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Evaluation of Asymmetric Immunoliposomal Nanoparticles for Cellular Uptake

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Abstract

Effective and targeted *in vivo* delivery of polynucleotide therapeutics is the key for the treatment of many diseases. Asymmetric immunoliposomes can be used as vehicles to deliver polynucleotides effectively because the two leaflets of the bilayer can have different compositions, which enhance the delivery capacity. The formation and *in vitro* cellular uptake of asymmetric immunoliposomes containing polynucleotide cargoes were studied here. Maleimide functionalized DSPE-PEG (2000) were incorporated into the outer leaflet to produce asymmetric liposomes capable of covalently attaching antibodies. Thiolated antibodies from both human and rabbit origin were conjugated to produce asymmetric pendant-type immunoliposomes that retain their specificity towards detection antibodies through the formation process. Human IgG conjugated asymmetric immunoliposomes were readily internalized (> 20 per cell) by macrophage, HEPG2, and CV-1 monkey kidney cells. The cells internalized the liposomal nanoparticles by the endocytic pathway. The immunoliposome-encapsulated endosomes were intact for at least 5 days and sequestered the plasmid from expression by the cell.

Keywords

inverse emulsion; maleimide functionalization; Human IgG conjugated; HEPG2; CV-1

Introduction

Effective *in vivo* delivery of polynucleotide therapeutics (*i.e.*, DNA and siRNA) is complex and challenging. We have previously shown that asymmetric liposomal nanoparticles (different inner and outer bilayer lipid leaflet composition) can be produced to encapsulate polynucleotides using an inverted emulsion technique. (Whittenton et al., 2008) In this asymmetric design, the inner leaflet is composed of cationic lipids, which are presumed to form an electrostatic interaction with the negatively charged polynucleotide cargo to increase encapsulation efficiency and liposomal nanoparticle stability. The outer leaflet is composed of neutral and/or anionic lipids to minimize elimination by the mononuclear

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phagocytic system (MPS), which readily recognizes and removes positively charged particles. This technique is advantageous over conventional aqueous lipid-cargo extrusion methods because it assembles a unilamellar lipid bilayer directly around the cargo, eliminating the need for recovering unencapsulated cargo, while allowing for the creation of liposomal nanoparticles with unique properties only achievable by an asymmetric design. (Pautot et al., 2003b, Pautot et al., 2003a) Eliminating the need to recover unencapsulated cargo is extremely important for expensive cargoes such as siRNA.

The inverse emulsion technique produces asymmetric liposomal nanoparticles through the use of oil-water interfaces by forming each leaflet of the bilayer independently. A 3-layer system is formed in which the top layer is the first lipid-oil solution containing nano-sized aqueous droplets with the polynucleotide inside (Figure 1). The lipids, because of their amphiphilic nature, will collect around the droplets with their hydrophobic tails pointed outward to form inverse emulsion nanoparticles. The lipids used in the inverse emulsion phase will form the inner leaflet of the asymmetric liposomal nanoparticles. The middle or intermediate phase is a second lipid-oil solution (of the same oil, but a different lipid), which forms the outer leaflet. The lipids in this phase collect at the oil-water interface of the intermediate phase and bottom aqueous phase with their hydrophobic tails pointing upward. Formation of the encapsulated asymmetric liposomes is performed by centrifuging the inverse emulsion nanoparticles downward, through the intermediate phase into the bottom aqueous phase. As the inverse emulsion nanoparticles pass through the intermediate and aqueous phase interface, they acquire the outer leaflet of lipid to form polynucleotide encapsulated asymmetric lipid bilayer vesicles.

Understanding how immunoliposomal and liposomal nanoparticles are internalized and processed within a cell is important to effectively deliver the nanoparticle's therapeutic content to the correct destination within the cell. For example, aminoglycoside antibiotics, which cause ribosomal miscoding, (Ortego et al., 2007) and siRNA, which targets mRNA for degradation, are only effective if delivered to the cytosol. (Aigner, 2006) A schematic of the immunoliposomal and liposomal internalization pathways are shown in Figure 2. In general, there are two uptake pathways, fusion and endocytosis.

