The Potential of Exosomes from Cow Milk for Oral Delivery

Jamie L. Betker[1], **Brittany M. Angle**[1], **Michael W. Graner**[2], **Thomas J. Anchordoquy**[1]

[1]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus Aurora, CO

[2]Dept. of Neurosurgery University of Colorado Anschutz Medical Campus Aurora, CO

Abstract

Many pharmaceuticals must be administered intravenously due to their poor oral bioavailability. In addition to issues associated with sterility and inconvenience, the cost of repeated infusion over a six-week course of therapy costs the healthcare system tens of billions of dollars per year. Attempts to improve oral bioavailability have traditionally focused on enhancing drug solubility and membrane permeability, and the use of synthetic nanoparticles has also been investigated. As an alternative strategy, some recent reports have clearly demonstrated that exosomes from cow milk are absorbed from the gastrointestinal tract in humans, and could potentially be used for oral delivery of drugs that are traditionally administered intravenously. Our previous work has shown that antibodies are present in exosome preparations, and the current work with milk exosomes suggests that absorption from the gastrointestinal tract occurs via the "neonatal" Fc receptor, FcRn. Furthermore, our results demonstrate that milk exosomes are absorbed from the gut as intact particles that can be modified with ligands to promote retention in target tissues.

Keywords

milk exosomes; oral delivery; FcRn; oral absorption

Introduction

Many drugs are not readily absorbed from the gastrointestinal tract, and therefore are typically administered via intravenous infusion. Accordingly, many patients undergoing therapy for cancer and microbial infection are required to receive multiple rounds of infusion as part of their treatment regimen. Because these drugs require infusion, sterile formulations must be prepared and administered by trained personnel. Not surprisingly, this significantly increases the cost associated with therapy, and 6 weeks of inpatient infusion therapy in the

To whom correspondence should be addressed: Thomas Anchordoquy, 12850 E. Montview Blvd, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Aurora, CO 80045, tom.anchordoquy@ucdenver.edu, phone: 303-724-6113.

hospital costs $50,980 [1]. Although the costs associated with infusion therapy are reduced significantly at extended care facilities ($31,072) or when administered in the home ($12,307), expenses associated with infusion of sterile solutions represents tens of billions of dollars each year [2]. In addition to the higher costs, the requirement for infusion therapy forces some patients to leave the comfort of their home, and be exposed to other sick patients during treatment. Considering that many of these patients are elderly and immune-suppressed, the risk of contracting hospital-acquired infections increases dramatically, which greatly compromises patient outcomes, significantly enhances the chances of mortality, and further increases costs [3–5].

In order for a drug to be orally bioavailable, the molecule must be inherently chemically-resistant or protected from the low pH and degradative enzymes encountered in the stomach [6]. Although encapsulation in a particulate delivery system can protect therapeutics from this harsh environment, absorption of the drug-containing particle from the gut and delivery into the circulation remains a formidable barrier [6–11]. In an attempt to understand the well-known phenomenon by which intact antibodies are passed into a baby's circulation via mother's milk, Brambell and colleagues first postulated and later identified a receptor that is capable of binding IgG in breast milk and transporting it into the blood circulation [12, 13]. Subsequent work has shown that the binding of IgG to the neonatal Fc receptor (FcRn) is strongly pH-dependent, which explains its high affinity in the acidified environment of the upper gastrointestinal tract [6, 8, 14]. It is important to recognize that this receptor is highly expressed in the human gastrointestinal tract throughout life, and thus the "neonatal" moniker is misleading [6–8, 15]. In fact, studies have shown that FcRn is heavily involved in fighting infections by allowing IgG to be readily transcytosed back-and-forth across the gastric epithelium [16, 17]. Furthermore, it is not generally appreciated that FcRn is located in the endosomal membrane of the vascular endothelium, where it plays a critical role in recycling and extending the circulation half-life of IgG [6, 8, 18, 19]. To this end, studies have clearly documented that the pH-dependent binding of IgG to FcRn causes antibodies to escape the lysosomal pathway and avoid cellular degradation [19].

In a seemingly unrelated area of drug delivery, the ability of exosomes to cross biological barriers (e.g., the blood-brain barrier) has received significant attention in recent years [20–23]. Accordingly, many laboratories (including ours) have attempted to harvest exosomes from various cell lines and use them as drug delivery vehicles [20–25]. Although many studies have reported remarkable results with exosomal carriers, issues associated with scale-up and immunogenicity represent significant barriers to the development of exosomes as therapeutic products [20, 22]. Put simply, harvesting exosomes from cell lines and/or patient samples is not compatible with commercial pharmaceutical production, and the protein component of exosomes will likely trigger immune responses when administered systemically. However, these obstacles could potentially be overcome if copious amounts of exosomes could be harvested from readily-available bulk sources, and if immune concerns could be mitigated/resolved. In this regard, it is intriguing that exosomes harvested from cow milk have recently been shown to be transported by human endothelial cells and exhibit cross-species tolerance without eliciting immune responses [26, 27]. Consistent with the potential for utilizing milk exosomes for oral drug delivery, a clinical trial has demonstrated that miRNAs from milk exosomes are absorbed into the blood of human patients at levels

sufficient to regulate genes in blood mononuclear cells [28, 29]. Additional studies have suggested that exosomes can be harvested from cow milk in scalable quantities, and that milk exosomes can be used to deliver drugs via oral administration [27, 30, 31]. While these studies were conducted in mice, research has shown that murine FcRn is very promiscuous and binds strongly to IgG from many species (including human and bovine). Although human FcRn is more discriminating, the binding of bovine IgG is approximately 28% of that observed for human IgG [32], suggesting that cow exosomes might have sufficient cross-reactivity to be used as delivery vehicles in human patients. The fact that most people in the U.S. consume milk as children, and that many consume dairy products that contain exosomes throughout their lifetime (e.g., milk, yogurt), it is likely that humans have developed an immune tolerance to the proteins in cow milk exosomes that would permit their use as oral delivery vehicles. This phenomenon has been extensively studied by Gupta and colleagues who have used cow milk exosomes to orally deliver chemotherapeutics in mice [27, 30]. In addition to this published work, our previous studies with exosomes have documented significant quantities of IgG in exosome preparations [33–35]. Accordingly, we performed experiments in mice to assess the potential of using cow milk exosomes for oral delivery, and test our hypothesis regarding the role of FcRn in gut absorption.

Materials and Methods

Materials

Raw milk from different cows was donated by Pam and Jeff Fiorino of Mucca Bella Dairy (Carr, Colorado). DIR (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide), DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine) and SYTO13 were obtained from Thermo Fisher Scientific, Carlsbad, CA. Egg phosphatidylcholine and cholesterol were acquired from Avanti Polar Lipids (Alabaster, AL). Bovine IgG was purchased from Pierce Protein Biology (Thermo Fisher, Rockford, IL). A cyclic iRGD peptide (CRGDRGPDC) was purchased from Peptides International (Louisville, KY), and specifically conjugated to the hydroxyl moiety of cholesterol via a carbamate linker with the terminal amine as previously described [36].

Exosome Isolation and characterization

Exosomes were freshly harvested from raw cow milk by the three-step centrifugation process according to the methods of Agrawal et al. [30]. Briefly, milk was centrifuged at 13,000 x g for 30 min, before passing through cheese cloth. Large fat globules, casein debris, large particles, and microvesicles were then removed by spinning at 100,000 x g for 60 min. Exosomes were isolated by centrifugation at 135,000 x g for 90 min at 4 °C on a Beckman Optima centrifuge (model # LE-80k, SW-28 rotor, K factor 257.1 at 135,000 x g) as previously described [30]. The size and zeta potential of exosomes was measured on a Zetasizer (Malvern, England). In addition, isolated exosomes were visualized via transmission electron microscopy. Four to five microliters of sample were applied to negatively charged formvar/carbon 300 mesh copper grids (EMS) for 20 seconds, then the excess was wicked off with filter paper. The grid was quickly rinsed by touching it to two drops of MilliQ water, wicking each time, and then stained with two drops of 0.75% uranyl formate. The grid was touched to the first drop of uranyl formate for a few seconds, wicked

with filter paper, and then applied to the second drop of uranyl formate for 20 seconds. After the excess stain was wicked off, the grid was allowed to dry for at least 10 minutes before it was imaged on the electron microscope. Images were collected on a FEI Tecnai G2 transmission electron microscope (Hillsboro, OR) at 80kV with an AMT digital camera (Woburn, MA). Exosomes were characterized for marker expression using Exo-Check Arrays (System Biosciences/SBI, Palo Alto, CA, USA) for eight purported exosome markers.

