Enhancing the Drug Encapsulation Efficiency of Liposomes for Therapeutic Delivery

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***Abstract*—Nano-delivery vehicles have been a staple in point of care innovations for nearly two decades. Chief among these are liposomes, which are one of the best developed FDA delivery systems. A critical step of the optimization process is the loading of drugs within the core of the nano-carrier, known as encapsulation. Several procedures have been developed to increase the encapsulation efficiency, however, none are universal and can often change the native form of the drug. Here, we highlight an approach for rapidly and efficiently loading liposomes with the chemotherapeutic, Doxorubicin. We then use this method to deliver liposomal drugs to cancer cells *in vitro*.**

Keywords: Liposomes, drug delivery, drug encapsulation

I. Introduction

Since their initial discovery in 1964[1], liposomes have been on the forefront of nanotechnology, and have been implemented in several point of care (POC) applications. These amphipathic nano-carriers have several advantages compared to other nano-vehicles such as polymeric nanoparticles, namely due to their biocompatibility and biodegradability[2], simplicity of production, and ability to be personalized for therapeutic applications. There are several methods for synthesizing liposomes on a large scale, including the injection[3], [4] and thin film deposition methods[5]. Both of these approaches allow for loading during the vesiculation (membrane closure) process, known as passive encapsulation. Using this technique, therapeutics are dissolved within an aqueous media. When introduced to the hydrophobic membrane constituents, the bilayer spontaneously forms and closes into a sphere, thereby encapsulating the surrounding media and the soluble therapeutics found in it. This method is simple and nearly universal, however the encapsulation efficiency (EE), calculated as the percentage of drug inside the nano-carrier compared to the total amount of drug initially available, is dependent on the reaction volume and lipid concentration used. However, in most cases the passive EE is only around 1%, leaving a large amount to waste[6].

Several attempts have been reported to increase encapsulation efficiency. Most notable has been remote loading, also known as active encapsulation. This approach is capable of encapsulating nearly 99% of small molecules[7] and can obtain lipid to drug ratios of 30% (w/w). The active method uses transmembrane gradients to drive the diffusion of therapeutics into the liposome core. The stability and diffusion from the core is often reduced due to the change in ionization, which alters the logP value of the therapeutic. In some circumstances, the therapeutics are chemically altered to precipitate within the liposome, retarding their escape from the even more. This approach has been used to successfully deliver concentrations of therapeutics to cells, however it has only been shown to work with certain small ionic molecules, limiting its use as a universal approach. Furthermore, the procedure alters the native structure of the therapeutic which may impact performance.

Here, we describe a novel approach for loading liposomes with therapeutics, known as passive equilibration. We then demonstrate its use by delivering Doxorubicin (DXR) to cancer cells *in vitro*.

II. Methods

A. Materials

Cholesterol and 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Sigma Aldrich (St. Louis, MO). Isopropyl Alcohol (IPA, 99% Pure), Ethanol (EtOH, 99% pure), Trypsin-EDTA, and 5-chloromethylfluorescein (CMFDA) were purchased from Fisher Scientific (Hampton, NH). Glass syringes were purchased from Hamilton (Reno, NV) and luer lock dispensing needles were purchased from Jensen Global (Santa Barbara, CA). Amicon Ultra 100kDa filter centrifuge tubes were purchased from EMD-Millipore (Billerica, MA). A NanoJet syringe pump from Chemyx Inc. (Stafford, TX) was used for all experiments. Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and Penicillin-Streptomycin antibiotic were purchased from VWR (Radnor, PA). MDA-MB-231 metastatic breast cancer cell line was acquired from ATCC (Manassas, Va).

B. Synthesis of Concentrated Liposomes

DSPC and Cholesterol were dissolved in alcohol (either isopropyl or ethyl) at a constant 2:1 molar ratio to a final concentration of 10M and 5M, respectively. The solutions were heated to 50 ^{O}C while being shaken at 270 rpm for 15 minutes to ensure adequate mixing of the chemicals. The alcoholic solution was aspirated by a glass syringe and injected into a vial containing distilled water heated to 50 ^{O}C using a syringe pump set to a constant infusion rate of 50 $\mu L \cdot min^{-1}$. The aqueous solution was constantly vortexed at 600 rpm during infusion and the vortexing continued for 3 minutes following the infusion. Following formation, liposomes were concentrated ten-fold by filter centrifugation for 60 minutes.

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C. Loading of Liposome and Purification of Product

Concentrated liposome slurries were incubated in sealed screw top tubes containing DXR for 3 hours at 55 ^{O}C. As a control, separate liposomes were similarly incubated with DXR, but at 4 ^{O}C. Liposomes were then diluted to 1x in PBS, and filtered using filter centrifuge tubes. The suspensions were centrifuged 8 times for 10 minutes each at 6k x g to remove any non-encapsulated DXR.