The vast majority of immunoliposomes/liposomes are internalized by the cell through endocytic pathways. (Mandal et al., 2003) These pathways include clathrin-dependent, clathrin-independent, and caveolae-mediate endocytosis. (Rejman et al., 2006) In clathrin-dependent endocytosis, the binding of plasma membrane receptors causes the assembly of AP-2 complexes on the internal side of the plasma membrane. The AP-2 complexes serve as nucleation sites for the polymerization of clathrin. As clathrin builds up on the internal side of the plasma membrane, the local area begins to invaginate. With the help of dynamin, a clathrin coated vesicle buds off from the plasma membrane into the cytosol. If an immunoliposome was responsible for the receptor binding, then that immunoliposome would have been invaginated into the clathrin coated vesicle. The clathrin coated vesicle then loses its clathrin coating, leaving behind what is now called an endosome. (Benmerah and Lamaze, 2007, Rappoport, 2008) Endosomes typically deliver their contents to lysosomes. Lysosomes are internal vesicles within the cell that contain digestive enzymes and have an internal pH of 4.5. They serve as a way for the cell to demolish worn organelles, and destroy virus particles and bacteria.

Immunoliposomes/liposomes trapped within lysosomes are not likely to escape and are ultimately believed to be degraded along with their cargo. Liposomes or polysomes have been designed in the past to allow their cargo to escape the lysosome. (Mandal et al., 2003) For example, hemolysin containing liposomes have been shown to escape lysosome degradation by hemolysin's lipid bilayer poration and disruption abilities. Liposomes made

of pH sensitive lipids, such as cholesteryl hemisuccinate (CHEMS), cause the lipid bilayer to become structurally unstable at low pH.(Tachibana et al., 2003) If the liposome's cargo escapes by lysosome disruption or membrane fusion, then the cargo is likely to be delivered to the cytosol.

Macropinocytosis is a clathrin-independent internalization pathway that involves the invagination of typically large particles (> 500 nm).(Gong et al., 2008) It occurs by large scale lamellar waves in the plasma membrane (edge and circular dorsal ruffles) that fold inward and close off. Until recently, macropinocytosis was considered to be a non-selective internalization process, however new evidence suggests that macropinocytosis is involved in antigen sampling of antigen-presenting cells.(Gong et al., 2008) Macropinocytosis also leads to the formation of endosomes and finally digestive lysosomes.

Caveolae-mediated endocytosis has some similarity to clathrin-dependent endocytosis, but with a major difference in regards to the fate of the immunoliposome/liposome. Caveolin proteins recognize lipid domains (lipid rafts) that contain a locally high concentration of cholesterol, saturated lipids, and signaling proteins, such as receptors, integrins, and kinases. (Benmerah and Lamaze, 2007) Caveolin proteins embed themselves into the inner leaflet of the plasma membrane forming hairpin structures. It is believed that these structures promote a positive curvature on the membrane to create a small invagination. Like that of clathrin-dependent endocytosis, dynamin also plays a role in cleaving off the invagination to form an endosome. Caveolae formed endosomes however, do not deliver their cargo to lysosomes, thus avoiding the fate of lysosome degradation. Instead they become what are called caveosomes, which have a neutral pH, and act as a trafficking intermediate.(Benmerah and Lamaze, 2007) Much is still unknown about caveosomes and their ability to sort and direct the trafficking of its contents. Designing immunoliposomes/liposomes to encourage caveolae-mediated endocytosis, thus avoiding lysosomal degradation, may prove more effective for polynucleotide and other cargo delivery (adjuvants in vaccination, signal enhancers in medical diagnostics, peptides for therapeutic use). (Lasic, 1995) Although immunoliposomes/liposomes almost exclusively enter the cell by the endocytic pathway, fusion of the immunoliposome/liposome with the plasma membrane is possible and even desirable to directly deliver the cargo to the cytosol and avoid lysosomal degradation. Work is being conducted to exploit lipid rafts on the cell membrane to promote direct fusion of compositionally specific liposomes.(Ikonen, 2001)

The goal of this work was to study the formation and *in vitro* cellular uptake of asymmetric immunoliposomes encapsulating a polynucleotide cargo. This was achieved by introducing terminally functionalized polyethylene glycol attached lipids (functionalized PEGylated lipids) into the intermediate phase of 3-layer oil-water system. After bilayer formation, antibodies were covalently attached to the end of the functionalized PEG. The internalization of these asymmetric immunoliposomes in HepG2 (hepatocarcinoma), RAW 264.7 (murine monocyte/macrophage), and CV-1 (monkey kidney cells) was studied. HepG2 was our target cell. RAW was used to demonstrate non-specific internalization. CV-1 was used to prove specificity. The HepG2 cell line displays several key surface receptors, including insulin IFG II, LDL and glypican-3. (Gherardi et al., 1992) The glypican-3 receptor is known to be overexpressed by hepatocellular carcinoma as compared to healthy liver cells, neighboring liver tissue, and other types of cancer, including intrahepatic cholangiocarcinoma, and gallbladder cancer. (Mast et al., 1997, Sung et al., 2003) Because of glypican-3's unique overexpression in hepatocellular carcinoma, it has been proposed that it could be used as a liver cancer biomarker. Specifically, glypican-3 is part of the glypican family of receptors, which are known to be involved in cell proliferation and differentiation. (Man et al., 2005) The mutation of glypican-3 in humans can cause Simpson-Golabi-Behmel syndrome, which is characterized by over-growth of craniofacial