Animal Experiments

DIR labelled exosomes.—Purified exosomes were labelled with DIR according to the protocol used in our previous studies [24]. Briefly, the dye is dissolved in ethanol at a concentration of 220 μg/mL. Five microliters of the dye is mixed with 220 μg/mL of exosomes (or control liposomes) in 100 ml PBS and incubated at room temperature for 1 h. Preparations were loaded on a sepharose column, and exosomes eluted with PBS to remove free dye [24]. DIR-labelled milk exosomes were administered to female balb/c mice (6–10 weeks old; acquired from Jackson labs, Bar Harbor, ME) via oral gavage at a dose of 40 mg exosome protein/kg in approximately 100 μl. For comparison, a single mouse was administered an equivalent dose of DIR-labelled liposomes (egg phosphatidylcholine, ≈ 100 nm). A single control mouse administered PBS was also included as a control for background fluorescence. Blood samples were collected at 30, 60, 120, 240 and 360 min from the sub-mandibular veins to evaluate both the extent and timing of absorption. A cotton swab was used to smear each blood sample onto a nitrocellulose membrane that was imaged on an Odyssey Imager (LI-COR Biosciences, Lincoln, NE). Immediately after sacrifice, organs (liver spleen, kidney, heart, lung, blood via cardiac puncture) were harvested and placed in 12 or 24- well plates for imaging. All animal procedures were approved by the University of Colorado Institute for Animal Care and Use Committee in accordance with guidelines from the National Institutes of Health.

Dual labelled exosomes.—Purified exosomes were labelled with SYTO 13 and DiD. Briefly, DiD was dissolved in DMSO to produce a 9.5 mM stock solution. SYTO 13 was supplied as a 5 mM stock solution in DMSO. Eight hundred micrograms of exosomal protein (as measured by BCA protein assay) were incubated with 200 μl of 50 μM SYTO 13 for 30 min at 37°C. Subsequently, 40 μl of 95 μM DiD were added to the exosomes and incubated for an additional 30 min at 37°C. Unincorporated dye was removed by passing the exosomes over a Sepharose CL-4B loaded spin column. Labeled exosomes were administered to female balb/c mice via oral gavage at a dose of 40 mg exosomal protein/kg. Blood was collected via cardiac puncture 4 hrs post-administration and allowed to clot overnight at 4°C to obtain serum. Exosomes were obtained from serum by differential centrifugation. Briefly, serum was centrifuged at 20,000 x g at 4°C for 30 min to remove debris. Samples was then centrifuged at 110,000 x g at 4°C for 70 min using a SW28 rotor and pellets were washed in 1X PBS. Samples were centrifuged again at 110,000 x g at 4°C for 70 min and pellets were resuspended in 1X PBS. Exosome samples were mounted and imaged via laser scanning confocal microscopy using a Zeiss LSM780 microscope.

In vivo bovine IgG competition.—Purified exosomes were labelled with DIR per the method described above, and bovine IgG purchased from Pierce Protein Biology (Thermo Fisher Rockford, IL) was administered via oral gavage. Bovine IgG was spin concentrated 3-fold using a 100 kDa Amicon Centrifugal filter (Millipore Burlington, MA). Exosomes (80 μg exosomal protein in approximately 100 μl) were administered by oral gavage immediately followed by bovine IgG at three doses (20 μg, 200 μg, 2000 μg). A similar experiment was conducted to determine the effect of a large oral dose of protein on exosome uptake. Accordingly, a protein that does not interact with FcRn (erythropoietin; 2000 μg) was administered immediately after exosomes. Blood samples were taken from the submandibular veins at 30, 60, 120, 180 minutes post-gavage. The mice were then sacrificed and blood and organs were harvested. The blood was again smeared on a nitrocellulose membrane and the organs were imaged in 12- or 24-well plates on the Odyssey Imager.

Studies with Tumor-Bearing Mice.—Because of the unexpectedly high levels of absorption and the signal saturation observed in our initial studies (Figs. 2 + 3), we reduced the amount of DIR used to label exosomes by 10-fold in further experiments. These exosomes were used to assess tumor accumulation in immunocompetent tumor-bearing mice. Briefly, female balb/c mice were inoculated in the flank with murine colon carcinoma cells (CT26), and tumors were allowed to grow to ≈ 100 mm^3. Triplicate mice were administered DIR-labelled milk exosomes, and tissues were harvested after 4 h. To assess the ability to target exosomes with ligands, we incorporated iRGD-cholesterol [36] into exosomes at a concentration of 0.1% using the same procedure described above for DIR loading. The term "iRGD" refers to a family of related peptide ligands that bind specifically to the α∨ β3/β5 integrin on tumor vasculature and promote transcytosis across tumor vasculature [37–40]. After translocation, the iRGD ligand is cleaved by an endogenous protease to yield a peptide that serves as a ligand for the neuropilin-1 receptor to promote uptake by cancer cells [37–40]. Although we did not determine the stability of the iRGD peptide in simulated gastric fluid, previous studies have shown that cyclic peptides can survive these conditions [7]. Accordingly, another set of triplicate mice were administered DIR-labelled, iRGD-targeted exosomes. In order to allow comparison with IV administration, single mice were IV injected with either free DIR or DIR-labelled milk exosomes. To simulate comparable dosing via the two routes of administration, we estimated the efficiency of oral absorption at 10%, and thus mice receiving IV injection received only one-tenth of the DIR dose that was administered via oral gavage. After 4 h, blood samples were collected and imaged as described above. In the experiments involving iRGD-containing exosomes, tissues were homogenized in homogenization buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris HCL pH 7.2 and protease inhibitor cocktail from Pierce Protein Biology Thermo Fisher, Rockford, IL) 4 h after oral gavage, and the fluorescent signal from accumulated DIR-labelled exosomes was measured with the Odyssey Imager. Values for DIR fluorescence (RLU) were standardized against the protein levels in each tissue homogenate. Exosome protein levels were determined by BCA assay (Pierce Thermo Fisher Rockford, IL). It is worth noting that several other methods for protein quantitation were tested including a Bradford (Biorad Hercules, CA), phase extraction, and a lysis protocol. Protein levels varied dramatically with each assay (data not shown), hence it is very critical to be cognizant of which assay is chosen when quantifying exosomal protein.

We chose the BCA assay in order to be consistent with previous studies by Gupta and colleagues with respect to measurements and dosing [27, 30].

Western Blot

Purified milk exosomes were precipitated with ExoQuick (System BioSciences, Palo Alto, CA); one batch was untreated (XOQ ppt), and the other batch was treated with 0.2 M glycine, pH 2.0 to strip antibodies. Glycine-treated exosomes (XOs strip) and eluted antibodies (eluted Abs) were separated by centrifugation through a 300 kDa cutoff membrane. Samples were electrophoresed, transferred to nitrocellulose (Western blot), and probed with goat anti-bovine IgG (H&L chain)-HRP, 1:2000 dilution (ThermoFisher Scientific, Grand Island, New York, USA; catalog #A18751). The blot was developed with chemiluminescence, and imaged with a FluroChem Q Imager III device (ProteinSimple, Santa Clara CA). Bovine γ-globulin (Bio-Rad Life Sciences Research, Hercules, CA, catalog #5000005) was included as a positive control, and "unbound" antibodies came from the ExoQuick supernatant.

Results

To characterize exosomes extracted from cow milk, we acquired fresh milk from a local dairy and harvested exosomes according to previously described protocols [27, 30]. As shown in Figure 1A, we were able to isolate vesicles that have sizes consistent with exosomes. As observed in our previous work with exosomes, dehydration of samples for electron microscopy causes some clumping and yields slightly smaller particle sizes as compared to the hydrated diameters measured by dynamic light scattering (127 nm) [25]. Consistent with other exosome preparations, the size distribution is quite uniform, and particles possess a negative charge (−15.2 mV). We also examined the variability of exosomes prepared from milk obtained from cows at different stages of lactation (early, mid, late). The size and zeta potential of exosomes harvested from four different cows were very consistent, and hydrated diameters ranged from 127 to 140 nm, and zeta potentials ranged from −15.2 to −7.6 mV.

