.

[145] P.J. Stevens, R.J. Lee, Formulation kit for liposomal doxorubicin composed of lyophilized liposomes, Anticancer Res. 23 (2003) 439–442.

[146] Full article: Preparation and Characterization of Lyophilized Liposomes with Incorporated Quercetin, (n.d.). https://www.tandfonline.com/doi/full/10.1080/08982100500528594?casa_token=1GdStm2RxIIAAAAA%3AVRdurySrx65ftzPPDjrEC5mVUU6lETpUTD7hKIYp__fJkuuunElVUblXKlm8mxS-fJG_pvQMo3RDF2g (accessed September 14, 2021).

[147] M. Glavas-Dodov, E. Fredro-Kumbaradzi, K. Goracinova, M. Simonoska, S. Calis, S. Trajkovic-Jolevska, A.A. Hincal, The effects of lyophilization on the stability of liposomes containing 5-FU, International Journal of Pharmaceutics. 291 (2005) 79–86. https://doi.org/10.1016/j.ijpharm.2004.07.045.

[148] T. Hernández-Caselles, J. Villalaín, J.C. Gómez-Fernández, Stability of Liposomes on Long Term Storage, Journal of Pharmacy and Pharmacology. 42 (1990) 397–400. https://doi.org/10.1111/j.2042-7158.1990.tb06578.x.

[149] P.R. Matias-Garcia, R. Wilson, V. Mussack, E. Reischl, M. Waldenberger, C. Gieger, G. Anton, A. Peters, A. Kuehn-Steven, Impact of long-term storage and freeze-thawing on eight circulating microRNAs in plasma samples, PLOS ONE. 15 (2020) e0227648. https://doi.org/10.1371/journal.pone.0227648.

[150] E.W. R., W. H., N. Y., C. J., Improving the Stability of Aptamers by Chemical Modification, Current Medicinal Chemistry. 18 (2011) 4126–4138.

[151] Q. Ge, Y. Zhou, J. Lu, Y. Bai, X. Xie, Z. Lu, miRNA in Plasma Exosome is Stable under Different Storage Conditions, Molecules. 19 (2014) 1568–1575. https://doi.org/10.3390/molecules19021568.

[152] ICMJE | Recommendations | Defining the Role of Authors and Contributors, (n.d.). http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html (accessed July 29, 2020).

[153] D.B. Resnik, A.M. Tyle, J.R. Black, G. Kissling, Authorship policies of scientific journals, J Med Ethics. 42 (2016) 199–202. https://doi.org/10.1136/medethics-2015-103171.

[154] E.E. Tarkang, M. Kweku, F.B. Zotor, Publication Practices and Responsible Authorship: A Review Article, J Public Health Afr. 8 (2017). https://doi.org/10.4081/jphia.2017.723.

[155] K. Strange, Authorship: why not just toss a coin?, Am J Physiol Cell Physiol. 295 (2008) C567–C575. https://doi.org/10.1152/ajpcell.00208.2008.