features and appendages.(Garganta and Bodurtha, 1992) The major aim of this work is to design and produce asymmetric immunoliposomes to target the delivery of siRNA to hepatocellular carcinoma *in vivo*. Towards this aim, the current study is to evaluate the cellular immunoliposomal uptake *in vitro* by hepatocellular carcinoma. The human hepatocellular carcinoma HepG2 cell line was used for this evaluation. The immunoliposomes were designed to specifically target the HepG2 cells by changing the conjugated antibody to a rabbit anti-human glypican-3 antibody. (Mast et al., 1997, Midorikawa et al., 2003)

Materials and Methods

Materials

Dodecane (Sigma, #D221104), mineral oil (Sigma, #330779), or squalene (Sigma, #S3626) were used as the oil phase. Tris-buffered saline (TBS) was prepared with 100 mM NaCl (Sigma, S5886) and 5 mM tris base (Promega, #H5131) at pH 7.4. The cargo used to produce the inverse emulsion contained either TBS or deionized water with Alexa Fluor 350 hydrazide sodium salt (Molecular Probes, #A10439) or 21-mer DNA oligo with 5' covalently bound Alexa Fluor 350 or Cy3 (MWG-Biotech) for fluorescent visualization. The 21-mer oligo is used here as a proxy for the more expensive siRNA, whose delivery is the goal of the overall project. POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), NBD-PC (1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine), POPS (1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine]), NBD-PS (1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phospho-L-serine), DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), and DSPE-PEG(2000)-MAL (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Maleimide (Polyethylene Glycol) 2000] (Ammonium Salt)) lipids were obtained in chloroform from Avanti Lipids. Lyophilized cholesterol was obtained from Sigma (#362794) and chloroform from EM Science (#CX1055-9). Thirty milliliter syringes were obtained from BD (#309650) and 15 mL centrifuge tubes from Corning (#430052). Fluorescent quenching was performed using sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) (#157953) from Sigma-Aldrich and liposome lysing was carried out using Triton-X-100 (#BP151) from Fisher Scientific. Thiolation of antibody was performed using Traut's reagent (2-Iminoethanol hydrochloride) from Sigma-Aldrich (#16256). Purification of thiolated antibody was carried out using PD-10 Sephadex G-25M desalting columns from Supelco. Human serum IgG (#14506) was from Sigma-Aldrich, rabbit glypican-3 antibody (#sc-11395) and FITC labeled goat anti-rabbit IgG (#sc-2012) was from Santa Cruz Biotechnology, and Cy3 labeled goat anti-human IgG (#109095003) was obtained from Jackson Immunology. Glass-bottom culture dishes (35 mm, #P35G-0-14-C) for microscopy were purchased from MatTek Corporation. The murine monocyte/macrophage cell line, RAW 264.7, was obtained from ATCC (TIB-71) and the monkey kidney cell line, CV-1, from ATCC (CCL-70). The enhanced green fluorescent plasmid (eGFP) was obtained from Santa Cruz Biotech (#sc-5046) and replicated to produce usable quantities at the Baylor College of Medicine, Texas Medical Center. The fluorescent water-soluble fluors Alexa 350 (#A10439) and 555 (#A20501MP) sodium hydrazide salts were obtained from Invitrogen.