Exosome marker characterization (using an Exo-Check Array) showed that bovine exosomes have markers consistent with other exosomes (Figure 1B). We note that ALIX is not apparent in our preparations, consistent with the protein's presence in exosomes from colostrum, but not mature milk [41]. We have also not found EPCAM identified on bovine milk exosomes in the literature.

Our next set of experiments involved assessing the extent to which exosomes are absorbed from the gastrointestinal tract. To track uptake, we utilized an infrared dye, DIR, that we have previously validated/optimized for tracking intravenously-administered exosomes [24]. DIR-labelled milk exosomes were administered to mice via oral gavage at a dose of 40 mg exosome protein/kg. For comparison, a single mouse was administered an equivalent dose of DIR-labelled liposomes (egg phosphatidylcholine, ≈ 100 nm), and a single control mouse administered PBS was also included as a control for background fluorescence. As shown in Figure 2, blood smears from mice administered PBS exhibited undetectable levels of background infrared signal. Blood from mice administered DIR-labelled liposomes had a

low infrared signal at 30 min, which increased at 60 min, diminished by 120 min, and was undetectable at later time points. In sharp contrast to the results with liposomes, the infrared signal of blood from mice administered exosomes saturated our detector, even on the lowest sensitivity setting (Fig. 2). The area of signal saturation increased after 30 min, but remained relatively constant (almost completely saturated) from 60–240 min, and then diminished slightly after 360 min in all mice. We feel these initial results are quite striking, and clearly demonstrate that molecules loaded into milk exosomes (in contrast to liposomes) are readily absorbed. In addition, the timing of absorption is surprisingly rapid, and sufficient quantities of dye are in the circulation after only 30 min. Furthermore, high levels of dye are sustained for at least 6 h. This initial experiment demonstrates that milk exosomes are capable of facilitating the transfer of significant quantities of molecular cargo into the blood after oral administration. Moreover, the fact that blood from mice administered DIR-labelled liposomes had markedly lower absorption suggests that the mechanism of absorption is not simply due to random particle uptake.

The mice described in the experiment above were sacrificed after 6 h, at which time the liver, spleen, kidney, heart and lungs were harvested and placed in 24-well plates for imaging. As shown in figure 3, background levels of IR signal were barely detectable in organs from mice administered PBS. The signal from mice administered DIR-labelled liposomes was clearly evident in the kidneys and liver. In contrast, organs from mice administered DIR-labelled milk exosomes displayed large regions in which the IR detector was saturated. Consistent with the high levels of absorption into the blood seen in figure 2, large amounts of dye accumulate in all organs 6 h after oral administration of milk exosomes (Fig. 3). It is important to point out that blood levels of dye are still high at this time point, and thus tissue accumulation will likely be greater at longer times. These data are consistent with previous studies showing that milk exosomes facilitate the transfer of cargo into the blood, which ultimately accumulates in tissues [27, 30].

Because of the unexpectedly high levels of absorption and the signal saturation observed above, we reduced the amount of DIR used to label exosomes by ten-fold in future experiments. These exosomes were then used to assess tumor accumulation in immunocompetent tumor-bearing mice after 4 h. In order to more effectively compare fluorescent signals from blood achieved after IV versus oral administration, IV doses were reduced an additional ten-fold. As shown in figure 4, no signal was seen in blood from mice administered free DIR by IV injection, suggesting that free dye is cleared from the blood within 4 h. Blood from mice administered DIR-labelled exosomes via IV injection had a strong IR signal that was just below the saturation limit of the detector. However, blood from mice dosed via oral gavage had a stronger signal that exceeded the saturation limit (Fig. 4). Furthermore, the incorporation of the iRGD ligand into exosomes did not substantially alter absorption. While these data are not quantitative, the fact that blood levels were higher after oral gavage, as compared to that from mice administered a ten-fold lower dose via IV injection, suggests that oral bioavailability may be greater than 10% under these conditions.

To assess tumor accumulation, tumors were harvested from mice after 4 h. In an effort to obtain semi-quantitative results, tumors were homogenized and the relative fluorescence units in the resulting suspension were quantified with the Odyssey imager and standardized

against the protein content. Figure 5 depicts the relative amounts of dye in tumors from mice receiving oral gavage as compared to mice dosed via intravenous injection. The results suggest that comparable levels of tumor accumulation were achieved via oral and intravenous administration (10% of the oral dose), and that the iRGD ligand significantly increased tumor accumulation. The fact that a ligand alters accumulation after oral absorption suggests that the dye remains associated with the ligand, potentially in an intact exosome. To test this hypothesis, we conducted an additional experiment wherein both the membrane and RNA encapsulated within milk exosomes were fluorescently-labelled, and these double-labelled exosomes were orally administered to three mice as described above. Blood from the mice was collected after 4 h, and exosomes were isolated from the pooled blood samples and analyzed by confocal microscopy. As shown in figure 6, some exosomes isolated from the blood of mice possessed both fluorescent labels, suggesting that milk exosomes can be absorbed from the gastrointestinal tract as intact particles. This suggestion is consistent with the greater tumor accumulation we observe with iRGD-containing exosomes (Fig. 5). It is important to note that previous studies have shown that particles can be altered during transcytosis, even if they remain intact [10].

In addition to the tumor, other organs were harvested after 4 h, homogenized, and analyzed for IR signal. As is commonly observed in drug delivery, liver accumulation was the highest of all the organs, consistent with clearance (Fig. 7). Interestingly, the organ accumulation of iRGD-containing exosomes was dramatically reduced despite enhanced tumor distribution and comparable absorption from the gastrointestinal tract. While this result is very encouraging in terms of limiting off-target effects, it suggests that iRGD-containing exosomes are excreted more efficiently and/or accumulate at a different site.

The results presented above are consistent with reports claiming that milk exosomes are absorbed from the gastrointestinal tract [27–30]. These findings can be viewed more broadly in terms of the ability of exosomes to cross other cell barriers (e.g., the blood-brain barrier). This phenomenon has gained a tremendous amount of interest, but no mechanistic explanation for this effect has been proposed [20–22]. However, previous studies have demonstrated the ability of FcRn to facilitate transport of Fc-targeted particles across the gastrointestinal tract [9, 42]. Furthermore, it is important to recognize that FcRn is expressed in the vascular endothelium as well as the gastrointestinal tract throughout life in humans [6–11]. Because both mouse and human FcRn bind bovine IgG [32], we proposed that interactions with this receptor might play a critical role in the ability of milk exosomes to be absorbed after oral administration. To test this hypothesis, mice were co-administered a constant amount of DIR-labeled exosomes with varying amounts of free bovine IgG. It follows that the free IgG should compete with exosomes for FcRn in the gastrointestinal tract, and should thereby reduce absorption. As before, blood samples were collected at different timepoints and imaged, and figure 8 clearly shows that the IR signal intensity diminishes at higher doses of co-administered IgG. To assess the potential for this effect to be simply due to the presence of increased protein in the gut, a parallel experiment was conducted in which erythropoietin was co-administered with exosomes, but no effect of this protein was observed (Fig. 8). Virtually identical effects on organ accumulation were observed (data not shown). These studies demonstrate that co-administration of bovine IgG reduces uptake, and that this effect is not generally observed with other proteins. Taken

together, these data strongly support our hypothesis that FcRn is involved in absorption of milk exosomes from the gastrointestinal tract. However, we suggest that the significant absorption of exosomes we observe despite swamping amounts of co-administered IgG indicates that other factors may contribute to gastrointestinal uptake.

It seems evident that utilization of the gut epithelial FcRn would require Fc ligand on milk exosomes; however, this has not been specifically explored. Other reports [41, 43–45] have identified immunoglobulin (Ig) components via proteomics of cow milk exosomes. Following a similar protocol as described previously [46], we detected the presence of both tightly-bound and elutable bovine IgG in/on cow milk exosomes (Fig. 9). Our results demonstrate that there are substantial amounts of bovine IgG in/on cow milk exosomes, and the majority of it remains associated with the exosomes following the glycine stripping. Densitometry determinations indicate that cow milk exosomes have 1–2 mg IgG per 50 mg of exosome protein. While this may seem like a high percentage, cow milk contains 300–600 mg/ml of IgG [47], so we feel that the exosome IgG concentrations are reasonable.

