University Honors Program

Capstone Approval Page

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Visionary Drug Delivery for Cancer Diseases

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Biochemistry
Vision’ary: Drug Delivery for Ocular Diseases

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Abstract Retinal ocular diseases such as age-related macular degeneration (AMD), clinically significant diabetic macular edema (DME), diabetic retinopathy (DR) and retinal vein occlusions are currently difficult to treat effectively with topical medications and eye drops. Intravitreal injections of pharmacological products are currently the most effective method of treatment. Frequent injections have been found to have adverse effects including infection, endophthalmitis and retinal detachment. Inconsistent patient compliance decreases the effectiveness of the pharmaceuticals and the fiscal impact also poses a problem with recurring intravitreal injections. Liposomes are nano-sized bi-layered vesicles that will encapsulate various hydrophilic or hydrophobic substances necessary for treatment of ocular diseases. Liposomes can be formulated to slowly release the drug thereby preventing frequent painful injections. Liposomes of various lipid to cholesterol concentrations were formulated using the ethanol-injection method and then extruded with a 100 nm pore sized polycarbonate filter. The liposomes were then characterized via confocal microscopy, dynamic light scattering, and transmission electron microscopy to analyze size, homogeneity, stability, and encapsulation efficiency.

I. INTRODUCTION

There are many barriers encountered when attempting ocular drug delivery to the retina. Drugs cannot be easily delivered to the retina by topical methods like eye drops because of structural barriers including the cornea and the lens. Figure 1 shows the anatomy of the eye illustrating the retina, the light sensitive layer of the eye. A localized portion of the retina is the macula, encasing the rods and cones, allowing sight in both the dark and in the light. Systemically administered drugs seldom enter the retina because of the blood/aqueous barrier and the inner/outer blood retinal barriers, consisting of retinal capillary endothelial cells and retinal pigment epithelium cells (RPE), therefore leaving intravitreal injection the current preferred method.1

Current drug delivery options include eye drops, intravitreal injections and liposomes. Eye drops that are administered for acute ocular diseases are often inconsistent in the effectiveness and can cause dry eye and allergic conjunctivitis. It has been reported that 80% of the drops administered exit the eye via the lacrimal duct after two minutes and only 5% of the free drug enters after tearing.1,2

Musin, produced by the tears, forms a hydrophilic barrier around the outer layer of the eye preventing foreign particles from adhering and entering the topical layers.4 The exterior of the eye is split into the epithelium, stroma, and endothelium.5 Figure 2 highlights the individual layers of the cornea.6 The exterior stroma and epithelium of the cornea and conjunctiva form a blockade.
Free drugs administered intraocularly tend to have short half-lives and frequent injections are necessary for satisfactory treatment. This half-life is dependent upon the route that the drug takes once inside the aqueous humor, the jelly-like solution inside the eye cavity. The drug may be diffused into the aqueous humor and eliminated via its rejuvenation or the blood. It may also cross the blood/brain barrier due to permeability or a transport protein.

AMD can be subdivided into wet and dry. Dry AMD is categorized by the formation of a defined, vascular, circular area forming on the retina with a pale color due to diminished pigment. Patients have a loss of vision in a localized area. The wet form is indicative of a fluid layer forming in the Bruch’s membrane and causing the RPE cells to detach. This interference with the photo receptors induces blurry vision called metamorphopsia and may lead to permanent blindness.

The liposomal preparation for our studies focuses on the disease process of wet AMD. For decades the treatment of ocular diseases via intravitreal injection has been used to administer steroids for the treatment of AMD. This particular ocular disease affects central vision and is caused by a deterioration of the photoreceptors in the macula. The first signs of AMD are hyper or hypo pigmentation of the retina, with a pale color due to diminished pigment. Patients have a loss of vision in a localized area. The wet form is indicative of a fluid layer forming in the Bruch’s membrane and causing the RPE cells to detach. This interference with the photoreceptors induces blurry vision called metamorphopsia and may lead to permanent blindness.

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patient compliance, decrease cost, and risk of infection.

The solution to these aforementioned problems may lie in the treatment of ocular diseases with liposomes. Liposomes are spherical vesicles made up of a lipid bi-layered membrane that plays an important role in the encapsulation of drugs in particular but also can enclose proteins, enzymes, antibiotics, hormones and viruses. A hydrophobic layer lies within the nonpolar tails of the bilayer where hydrophobic drugs can be positioned and a hydrophilic core where hydrophilic drugs can be contained, allowing the liposome to have an amphphilic quality.