Liposome Formation

Neutral and cationic lipids (DMPC, DOTAP) to comprise the inner leaflet of the asymmetric bilayer were dissolved in oil (dodecane, mineral oil, or squalene) to a concentration of 0.13 to 1.0 mM. The aqueous phase containing the fluorescently-tagged DNA oligo (0.05 to 0.1 mM) and/or Alexa Fluor 350 salt (0.57 to 0.72 mM) were mixed with the oil to form an inverse emulsion with water droplets (0.5-5 volume%) suspended in the continuous oil phase in an ultrasonic bath. As the water droplets form, the lipid molecules adsorb at the oil-

water interface of the droplets to form a monolayer with their hydrophilic head groups pointed inward and the hydrophobic tails pointed outward. The lipid monolayer helps stabilize the inverse emulsion by reducing droplet coalescence. To control inverse emulsion particle size, the inverse emulsion was sonicated for 10-60 min and then extruded by hand through a polycarbonate membrane of small pore size (e.g., 0.2-5 μm). A second lipid-oil solution, termed the intermediate phase, was prepared separately by dissolving neutral lipids to comprise the outer leaflet of the bilayer (DMPC, POPC with NBD-PC, etc.) in the same oil used for the inverse emulsion. The intermediate phase was placed on top of an aqueous phase as shown in Figure 1 and allowed to equilibrate. Since the oil is less dense than water, it remains above the aqueous phase. The outer leaflet lipid molecules in the oil phase diffuse from the bulk to the oil-water interface, again forming a monolayer with the heads groups pointed downward and the fatty acid tails pointed upward. The inverse emulsion is then gently poured on top of the intermediate phase (Figure 1). The water droplets slowly fall through the oil phase and approach the oil-water interface owing to their higher density. Upon crossing the interface, the emulsion droplets pick up a second layer of lipid forming a bilayer and transforming the emulsion droplets into unilamellar vesicles. (Pautot et al., 2003b) The above process is hastened by centrifugation. The liposome size distribution was characterized by dynamic light scattering (DLS) with Malvern ZET 5004 ($\lambda = 633 \text{ nm}$) and ALV-5000 ($\lambda = 514.5 \text{ nm}$).

Asymmetric Immunoliposome Antibody Conjugation

Asymmetric immunoliposomal nanoparticles were formed by adding a commercially available maleimide functionalized PEGylated lipid (1 mol% MAL-PEG-DSPE, see Figure 3a) into the intermediate phase of the inverse emulsion as described above and in our previous work. (Whittenton et al., 2008) The maleimide functional group reacts readily in an aqueous environment with thiols. Therefore, to conjugate the antibodies to the end of the PEG chains, thiol groups were added to the antibodies by converting the primary amines contained natively within the antibody to thiols. The reaction scheme for the antibody thiolation and conjugation to the MAL-PEGylated lipids is shown in Figure 3b. The thiolated antibodies are added directly to the maleimide functionalized PEGylated liposomes. A covalent thiol-ether linkage then readily forms between the antibody and MAL-PEGylated liposome to form a pendant-type immunoliposome.

In-vitro test of liposome activity

The liposomes/immunoliposomes ability for internalization and biofunctionality of their polynucleotide cargo was assessed in three cell lines: HepG2 (hepatocellular carcinoma), RAW 264.7 (monocyte/macrophage), and CV-1 (monkey kidney cells). Unless otherwise stated, cells were seeded 24 h prior to use in 35 mm glass-bottom tissue culture dishes at a density of approximately $2 \times 10^5 - 2 \times 10^6$ cells per dish in 2 mL of complete media. Before addition of liposome/immunoliposome sample, the culture media was removed by aspiration and the culture dishes were rinsed with complete media without FBS and antibiotics and then filled with less than 2 mL of the same media. Cell solution was then added to each culture dish to provide the desired number of cells with a total media volume of 2 mL. The cultures were incubated overnight at 37° C and 5% CO₂. The FBS and antibiotics were removed from the media to ensure their presence would not negatively impact uptake.

After 24 h incubation with the liposome/immunoliposome samples (immunoliposome to cell ratio = 100:1), the cells were examined by transmitted light and fluorescent microscopy. To verify internalization of immunoliposomes the media was removed by aspiration, rinsed and 2 mL of fresh media containing 10% FBS, antibiotics, Hoechst 33342 (5 $\mu\text{L/mL}$), and a second (Cy3 or FITC) fluorescently labeled goat anti-rabbit or goat anti-human IgG detection antibodies (5 $\mu\text{L/mL}$) was added and the cultures were incubated for

approximately 20 min. If the immunoliposomes were internalized, then the detection antibody would be inaccessible to the immunoliposomes and therefore unable to bind to their antibody conjugated surface for fluorescent visualization. The Hoechst stain was used to visualize the nuclei of cells.