Discussion

Although oral dosage forms are clearly preferable for patients, many drugs require parenteral administration due to poor oral bioavailability. The reasons for poor oral bioavailability can be multiple, but typically involve low aqueous solubility, instability under the conditions in the gastrointestinal tract, low absorption through the gut epithelium, and/or the first-pass effect in the liver [48]. The use of particulate delivery systems for oral delivery has the potential to circumvent many of these issues, but uptake of particles from the gastrointestinal tract is the predominant barrier. Indeed, previous work with lipid-based formulations and emulsions have been used to improve the solubility of lipophilic drugs and enhance permeability of the intestinal endothelium [49, 50]. It is conceivable that successful oral delivery could be achieved by using particles that release drug into the intestinal epithelial cells and rely on subsequent diffusion to access the systemic circulation. This scenario would appear more feasible, albeit potentially less efficient, because it does not require uptake of intact delivery systems from the gastrointestinal tract and subsequent transport across the epithelium.

It is well established that exosomes play a role in many biological processes and possess the ability to transfer molecules among cells. Previous research from our laboratory and others have attempted to harvest exosomes from cells in culture for use as delivery vehicles [20–25, 51]. While some promising results have been reported, the inability to produce and harvest exosomes on a commercial scale has always been a concern associated with exosome-mediated delivery [20, 21, 24]. In this regard, the isolation of small quantities of exosomes needed for *in vitro* experiments is labor-intensive, but feasible. However, *in vivo* experiments and potential clinical studies require that large amounts of exosomes be harvested from a readily-available source [24, 25, 29]. It is now recognized that all bodily fluids (e.g., blood, urine, lymph, milk) contain exosomes that are secreted by every cell type [52, 53]. Not surprisingly, exosomes from mother's milk have evolved to resist conditions encountered in the stomach in order to transfer molecules to the baby. In fact, studies have shown that exosomes from cow milk are capable of withstanding simulated gastric

conditions, maintaining and transferring their contents from the gastrointestinal tract into the blood [27, 28, 54]. With particular relevance to the clinical application of this approach, it is important to point out *that a human study documented that functional miRNAs from cow milk are absorbed into the circulation* [28]. Furthermore, previous studies in mice have demonstrated that exosomes can be isolated from cow milk and used to transfer molecules across the gastrointestinal epithelium [27, 29, 30, 54]. Our results are consistent with these previous studies, and suggest that significant quantities of exosomes are absorbed from the gut within 30 min, and blood levels remain high for at least 6 h in mice (Fig. 2). In addition, significant levels of orally-administered exosomes were observed in all tissues after 6 h (Fig. 3).

Our experiments in tumor-bearing mice suggest that ligands can be incorporated into orally-delivered exosomes to enhance accumulation in tumors (Fig. 5). These findings are consistent with previous studies by Munagala et al. [27] who reported improved tumor shrinkage by incorporating folic acid into drug-loaded milk exosomes. In addition to enhanced tumor accumulation, we observed a reduced accumulation in other organs as compared to exosomes lacking the iRGD ligand (Fig. 7). The ability of the ligand to alter the distribution profile suggests that the ligand remains associated with the exosome throughout absorption and distribution via the systemic circulation. This implies that milk exosomes can be absorbed as intact particles from the gastrointestinal tract, and previous studies have suggested that some level of repackaging may occur during passage through the intestinal epithelium [29, 55]. While we did not attempt to characterize molecular-level changes to orally-administered exosomes upon absorption, our results from the double-label experiment demonstrate that at least some exosomes are absorbed as intact particles (Fig. 6).

Although previous studies have utilized milk exosomes for oral delivery, a plausible mechanism by which exosomes (especially intact exosomes) could be absorbed from the gastrointestinal tract has been lacking. Considering the ability of FcRn to salvage IgG and transport it from the gastrointestinal tract into the blood, researchers have attempted to exploit this natural mechanism to transcytose particulate delivery systems across the gut after oral administration. Early studies conducted with liposomes demonstrated that IgG could be used as a ligand to facilitate uptake from the gut into the circulation [9]. More recent studies have utilized a similar approach with Fc-targeted PLA nanoparticles in an attempt to develop an orally-administered form of insulin [42]. Similar to the earlier studies with liposomes, the researchers demonstrated significant oral bioavailability with Fc-targeted particles, and achieved very high mean absorption efficiencies of $13.7 \pm 1.3\%$/hr. In contrast, non-targeted particles exhibited an oral absorption efficiency of $1.2 \pm 0.2\%$/hr [42]. As mentioned earlier, our proteomic work has documented the presence of antibody fragments on purified exosomes [33–35], and we hypothesized that this may play a role in uptake via FcRn. Our experiments demonstrating that co-administration of bovine IgG substantially reduces the levels of exosomes absorbed into the blood is consistent with this hypothesis (Fig. 8). The fact that co-administration of another protein (i.e., erythropoietin) did not affect absorption indicates that this effect is specific to IgG, consistent with uptake involving FcRn.

In considering the absorption of bovine exosomes across the intestinal epithelium of mice or humans, it is important to recognize that studies quantifying the relative binding affinities of bovine IgG for murine and human FcRn have documented significant cross-reactivity among these species [32]. More specifically, the affinity of bovine IgG for the mouse receptor is comparable to that of murine IgG. With regards to uptake in humans, the relative binding of bovine IgG for the human receptor is approximately 28% of that for human IgG [32]. Considering that lactating cows have evolved mechanisms to pass materials through the intestinal epithelium of their calves, it is conceivable that the cross-reactivity with human FcRn could permit similar transport, albeit with reduced efficiency. We feel that these reports are consistent with a role for FcRn in the absorption of milk exosomes observed in both humans and mouse models, presumably via transcytosis [6–8, 10, 56]. However, we cannot rule out that additional factors may be involved in transport across the intestinal epithelium. In fact, previous studies have suggested that additional components are likely involved in FcRn-mediated transcytosis, and even postulated that a yet unknown "Factor X" provides an additional function, working in collaboration with FcRn, that results in highly-efficient transport across epithelia [6, 8]. Future studies are planned with transgenic mice to provide further insight into the mechanism of gastrointestinal transport.

It is worth considering differences between exosome-mediated delivery and conventional nanoparticles in terms of drug release and tumor delivery. As shown in figure 4, high blood levels of exosome-bound dye are observed 4 h after exosome-mediated delivery regardless of the route of administration (e.g., oral or intravenous). In contrast, intravenous administration of free dye resulted in undetectable blood levels under the same conditions, indicating that dye remains sequestered within exosomes after gastrointestinal uptake. This is in agreement with the findings in figure 6 demonstrating that exosomes are transported across the gastrointestinal epithelium as intact particles that are dissociated after internalization by the recipient cell. This scenario is consistent with the biological role of exosomes in the intercellular transfer of microRNAs that would likely not survive exposure to the extracellular environment. Taken together, these data indicate that dye release from circulating exosomes is minimal, and that drugs encapsulated *within* exosomes may be most effective against intracellular targets. However, the data in figures 5 + 7 suggest that molecules present on the exterior surface of exosomes are able to access cell receptors (i.e., the mechanism of iRGD targeting involves binding to the αv $\beta3/\beta5$ integrin on tumor vasculature [36]), indicating that it may be possible to achieve both targeting via surface moieties and efficient intracellular delivery after accessing the circulation. This could prove especially advantageous for reducing the systemic distribution and off-target effects of chemotherapy if efficacious tumor targeting can be achieved. Our results showing increased tumor accumulation and reduced organ accumulation by employing iRGD-mediated targeting are very promising in this regard.