There exist various lipid compositions both in nature and bioengineered liposomes. Phosphatidylcholine (PC), Fig. 4, has been found to be a viable lipid used in the formation of liposomes. This lipid is the makeup of many biological membranes and is important in cell membrane signaling. It is the most abundant phospholipid in animals, plants and can be found in egg yolk. Because of its cylindrical shape, PC spontaneously forms a bilayer so it is ideally suited for the bulk of the plasma membrane or, in this case, the liposome bilayer. This molecule contains a choline polar head group with hydrocarbon non-polar tails and is categorized as a glycerophospholipid. The unsaturated acyl chains are linked and confer fluidity on the membrane. The amphipathic bilayer not only surrounds the hydrophilic core where hydrophilic drugs can be contained, but hydrophobic drugs can also be dissolved in between the non-polar bilayer.

To prevent the liposome from being too fluid, prevent leakage and reduce permeability, cholesterol (CH) can be added to the liposome formulation, filling in the free space between the lipids. The molecular structure of cholesterol is depicted in Fig. 5. The body of cholesterol consists of a series of fused rings that make the molecule quite rigid. At one end of this planar ring system is a hydroxyl group and at the other end is a hydrocarbon tail, so cholesterol, like other membrane lipids, has both hydrophilic and hydrophobic poles that determine its positioning within the lipid bilayer. When the hydroxyl group is next to the phospholipid ester carbonyl, the rigid body of cholesterol is situated alongside the fatty-acid tails of neighboring phospholipids and can help to order these tails.

The main parameters of our liposomes are slow release and stability. We want to utilize them as a slow release vesicle. Encapsulation stability and entrapment is extremely important in our formulations. The quality must be maintained so no leaking occurs of the drug prematurely. Encapsulation prevents the drug from interacting with surrounding molecules and being metabolized by enzymes and thereby reducing the drug’s effectiveness.

The actual manufacturing method and preparation also needs to be taken into account when thinking wide scale production. Proper materials need to be used to prevent any cytotoxicity and cell death to the fragile tissues of the eye. Systemic affects also need to be considered when administering any form of the drug.

Liposomes are also currently being researched and developed for the treatment of cancer and respiratory diseases by adding polyethylene glycol (PEG) to the liposome formulation to improve encapsulation efficiency and to attach PEG conjugates for specific binding and uptake by the target cell. PEG liposomes are also proven to prevent fusion and aggregation. Studies are also in effect to deliver encapsulats drugs via eye drops although no substantial evidence is confirmed that liposomes can penetrate the outer surface.

Size plays an important role in the preparation of liposomes. Liposomes must be in the nano-size range to be a potential drug carrier targeting the retina. The larger the particle, the more light scattering will occur, interfering with vision. Nanoparticles up to 500 nanometers in diameter that bypass the possible obstruction of steric hindrance in the vitreous humor, having no PEG attachments have been...
found to be distributed quickly through the internal eye. Particles larger than 500 nanometers can interact with the vitreous and remain in the gelatin like material, never reaching the retina. An advantage of liposomes without PEG attachments eliminates the need for covalent interactions which may decrease the activity of the PEGylated molecule.

The surface area of each particle is very large compared to the volume and the size making them more soluble. Therefore the drug can dissolve more readily and become bioavailable in the body. They also need to have the ability to hold a homogeneous liposome size that are stable and can hold their encapsulated payload. These are the primary key elements to be studied for an effective liposome carrier.

Due to the size of small uni-lamellar vesicle (SUV, 20-50 nanometers) or large uni-lamellar vesicle (LUV, 100 nanometers), direct uptake by the eye has been shown ineffective. Studies state that SUV have a shorter half-life than LUV.

In order to determine optimal release stability we formulated and produced liposomes with various lipid to cholesterol molar ratios and formed liposomes via ethanol-injection method and extrusion to observe the resulting stability, size, aggregation, and encapsulation efficiency via transmission electron microscopy (TEM), confocal microscopy (CM), and dynamic light scatterer (DLS).

II. MATERIALS AND METHODS

A. Materials

The phosphatidylcholine, cholesterol, 100% ethanol, florescein and vitamin E were purchased from Sigma. The glass syringes used for the ethanol injection were Hamilton 500 microliters or 100 microliters. The Lipex extruder was purchased from Northern Lipids. The polycarbonate filters are Whatman. The injection machine is Hamilton. The DLS is Brookhaven and the confocal is Nikon.