Biofunctionality of the polynucleotide cargo was assessed by encapsulating an enhanced Green Fluorescent Protein plasmid (eGFP). The plasmid is 7.3 kb, uses a cytomegalovirus (CMV) mammalian expression promoter, and contains kanamycin resistance gene for selection in *E. coli* and neomycin resistance gene for selection in stable mammalian systems. (Biotechnology, 1999) The eGFP plasmid was used at concentrations of 0.1 to 1.0 $\mu\text{g}/\mu\text{L}$ in the inverse emulsion cargo. If mammalian transfection of the eGFP plasmid occurs, fluorescent levels of GFP (ex: 488 nm, em: 508 nm) should be visible within 24-72 h.

Results

Liposome Size Distribution

The liposome product produced a trimodal distribution. The first and most prominent mode has a mean radius of 44 nm followed by a mode of 188 nm and 489 nm. In terms of size stability however, the three modes remain relatively constant over a 160 hr period, especially in comparison to the stability of the inverse emulsion. The increased liposome stability is expected because the lipid bilayer vesicles are thermodynamically more stable than lipid monolayer inverse emulsions (Whittenton et al., 2008).

Antibody Conjugation

To confirm that the antibody conjugation process was successful, an experiment was performed in which asymmetric liposomes were produced with 85 mol% POPC and 15 mol % DOTAP (cationic lipid) in the inner leaflet and 99 mol% POPC and 1 mol% MAL-PEG-DSPE in the outer leaflet. Squalene was the oil used and the inverse emulsion was extruded with a 400 nm pore size polycarbonate membrane. After the MAL-PEG liposomes were made and concentrated using diafiltration, thiolated human IgG antibody was added in stoichiometric excess and incubated at room temperature for approximately 1 hour. Unconjugated human IgG was removed by a second diafiltration. To detect the success of the conjugation, goat anti-human IgG detection antibody with Cy3 tag (ex: 552, em: 565 nm) was added in dilute concentration (30 $\mu\text{g}/\text{mL}$) to the immunoliposome sample and imaged. The bright objects in Figure 4 prove the specific binding of the detection antibody to the complementary human IgG conjugated onto the asymmetric liposomes' surface. The immunoliposomes in the figure appear larger than the size at which they were extruded for several reasons: (1) the inverse emulsion after extrusion may have coalesced to increase its size; (2) some of the liposomes in the view field may not be precisely in the focal plane, which can exaggerate their apparent size; and (3) since the immunoliposomes are point sources of light, scattering of emitted light can create a false appearance of larger size.

Evaluation of Asymmetric Immunoliposomal Nanoparticles for Cellular Uptake

The *in vivo* delivery of polynucleotides using liposomal encapsulation is a multistep process. The first step is targeted delivery of the liposomes to their destination. The second is cellular uptake of the DNA-encapsulated liposomes. The last is the effective expression of the delivered polynucleotide. In developing an *in vivo* delivery vector, it is first prudent to test its ability to be taken up by its target cell *in vitro*. Three cell lines were used to evaluate the asymmetric immunoliposome's ability for uptake: monocyte/macrophage (RAW 264.7), hepatocellular carcinoma (HepG2), and kidney cells (CV-1).

Cellular Uptake in vitro by Murine Monocyte/Macrophage Cells

Macrophage cells provide a convenient baseline to compare asymmetric immunoliposomes internalization because of their inherent ability to phagocytose foreign particles. (Steinberg et al., 2007, Madigan et al., 2000) Immunoliposomes were conjugated with Human IgG antibody because it is known to trigger murine monocyte/macrophage uptake. (Steinberg and Grinstein, 2007) A Cy3-labeled anti-Human IgG antibody was added in dilute concentration to the immunoliposomes prior to addition to the macrophages to fluorescently tag the Human IgG immunoliposomes for imaging.

Fluorescent images of macrophage uptake of the immunoliposomes are shown in Figure 5. Two macrophage cells are shown in the figure and the bright objects are Cy3 fluorescent immunoliposomes. It is evident that the immunoliposomes are inside the cells because each image slice represents a different focal plane in the z-direction, and as the focal plane moves, new immunoliposomes come into focus, confirming that the immunoliposomes are imbedded within the macrophage and not just adsorbed to the macrophages' outer surface. The nucleus of the lower macrophage is evident in slices #2-4, which indicates that the immunoliposomes have not entered into the nucleus.