In conclusion, our data are in agreement with previous studies showing uptake of cow milk exosomes in both mice and humans [27–30, 55]. In addition, we propose that milk exosomes can be taken up as intact particles via a process involving FcRn. It is important to recognize that FcRn is expressed in humans at high levels throughout life [6–8, 11, 15]. It is also noteworthy that analyses have indicated that the isolation of milk exosomes could be scaled up to yield large quantities for pharmaceutical applications [31]. It follows that many drugs

that are currently administered intravenously due to poor oral bioavailability (e.g., antibiotics, chemotherapeutics) could potentially be incorporated into milk exosomes for oral administration. In that regard, oral administration allows considerable flexibility in terms of dosing, such that large quantities (hundreds of grams) could be consumed even if drug loading is inefficient. For example, if only 1% drug loading is achieved and our estimate of 10% oral bioavailability is accurate, blood levels equivalent to a 100-mg intravenous dose should be possible via this approach. This is a conservative estimate considering that studies have shown drug loading to be considerably higher for chemotherapeutic agents (10–40%) [27]. This general strategy is potentially applicable to the oral administration of larger molecules (e.g., peptides, proteins, DNA, RNA) if loading procedures that overcome the formidable barriers of maintaining the integrity and function of the exosome and its therapeutic cargo can be developed [57]. It is also not clear that there would be a benefit to loading antibodies in or on exosomes because they might interfere with FcRn binding and/or transcytosis. In addition, therapeutic antibodies typically target cell surface and/or extracellular proteins. As mentioned above, we believe that the milk exosome delivery system has evolved predominantly for intracellular delivery, but could also be used to accommodate proteins or peptides that are below the renal threshold for clearance and/or easily-degraded nucleic acid entities. Considering the enormous quantities of exosomes that can be harvested from ordinary cow milk that humans have routinely consumed for thousands of years [58], it is conceivable that this process could be scaled rapidly, and be approved for treatment with minimal regulatory hurdles. Although we observe consistent exosome properties isolated from cows at different stages of lactation, variability among preparations will need to be considered during product development. Furthermore, current studies indicate that milk exosomes can be stored frozen without significant changes in their physical properties, and it may also be possible to lyophilize drug-loaded exosomes using conventional methods developed for lipid-based nanoparticles [59–64]. The application of existing stabilization technology could potentially provide dehydrated preparations with shelf-lives compatible with pharmaceutical products. Considering all these factors, we feel that the benefits of oral administration in terms of both improved health and convenience for many patients, in addition to the reduced costs and potential for commercial production, support further investigations into oral dosage forms employing milk exosomes.

Acknowledgments

Literature Cited

[1]. Odom EB, Patel KB, Odom DC, Inpatient care versus subacute care for long term intravenous antibiotics: cost from the patient perspective, American Journal of Hospital Medicine 8(3) (2016).

[2]. Shelley S, Home infusion providers struggle with unfriendly reimbursement policies., Pharmaceutical Commerce 2009(10 10) (2009).

[3]. Roberts RR, Hota B, Ahmad I, Scott RD, Foster SD, Abbasi F, Schabowski S, Kampe LM, Ciavarella GG, Supino M, Naples J, Cordell R, Levy SB, Weinstein RA, Hospital and Societal Costs of Antimicrobial-Resistant Infections in a Chicago Teaching Hospital: Implications for Antibiotic Stewardship, Clin Infect Dis 49(8) (2009) 1175–1184. [PubMed: 19739972]

[4]. Roberts RR, Scott RD, Hota B, Kampe LM, Abbasi F, Schabowski S, Ahmad I, Ciavarella GG, Cordell R, Solomon SL, Hagtvedt R, Weinstein RA, Costs Attributable to Healthcare-Acquired Infection in Hospitalized Adults and a Comparison of Economic Methods, Med Care 48(11) (2010) 1026–1035. [PubMed: 20940650]

[5]. Scott RD, The direct medical costs of healthcare-associated infections in U.S. hospitals and the benefits of prevention, Centers for Disease Control and Prevention 10 10 (2009).

[6]. Sockolosky JT, Szoka FC, The neonatal Fc receptor, FcRn, as a target for drug delivery and therapy, Adv Drug Deliver Rev 91 (2015) 109–124.

[7]. Lundquist P, Artursson P, Oral absorption of peptides and nanoparticles across the human intestine: Opportunities, limitations and studies in human tissues, Adv Drug Deliver Rev 106 (2016) 256–276.

[8]. Martins JP, Kennedy PJ, Santos HA, Barrias C, Sarmento B, A comprehensive review of the neonatal Fc receptor and its application in drug delivery, Pharmacol Therapeut 161 (2016) 22–39.

[9]. Patel HM, Wild AE, Fc Receptor-Mediated Transcytosis of Igg-Coated Liposomes across Epithelial Barriers, Febs Lett 234(2) (1988) 321–325. [PubMed: 3292290]

[10]. Thuenauer R, Muller SK, Romer W, Pathways of protein and lipid receptor-mediated transcytosis in drug delivery, Expert Opin Drug Del 14(3) (2017) 341–351.

[11]. Israel EJ, Taylor S, Wu Z, Mizoguchi E, Blumberg RS, Bhan A, Simister NE, Expression of the neonatal Fc receptor, FcRn, on human intestinal epithelial cells, Immunology 92(1) (1997) 69–74. [PubMed: 9370926]

[12]. Brambell FW, the transmission of immune globulins from the mother to the foetal and newborn young., Proc Nutr Soc 28(1) (1969) 35–41. [PubMed: 4182340]

[13]. Brambell FW, Hemmings WA, Morris IG, A theoretical model of gamma-globulin catabolism, Nature 203 (1964) 1352–1354. [PubMed: 14207307]

[14]. Rodewald R, Ph-Dependent Binding of Immunoglobulins to Intestinal-Cells of Neonatal Rat, J Cell Biol 71(2) (1976) 666–670. [PubMed: 11223]

[15]. Hornby PJ, Cooper PR, Kliwinski C, Ragwan E, Mabus JR, Harman B, Thompson S, Kauffman AL, Yan ZY, Tam SH, Dorai H, Powers GD, Giles-Komar J, Human and Non-Human Primate Intestinal FcRn Expression and Immunoglobulin G Transcytosis, Pharm Res-Dordr 31(4) (2014) 908–922.

[16]. Ben Suleiman Y, Yoshida M, Nishiumi S, Tanaka H, Mimura T, Nobutani K, Yamamoto K, Takenaka M, Aoganghua A, Miki I, Ota H, Takahashi S, Matsui H, Nakamura M, Blumberg RS, Azuma T, Neonatal Fc receptor for IgG (FcRn) expressed in the gastric epithelium regulates bacterial infection in mice, Mucosal Immunol 5(1) (2012) 87–98. [PubMed: 22089027]

[17]. Rath T, Kuo TT, Baker K, Qiao SW, Kobayashi K, Yoshida M, Roopenian D, Fiebiger E, Lencer WI, Blumberg RS, The Immunologic Functions of the Neonatal Fc Receptor for IgG, J Clin Immunol 33 (2013) S9–S17. [PubMed: 22948741]

[18]. Ober RJ, Martinez C, Lai XM, Zhou AC, Ward ES, Exocytosis of IgG as mediated by the receptor, FcRn: An analysis at the single-molecule level, P Natl Acad Sci USA 101(30) (2004) 11076–11081.

[19]. Ward ES, Zhou JC, Ghetie V, Ober RJ, Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans, Int Immunol 15(2) (2003) 187–195. [PubMed: 12578848]

[20]. Akao Y, Iio A, Itoh T, Noguchi S, Itoh Y, Ohtsuki Y, Naoe T, Microvesicle-mediated RNA Molecule Delivery System Using Monocytes/Macrophages, Mol Ther 19(2) (2011) 395–399. [PubMed: 21102562]

[21]. Alvarez-Erviti L, Seow YQ, Yin HF, Betts C, Lakhal S, Wood MJA, Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes, Nat Biotechnol 29(4) (2011) 341–U179. [PubMed: 21423189]

[22]. Hagiwara K, Ochiya T, Kosaka N, A paradigm shift for extracellular vesicles as small RNA carriers: from cellular waste elimination to therapeutic applications, Drug Deliv Transl Re 4(1) (2014) 31–37.

[23]. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT, Carter BS, Krichevsky AM, Breakefield XO, Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers, Nature Cell Biology 10(12) (2008) 1470–U209. [PubMed: 19011622]

[24]. Smyth T, Kullberg M, Malik N, Smith-Jones P, Graner MW, Anchordoquy TJ, Biodistribution and delivery efficiency of unmodified tumor-derived exosomes, Journal of Controlled Release 199 (2015) 145–155. [PubMed: 25523519]

[25]. Smyth TJ, Redzic JS, Michael WB, Anchordoquy TJ, Examination of the specificity of tumor cell derived exosomes with tumor cells in vitro, Bba-Biomembranes 1838(11) (2014) 2954–2965. [PubMed: 25102470]

[26]. Kusuma RJ, Manca S, Friemel T, Sukreet S, Nguyen C, Zempleni J, Human vascular endothelial cells transport foreign exosomes from cow's milk by endocytosis, Am J Physiol-Cell Ph 310(10) (2016) C800–C807.