B. Preparation of Liposomes

Vesicles were prepared using dry PC and CH with 10:1, 8:6, 8:4, and 8:2 PC/CH molar concentration mixtures and were hydrated with 3mL of 100% ethanol. Liposomes were stored at 4 ºC. The samples were alliquotted to 500 µL. Vitamin E was added (1% by volume) to each 500 µL sample to prevent oxidation and aggregation of the sample. The ethanol-injection method was then used at .5, 2, 4, 6, 8, and 10 µL per hour. A 50 or 100 mL glass syringe was used and the 500 µL sample was then drawn up with the syringe. The needle was loaded into a pump that regulated the solutions ejection rate. The bezzle of the needle was placed directly underneath the surface of a 10 mM florescein solution in a round bottom flask. A spinner bar magnet was put in the round bottom flask and the round bottom flask was placed on a magnetic plate at a low stir rate. The injected samples were dried with an argon gas to remove any additional ethanol not encapsulated within the liposomes. The round bottom flask remained on the magnetic stir plate. The argon gas was an attempt to remove any cytotoxic solvent that would be damaging to the eye. The liposomes were then extruded using an extruder with nitrogen gas 10 times through a .4 µm polycarbonate filter, 10 times with a .2 µm filter and 10 times with a .1 µm filter using aseptic technique.

C. Characterization of liposomes

Each sample was then diluted to 10 mM in order to characterize using fluorescence microscopy (Fig. 7). The liposomes were then viewed using UV (Fig. 8). The TEM (Fig. 9) was used to view the liposomes and note any aggregation or large size differential. Images of the liposomes (Fig. 10-13) are from the 40 mg/mL sample in the 10:1, 8:2, 8:4, and 8:6 ratios. Each sample was also characterized using the DLS (Fig. 14) which determined the overall size of the liposomes as well as the homogeneity.

1) Evaluation with fluorescence microscopy

Fig. 7 Fluorescence Microscopy

a. Fluorescence images

Fig. 8 UV image of liposomes
2) *Evaluation with transmission electron microscopy (TEM)*

**Fig. 9** TEM Instrument

a. **TEM images**

**Fig. 10** 40 mg/mL 10:1

**Fig. 11** 40 mg/mL 8:6

**Fig. 12** 40 mg/mL 8:4

3) *Evaluation with dynamic light scatterer*

**Fig. 13** 40 mg/mL 8:2

**Fig. 14** Dynamic Light Scatterer Instrument

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### III. RESULTS

Based on the results of the characterization, there is a large deviation in the size homogeneity of the 8:6 and the 10:1 ratios overall. The 8:2 lipid to cholesterol ratio seems to be the most stable with the smallest standard deviation in the 40mg/mL sample (Fig. 15). In the 50mg/mL sample the 8:2 and the 8:4 ratios are the most stable (Fig. 16). The 60 mg/mL 8:4 and 8:2 ratios are currently still being characterized, but from the data obtained the trend observed in the previous concentrations is consistent (Fig. 17). Overall, the 8:2 ratio of the 50 mg/mL sample has the smallest size with the least standard deviation (Fig. 18). The injection rate analysis indicated that the 10 mL/hr had the smallest size liposome whereas the 4 mL/hr had the most consistent sizing throughout the 40 and 50 mg/mL concentrations.
IV. DISCUSSION

The ongoing project is geared towards a number of different target studies with a goal to ensure the stability of the liposomes is sufficient for drug delivery to humans. The current research is being conducted in order to synthesize and mass produce a stable liposome that is able to release the encapsulated drug at a particular duration of time based on the drugs specifications.

Fluorescence lifetimes would be beneficial to observe the encapsulation efficiency of these structures. Additional samples need to be synthesized between the 40-50 mg/mL range to fine tune the best possible ratio as well as observing the effects of integrating various combinations of lipids into the liposome. Other characterization methods may include dissolution, to monitor the rate of release, or zeta potential, to monitor the electrical charge on the liposomes and thus its plausibility to form aggregates. Based on the successful results of a particular method for synthesis of the liposome used during present research, the efficiency and effectiveness to mass produce liposomes will be analyzed. The capability for a particular method of synthesis of the liposome will be analyzed for the ease to be mass produced efficiently and cost productively.

Further studies are necessary in order to improve upon current practices in ocular healthcare. The formation of liposomes as a drug delivery method for ocular diseases is the future of ocular health and will decrease many of the current complications with current drug delivery methods. Due to the increasing age and size of the population, improved health and ocular care of age related diseases is crucial. Due to this increasing elderly population and the number of American’s being affected by these ocular diseases, a more effective mode of drug delivery needs to be implemented to lower the cost, decrease adverse effects, increase patient compliance, and increase effectiveness.

APPENDIX

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