In a second experiment, human IgG conjugated asymmetric immunoliposomes were again presented to macrophage cells for 24 h. The cells were then rinsed to remove any non-internalized liposomes and a second human IgG detection antibody, tagged with FITC (ex: 494, em: 519 nm), was added in dilute concentration (30 μ g/mL). The purpose of this experiment was to further demonstrate that the immunoliposomes were internalized. Since the Cy3-labeled anti-Human IgG antibody was added in dilute concentration, the newly added FITC detection antibody will bind to the free binding sites on immunoliposomes if the immunoliposomes would be external to the cells. If however, the immunoliposomes were internalized by the cells, then the second FITC detection antibody could not bind to the immunoliposomes because of the physical separation by the cell membrane. Transmitted light and fluorescent images of the macrophage internalized immunoliposomes are shown in Figure 6. The left image shows the placement and morphology of the macrophages using differential interference contrast microscopy (DIC) of transmitted light. The right image is the fluorescent image of the same field of view and shows the Cy3 detection antibody bound immunoliposomes, which appear to be on or inside the macrophage. FITC-tagged immunoliposomes (not shown) were not visible indicating the immunoliposomes were internalized by the macrophages.

Cellular Uptake in vitro by Hepatocellular Carcinoma Cells

One application of these asymmetric immunoliposomes is the treatment of hepatocellular carcinoma by the delivery of the polynucleotide siRNA, which can be specific for the knock down of protein expression necessary for the carcinoma's proliferation. Towards this application, asymmetric immunoliposomes were constructed with the glypican-3 antibody, which is specific for the glypican-3 surface receptor and known to be overexpressed by hepatocellular carcinoma, making it adept for hepatocellular carcinoma targeting. (Mast et al., 1997, Midorikawa et al., 2003)

HepG2 cells were incubated with anti-glypican-3 immunoliposomes. The immunoliposomes prepared here were not stained with Cy3-labeled detection antibody prior to cell exposure. Instead, to visualize the immunoliposomes, the membrane impermeable Alexa Fluor 555 hydrazide salt (0.5 mg/mL) was added to the aqueous droplets used to make the inverse emulsion so that the immunoliposomes encapsulated a fluorescent cargo. Control experiments were also carried out in which asymmetric anti-human IgG conjugated

immunoliposomes and non-antibody conjugated asymmetric liposomes were added to HepG2 cells and incubated for 24 h.

Fluorescent images of HepG2 uptake of the anti-glypican-3 and anti-human IgG immunoliposomes and non-antibody conjugated asymmetric liposomes (still containing 1 mol% DSPE-PEG(2000)-MAL) are shown in Figure 7. Each row of images shows the same field of view for each asymmetric immunoliposome/liposome preparation. The first column of images shows the Hoechst stained nuclei of the cells in each view field (> 20 cells/view field). The second column shows the Alexa Fluor 555 encapsulated immunoliposomes/liposomes in each view field. No FITC-labeled detection antibody bound immunoliposomes/liposomes were visible (not shown) because all the immunoliposomes present in the second column view field have been internalized. For a control, a cluster of non-internalized immunoliposomes was found in the culture and imaged as shown in Figure 8. The figure demonstrates that non-internalized immunoliposomes are clearly illuminated by the FITC-labeled detection antibody. The immunoliposomes in the figure were likely not internalized because they were aggregated. Aggregation was observed after antibody conjugation was performed and was likely caused by liposome-antibody-liposome cross-linking. Such cross-linking can be diminished by further optimizing reagent concentration and reaction time during antibody conjugation.

The results of Figure 7 indicate that HepG2 cells readily internalized asymmetric liposomes/immunoliposomes by the endocytic pathway. They also showed internalization whether or not a specific or non-specific targeting antibody is conjugated to the liposome surface. In terms of polynucleotide delivery to hepatocellular carcinoma, this may be advantageous because the carcinoma may easily take up the unmodified asymmetric liposomal delivery vector. However, if other cells types also easily internalize the asymmetric immunoliposomes, then their targeting specificity is lost and their therapeutic capacity is diminished.

To investigate the effect of PEG surface modification on liposome uptake the same above experiment was repeated, but with the incorporation of 5 mol% DSPE-PEG(2000) to the outer leaflet. A value of 5 mol% was chosen based on literature.(Woodle and Lasic, 1992, Qiu et al., 2008, Plassat et al., 2007, Woodle et al., 1992, Klivanov et al., 1991, Litzinger and Huang, 1992) In addition, the cargo also contained 0.11 $\mu\text{g}/\mu\text{L}$ eGFP plasmid to assess the biofunctionality of the polynucleotide cargo once delivered internally to the cell. The results of the PEGylated liposome internalization and eGFP expression 5 days after the addition of the liposomes are shown in Figure 9a. Similar to the results shown in Figure 7, Glypican-3 antibody tagged liposomes are all internalized in HepG2 liver cancer cells. No FITC-labeled detection antibody bound immunoliposomes/liposomes are visible (not shown) because all the liposomes present in the right view field have been internalized. No eGFP fluorescence is visible (not shown), indicating no plasmid expression.