[27]. Munagala R, Aqil F, Jeyabalan J, Gupta RC, Bovine milk-derived exosomes for drug delivery, Cancer Lett 371(1) (2016) 48–61. [PubMed: 26604130]

[28]. Baier SR, Nguyen C, Xie F, Wood JR, Zempleni J, MicroRNAs Are Absorbed in Biologically Meaningful Amounts from Nutritionally Relevant Doses of Cow Milk and Affect Gene Expression in Peripheral Blood Mononuclear Cells, HEK-293 Kidney Cell Cultures, and Mouse Livers, J Nutr 144(10) (2014) 1495–1500. [PubMed: 25122645]

[29]. Zempleni J, Aguilar-Lozano A, Sadri M, Sukreet S, Manca S, Wu D, Zhou F, Mutai E, Biological Activities of Extracellular Vesicles and Their Cargos from Bovine and Human Milk in Humans and Implications for Infants, J Nutr 147(1) (2017) 3–10. [PubMed: 27852870]

[30]. Agrawal AK, Aqil F, Jeyabalan J, Spencer WA, Beck J, Gachuki BW, Alhakeem SS, Oben K, Munagala R, Bondada S, Gupta RC, Milk-derived exosomes for oral delivery of paclitaxel, Nanomed-Nanotechnol 13(5) (2017) 1627–1636.

[31]. Aqil F, Munagala R, Jeyabalan J, Gupta RC, Milk derived exosomes: Scalable source of biologically active drug delivery nanoparticles, Cancer Res 74(19) (2014).

[32]. Ober RJ, Radu CG, Ghetie V, Ward ES, Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies, Int Immunol 13(12) (2001) 1551–1559. [PubMed: 11717196]

[33]. Epple LM, Griffiths SG, Dechkovskaia AM, Dusto NL, White J, Ouellette RJ, Anchordoquy TJ, Bemis LT, Graner MW, Medulloblastoma Exosome Proteomics Yield Functional Roles for Extracellular Vesicles, Plos One 7(7) (2012).

[34]. Graner MW, Alzate O, Dechkovskaia AM, Keene JD, Sampson JH, Mitchell DA, Bigner DD, Proteomic and immunologic analyses of brain tumor exosomes, Faseb J 23(5) (2009) 1541–1557. [PubMed: 19109410]

[35]. Hellwinkel JE, Redzic JS, Harland TA, Gunaydin D, Anchordoquy TJ, Graner MW, Glioma-derived extracellular vesicles selectively suppress immune responses, Neuro-Oncology 18(4) (2016) 497–506. [PubMed: 26385614]

[36]. Fete MG, Betker JL, Shoemaker RK, Anchordoguy TJ, A Novel Method for Conjugating the Terminal Amine of Peptide Ligands to Cholesterol: Synthesis iRGD-Cholesterol, Therapeutic Delivery in press (2018).

[37]. Nie X, Zhang JK, Xu Q, Liu XG, Li YP, Wu Y, Chen CY, Targeting peptide iRGD-conjugated amphiphilic chitosan-co-PLA/DPPE drug delivery system for enhanced tumor therapy, Journal of Materials Chemistry B 2(21) (2014) 3232–3242.

[38]. Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Girard OM, Hanahan D, Mattrey RF, Ruoslahti E, Tissue-Penetrating Delivery of Compounds and Nanoparticles into Tumors, Cancer Cell 16(6) (2009) 510–520. [PubMed: 19962669]

[39]. Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Greenwald DR, Ruoslahti E, Coadminstration of a Tumor-Penetrating Peptide Enhances the Efficacy of Cancer Drugs, Science 328(5981) (2010) 1031–1035. [PubMed: 20378772]

[40]. Teesalu T, Sugahara KN, Kotamraju VR, Ruoslahti E, C-end rule peptides mediate neuropilin-1-dependent cell, vascular, and tissue penetration, P Natl Acad Sci USA 106(38) (2009) 16157–16162.

[41]. Samuel M, Chisanga D, Liem M, Keerthikumar S, Anand S, Ang CS, Adda CG, Versteegen E, Jois M, Mathivanan S, Bovine milk-derived exosomes from colostrum are enriched with proteins implicated in immune response and growth, Sci Rep-Uk 7 (2017).

[42]. Pridgen EM, Alexis F, Kuo TT, Levy-Nissenbaum E, Karnik R, Blumberg RS, Langer R, Farokhzad OC, Transepithelial Transport of Fc-Targeted Nanoparticles by the Neonatal Fc Receptor for Oral Delivery, Sci Transl Med 5(213) (2013).

[43]. Reinhardt TA, Lippolis JD, Nonnecke BJ, Sacco RE, Bovine milk exosome proteome, J Proteomics 75(5) (2012) 1486–1492. [PubMed: 22129587]

[44]. Reinhardt TA, Sacco RE, Nonnecke BJ, Lippolis JD, Bovine milk proteome: Quantitative changes in normal milk exosomes, milk fat globule membranes and whey proteomes resulting from Staphylococcus aureus mastitis, J Proteomics 82 (2013) 141–154. [PubMed: 23459212]

[45]. Yang M, Song DH, Cao XY, Wu RN, Liu B, Ye WH, Wu JR, Yue XQ, Comparative proteomic analysis of milk-derived exosomes in human and bovine colostrum and mature milk samples by iTRAQ-coupled LC-MS/MS, Food Res Int 92 (2017) 17–25. [PubMed: 28290293]

[46]. Graner MW, Epple LM, Dusto NL, Lencioni AM, Nega M, Herring M, Winston B, Madsen H, Bemis LT, Anchordoquy TJ, Circulating exosomes as new biomarkers for brain disease and injury, Proc SPIE 8723, Sensign Technologies for Global Health Military Medicine, and Environmental Monitoring !!! (2013).

[47]. El-Loly MM, Bovine milk immunoglobulins in relation to human health, International Journal of Dairy Science 2 (2007) 183–195.

[48]. Thanki K, Gangwal RP, Sangamwar AT, Jain S, Oral delivery of anticancer drugs: Challenges and opportunities, Journal of Controlled Release 170(1) (2013) 15–40. [PubMed: 23648832]

[49]. Kalepu S, Nekkanti V, Insoluble drug delivery strategies: review of recent advances and business prospects, Acta Pharm Sin B 5(5) (2015) 442–453. [PubMed: 26579474]

[50]. Porter CJH, Trevaskis NL, Charman WN, Lipids and lipid-based formulations: optimizing the oral delivery of lipophilic drugs, Nat Rev Drug Discov 6(3) (2007) 231–248. [PubMed: 17330072]

[51]. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nature Cell Biology 9(6) (2007) 654–U72. [PubMed: 17486113]

[52]. Admyre C, Grunewald J, Thyberg J, Gripenback S, Tornling G, Eklund A, Scheynius A, Gabrielsson S, Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid, Eur Respir J 22(4) (2003) 578–583. [PubMed: 14582906]

[53]. Thery C, Zitvogel L, Amigorena S, Exosomes: Composition, biogenesis and function, Nat Rev Immunol 2(8) (2002) 569–579. [PubMed: 12154376]

[54]. Benmoussa A, Lee CHC, Laffont B, Savard P, Laugier J, Boilard E, Gilbert C, Fliss I, Provost P, Commercial Dairy Cow Milk microRNAs Resist Digestion under Simulated Gastrointestinal Tract Conditions, J Nutr 146(11) (2016) 2206–2215. [PubMed: 27708120]

[55]. Wolf T, Baier SR, Zempleni J, The Intestinal Transport of Bovine Milk Exosomes Is Mediated by Endocytosis in Human Colon Carcinoma Caco-2 Cells and Rat Small Intestinal IEC-6 Cells, J Nutr 145(10) (2015) 2201–2206. [PubMed: 26269243]

[56]. Bian F, Xiong B, Yang XY, Jin S, Lipid rafts, ceramide and molecular transcytosis, Front Biosci-Landmrk 21 (2016) 806–838.

[57]. Anchordoquy TJ, Simberg D, Watching the gorilla and questioning delivery dogma, Journal of Controlled Release 262 (2017) 87–90. [PubMed: 28713040]

[58]. Kurlansky M, Milk, Bloomsbury Publishing, New York, New York, 2018.