The Alexa 555 hydrazide salt, which is dissolved in the aqueous cargo of the liposomes, has remained localized within the liposomes as seen by the small intense spherical dots ($\sim 1 \mu\text{m}$) in the right column of image. It is likely that the endosomes, which are formed by internalizing the liposomes through the endocytic pathway, are still intact and still encapsulate the eGFP-plasmid containing liposomes. In order for the plasmid to be expressed it must be released from both the liposomes and endosomes into the cytosol where the cell's internal machinery can transcribe and translate the plasmid sequence to a GFP product.

Cellular Uptake *in vitro* by CV-1 Monkey Kidney Cells

The specificity of an *in vivo* immunoliposome delivery vector is critical for minimizing patient toxicity and harmful side effects. Liposome internalization was also investigated with the CV-1 monkey kidney cell line in contrast to macrophage (which actively phagocytose) and hepatocarcinoma (which is the target cell type). CV-1 cells are known not to display the glypican-3 surface receptor, which is overexpressed in HepG2. CV-1 cells were incubated with Cy3 tagged immunoliposomes, and the images in Figure 9b were acquired 5 days after liposome addition.

Like the macrophage and HepG2 cells, the CV-1 cells also internalized the liposomes to an equal extent regardless of the type of antibody attached (human IgG, rabbit anti-glypican-3). It is plausible that the similar uptake was observed by antibody-targeted liposomes (to HepG2 cells) versus non-targeted liposomes (to CV-1 cells) because the antibody conjugation process denatured the anti-glypican-3 antibody and/or thiolation occurred of an amine group vital for antigen binding. Western blot of conjugated and unconjugated anti-glypican-3 antibody could confirm the retention of antibody specificity to the glypican receptor through the conjugation process.

Internalization by CV-1 cells indicates that the immunoliposomes in their current configuration lack specificity *in vitro* and would be readily internalized by many cell types. Like that of the HepG2 cells, the Alexa 555 fluorescent cargo of the liposomes remained localized within the CV-1 cells, suggesting the endosomes are still intact and sequestering the eGFP plasmid from expression. There were liposomes/immunoliposomes that were external to the CV-1 as visible in the images in the right column of Figure 9b. These vesicles were likely not internalized because of their large size ($> 1 \mu\text{m}$).

Conclusions

Production of liposomes from inverse emulsion allows the design and construction of compositionally different (asymmetric) inner and outer leaflets of the lipid bilayer liposome. Maleimide functionalized DSPE-PEG(2000) can be incorporated into the intermediate phase to produce asymmetric liposomes capable of covalently attaching antibodies, and thiolated antibodies from both human and rabbit origin can be conjugated to produce asymmetric pendant-type immunoliposomes that retain their specificity towards detection antibodies through the formation process. Yet the conjugation conditions used here produced a large number of liposome aggregates, likely by way of liposome-antibody-liposome cross-linking. Further optimization of the reaction conditions will minimize aggregates.

The immunoliposomes' ability for cellular uptake was assessed in 3 cell types: murine monocyte/macrophage (RAW 264.7), human hepatocellular carcinoma (HepG2), and monkey kidney (CV-1). Macrophage cells actively phagocytose human IgG conjugated asymmetric immunoliposomes (> 20 per cell). The cellular uptake is by the endocytic pathway because intact liposomes are seen inside the cells.

To provide specificity and encourage internalization to HepG2 cells, the immunoliposomes were prepared with anti-glypican-3 antibodies to interact with the overexpressed glypican-3 surface receptors. It was also observed that HepG2 cells would internalize the liposomal nanoparticles regardless of the antibody used for targeting, or even without any antibody. A liposomal delivery vector that is easily internalized by the target cell is advantageous, but would be problematic if also easily internalized by non-targeted cell types and the mononuclear phagocytic system (MPS).