[59]. Allison SD, Molina MDC, Anchordoquy TJ, Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis, Bba-Biomembranes 1468(1–2) (2000) 127–138. [PubMed: 11018658]

[60]. Molina MD, Armstrong TK, Zhang Y, Patel MM, Lentz YK, Anchordoquy TJ, The stability of lyophilized lipid/DNA complexes during prolonged storage, J Pharm Sci-Us 93(9) (2004) 2259–2273.

[61]. Molina MDC, Allison SD, Anchordoquy TJ, Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: Insight from other structural indicators, J Pharm Sci-Us 90(10) (2001) 1445–1455.

[62]. Molina MDC, Anchordoquy TJ, Formulation Strategies to Minimize Oxidative Damage in Lyophilized Lipid/DNA Complexes During Storage, J Pharm Sci-Us 97(12) (2008) 5089–5105.

[63]. Molina MDC, Anchordoquy TJ, Degradation of lyophilized lipid/DNA complexes during storage: The role of lipid and reactive oxygen species, Bba-Biomembranes 1778(10) (2008) 2119–2126. [PubMed: 18445474]

[64]. Payton NM, Wempe MF, Xu YM, Anchordoquy TJ, Long-Term Storage of Lyophilized Liposomal Formulations, J Pharm Sci-Us 103(12) (2014) 3869–3878.
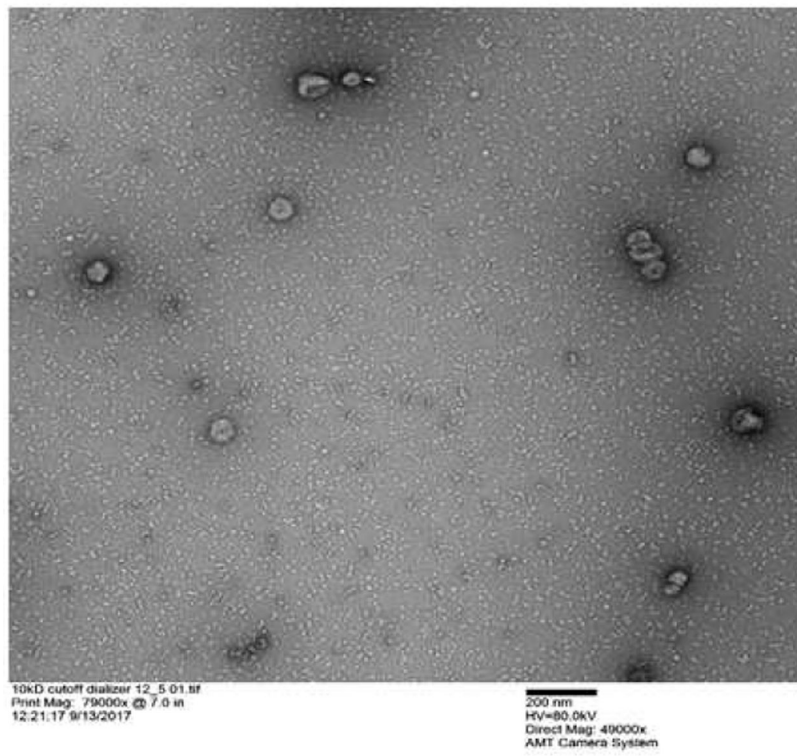
Figure 1A.
Transmission electron micrograph of exosomes isolated from milk.
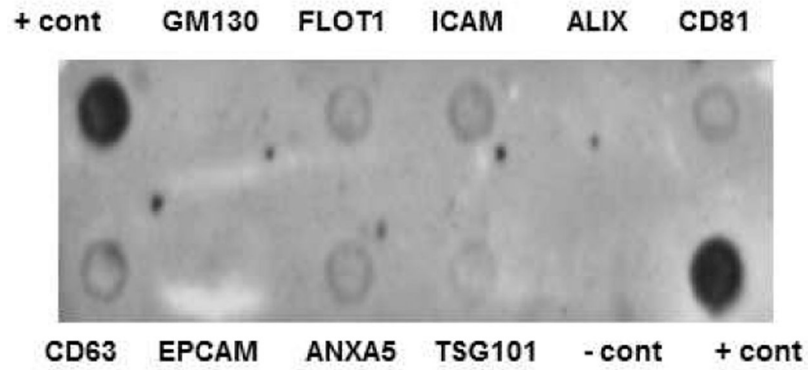
Figure 1B:

Bovine milk exosomes contain standard markers of exosome-like vesicles. ExoCheck Array was probed with lysed milk exosomes, showing positive staining for typical exosome markers (CD63, CD81, ANXA5, FLOT1, ICAM, and TSG101) while negative for the putative contaminant GM130. "+ cont+ = positive control for secondary antibody reactivity; "-cont" = negative control spot.
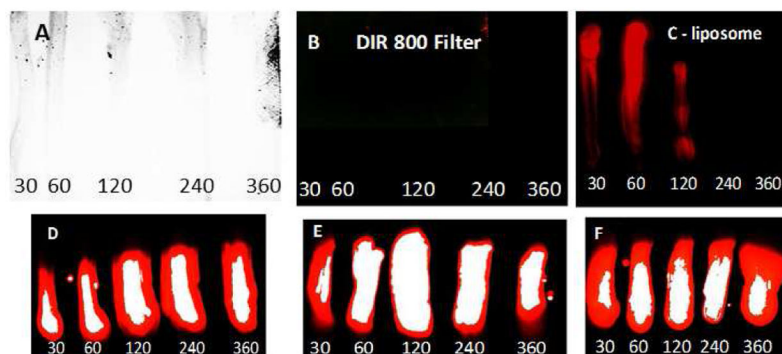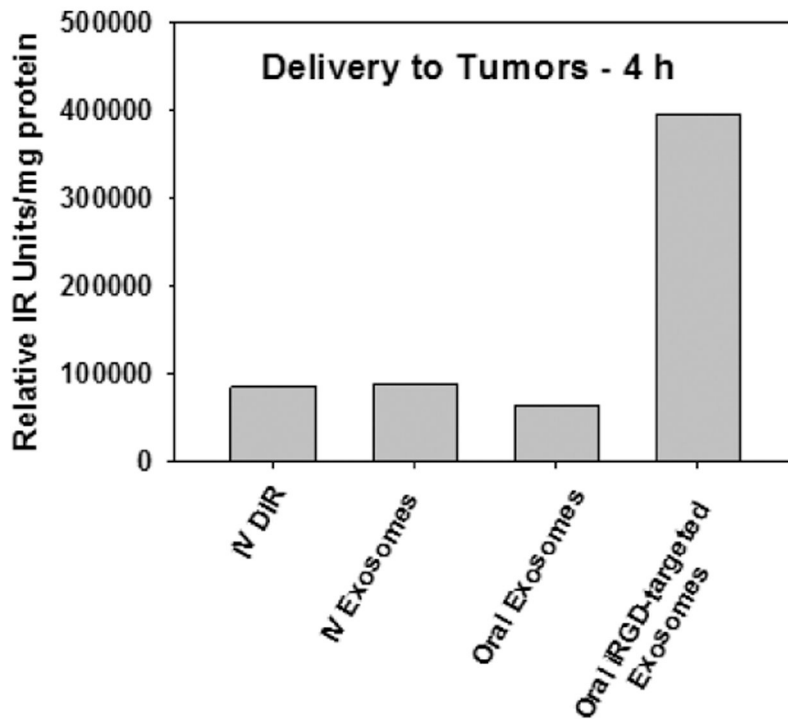
Figure 2.
Absorption from the Gastrointestinal Tract after Oral Gavage. Blood samples collected from mice at 30, 60, 120, 240, and 360 min were applied to a membrane (A) and imaged with an infrared detector (B-F). Samples from mice administered PBS (B) had no detectable levels of IR signal. Blood from mice administered DIR-labelled liposomes (C) showed low levels of IR signal which was undetectable after 120 min. In contrast, blood from mice administered DIR-labelled milk exosomes (D-F) exhibited high IR signal which saturated the detector even on the lowest sensitivity. High signal is maintained in the blood for the duration of the experiment (6 h).
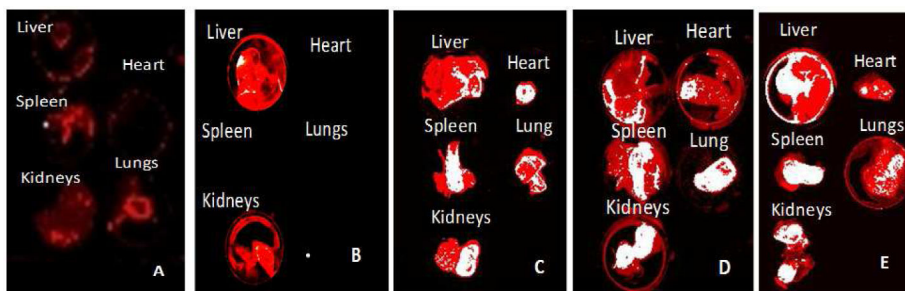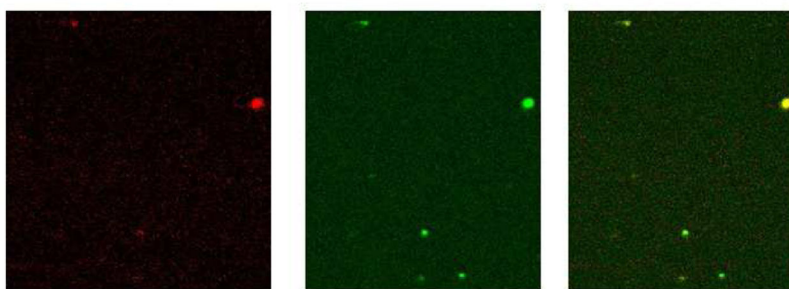
Figure 3.
Tissue Accumulation after Oral Administration. Organs harvested (6 h) from mice administered PBS (A), DIR-labelled liposomes (B), and DIR-labelled milk exosomes (C-E) were imaged. High accumulation of dye in all organs from mice administered exosomes was observed.
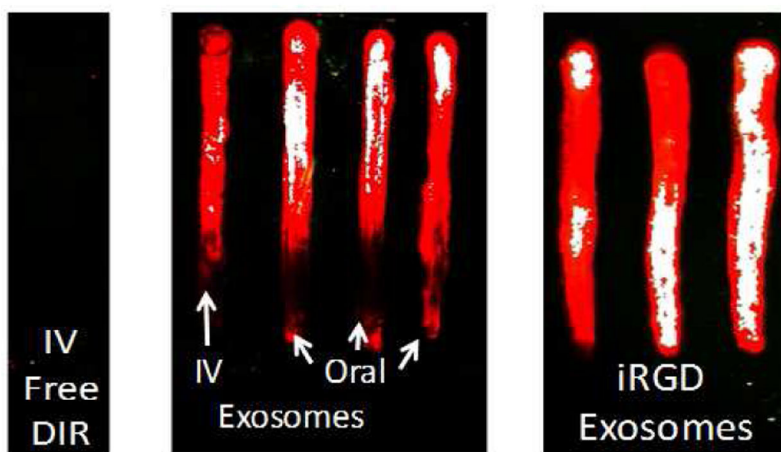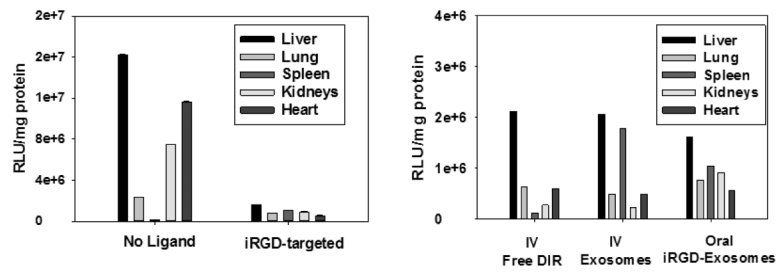
Figure 4.
Bioavailability in Tumor-bearing Mice. Blood from mice administered free DIR or DIR-labelled milk exosomes via IV injection is compared to that from mice administered DIR-labelled exosomes (untargeted or targeted w/ iRGD) via oral gavage after 4 h. Note that the IV dose was 10% of the oral dose.

Figure 5.
Effects of Targeting and Route of Administration on Tumor Accumulation. Tumors from mice administered free DIR or labelled exosomes via IV injection accumulated comparable levels of delivery to oral administration. Note that IV doses were 10% of that administered orally. iRGD-targeting significantly increased tumor delivery. (n=3 for oral administration; std. error < 2%).
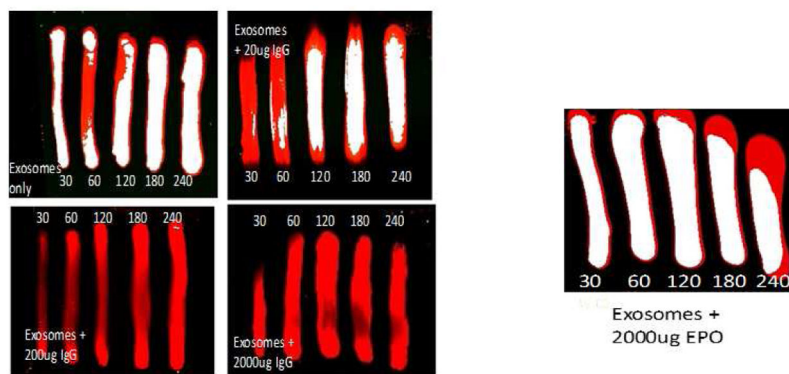
Figure 6.
Double-labelled Exosomes Isolated from Blood after Oral Administration. The image shows that the membrane dye (left) and the RNA dye (middle) are present in the same exosomes (merged, right) after oral administration.
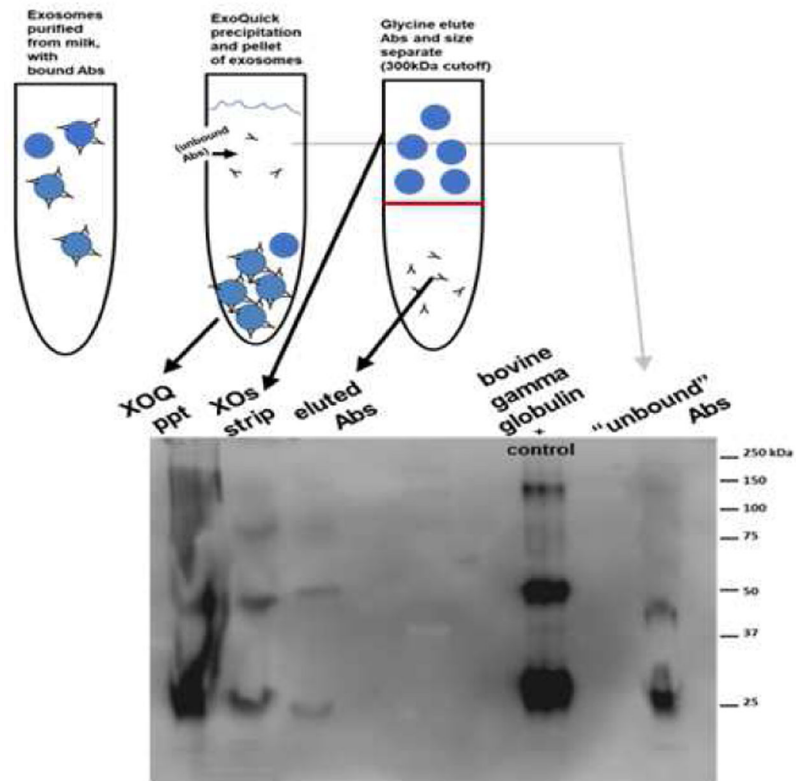
Figure 7.
Effect of Incorporating the iRGD Ligand on Organ Accumulation. Relative infrared signal in different organs after intravenous injection of untargeted and targeted exosomes (left panel). IV-administered free DIR and untargeted exosomes compared to orally-administered targeted exosomes (right panel). Note difference in scales between panels.

Figure 8.
Co-administration of IgG Reduces Absorption. DIR-labelled exosomes were co-administered with different amounts of bovine IgG via oral gavage and blood samples were imaged at 0.5, 1, 2, 3, and 4 h (left). Co-administration of erythropoietin (EPO) did not reduce absorption (right).

Figure 9.
Western blot demonstrating that bovine IgG is present in/on cow milk exosomes. Purified milk exosomes were untreated (XOQ ppt) or treated to strip antibodies (XOs strip). Eluted antibodies (eluted Abs), bovine g-globulin as a positive control, and "Unbound" Abs from the supernatant are also shown.