Lastly, the immunoliposomes' ability to deliver a polynucleotide cargo that remains biofunctionally active after internalization was assessed. Anti-glypican-3 and human IgG asymmetric immunoliposomes were produced to encapsulate an enhanced Green Fluorescent Protein (eGFP) plasmid. The immunoliposomes were internalized by both HepG2 and CV-1 cells. After 5 days, no GFP fluorescence was detected, indicating the eGFP plasmid was not expressed. A fluorescent dye (Alexa 555 hydrazide salt), also present in the immunoliposomes' cargo, revealed that the internalized immunoliposomes remained as localized points with the cells. It is likely that the immunoliposome-encapsulated endosomes, formed by endocytic uptake, were still intact and sequestering the plasmid from expression by the cell. In the past, liposomal and polysomal (polymer based vesicles) vectors have been designed to be pH sensitivity, so that when the internal pH of the endosome drops, as it does in the endocytic process, the vesicle lyses to release its contents. Such an approach could be incorporated into the asymmetric immunoliposomes to better achieve biofunctionality of its internalized polynucleotide cargo.

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Asymmetric Liposome Production

Figure 1.
A sketch of asymmetric liposome/immunoliposome production from inverse emulsion.

Figure 2.
Immunoliposomal and liposomal membrane fusion and endocytic cellular uptake pathways.

Figure 3.

(a) Chemical structure of the maleimide functionalized PEGylated DSPE lipid used in the outer leaflet of the asymmetric immunoliposomes for conjugation to thiolated antibodies. (b) Antibody and maleimide-PEG-DSPE lipid conjugation chemistry. Free amine groups on the antibody are thiolated with iminothiolane (Traut's reagent). The thiolated antibody then reacts directly with the maleimide functional group at the end of a polyethylene glycol chain attached to the head group of a DSPE lipid.

Figure 4. Human IgG conjugated asymmetric immunoliposomal nanoparticles. Conjugation was detected by adding rabbit anti-human IgG antibody labeled with Cy3 fluorophore.

Figure 5.

Murine monocyte/macrophage (RAW 264.7) cellular uptake of Cy3-labeled human IgG conjugated asymmetric immunoliposomal nanoparticles. Each image represents a different vertical slice of the immunoliposome containing macrophages. Cells were incubated at 37°C and 5% CO₂ in absence of FBS with immunoliposomes 24 h prior to imaging.

Figure 6.

Mouse monocyte/macrophage (RAW 264.7) cellular uptake of Cy3-labeled human IgG conjugated asymmetric immunoliposomal nanoparticles. Left image shows the placement of the cells. Right image shows the internalized Cy3-labeled immunoliposomes. FITC-labeled immunoliposomes (not shown) were not visible, demonstrating immunoliposomes are internal to the cells. Cells were incubated at 37° C and 5% CO₂ in absence of FBS with immunoliposomes 24 h prior to imaging.

Figure 7. HepG2 liver cancer cell uptake of asymmetric liposomes/immunoliposomes. Top row shows uptake of anti-glypican-3 immunoliposomes. Middle row shows uptake of anti-human IgG immunoliposomes. Bottom row shows uptake of non-antibody conjugated asymmetric liposomes (containing 1 mol% unconjugated DSPE-PEG(2000)-MAL). Cells were incubated overnight with FBS and antibiotic free media in the presence of asymmetric liposomes/immunoliposomes encapsulating Alexa Fluor 555 hydrazide salt. Cells were rinsed and stained with Hoechst 33342 to illuminate the nuclei and FITC-tagged detection antibodies complementary to glypican-3 and human IgG to illuminate non-internalized immunoliposomes. Any fluorescence crossover between the images was systematically removed.

Cy3-Detection Antibody Labeled Immunoliposomes

FITC-Detection Antibody Labeled Immunoliposomes

Figure 8. Detection antibody labeling of non-internalized anti-glypican-3 conjugated immunoliposomes. Figure shows a cluster of aggregated immunoliposomes that were treated with Cy3-labeled and FITC-labeled detection antibodies. Any fluorescence crossover between the images was systematically removed.

Figure 9.

(a) HepG2 liver cancer cell uptake of PEGylated asymmetric anti-glypican-3 immunoliposomes. (b) CV-1 monkey kidney cell uptake of PEGylated asymmetric anti-glypican-3 immunoliposomes. In both cases, cells were incubated overnight with FBS and antibiotic free media in the presence of asymmetric immunoliposomes encapsulating Alexa Fluor 555 hydrazide salt and eGFP plasmid. Cells were rinsed and stained with Hoechst 33342 to illuminate the nuclei and FITC-tagged detection antibodies complementary to anti-glypican-3 and human IgG to illuminate non-internalized immunoliposomes. Images were taken 5 days after immunoliposome addition. Any fluorescence crossover between the images was systematically removed. HepG2 cells show internalization of liposomes,

however eGFP expression doesn't occur. Image of External IgG-conjugated liposomes not shown.