D. Cell Culture and Delivery

MDA-MB-231 metastatic breast cancer cells were grown to 70% confluency, removed via trypsin, and cultured in 24 well plates. The cells were grown for 2 days in complete media prior to any experiments. Processed liposomes were UV sterilized prior to their use. Cells were incubated for 1 hour in serum free media to remove any proteins that may interfere with liposome-cell interactions. The liposomes were then added to the cells and incubated for 2 days. On the second day, non-viable cells and unused liposomes were removed from the wells using PBS. Nuclei were counterstained with Hoescht stain. All imaging was done in using an Evos FL Auto epifluorescent microscope with on stage incubator to inhibit further cell death.

III. Results

A. Synthesis and Loading of Small Unilamellar Liposomes

Liposomes are one of the most used nano-delivery methods in POC applications. They are incredibly amendable to customization, and solely by altering the synthesis procedures or membrane constituents, liposomes can be personalized for a variety of tasks. We synthesized liposomes using the injection method. This protocol uses a water miscible organic solvent, such as isopropyl alcohol, to dissolve membrane components. The alcohol is then injected through a thin needle into water, creating an abrupt phase change. During this change, the membrane components spontaneously arrange to form small monodisperse populations of liposomes. We chose DSPC, a synthetic lipid, as our main membrane component due to its elevated transition temperature (T_m = 55 ^{O}C). Liposomes were synthesized and concentrated using filter centrifuge tubes. This approach left us with a highly concentrated solution of small unilamellar liposomes (Fig. 1a). Liposome size decreased marginally from 109nm to 100nm following filtration. We attribute this to the loss of poorly formed large liposomes, either by disintegration or adhesion to the filter membrane.

Because DSPC has a high T_m, liposomes are able to withstand elevated temperatures, more so than other natural lipids, which typically have T_m of near body temperature (37 ^{O}C). To test this, liposomes were incubated at varying temperatures (Figure 2): physiological (37 ^{O}C), transition temperature (55 ^{O}C), and beyond transition temperature (65 ^{O}C). The liposomes' size and polydispersity did not vary significantly ($p>0.05$) at physiological and transition temperatures up to three hours. Above the transition temperature (65 ^{O}C), liposome diameter increased after one hour from 110 nm to over 2 µm. The polydispersity also followed this pattern, however the polydispersity doubled from 0.136 to 0.285. We believe this is due to the liposomes reaching the liquid phase, which promotes lipid disorder and

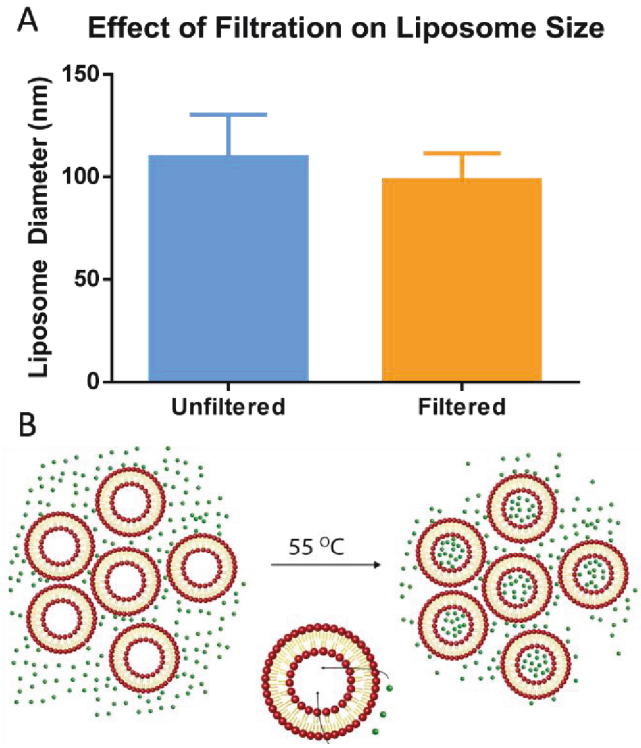

Figure 1: Concentration and Loading of Liposomes. (A) Liposomes were synthesized by the injection method. Following synthesis, liposomes were filter centrifuged to concentrate the suspension tenfold. This has the effect of slightly decreasing the average liposome diameter. (B) Once concentrated, liposomes can be efficiently loaded by incubation at 55 ^{O}C (T_m of lipid) with a drug of choice. The drug diffuses into the core of the liposome, eventually equilibrating with the outside media.

increases the chance of multiple liposomes fusing together to create large polydisperse liposome populations.

Before small molecules can reach the liposome core, they must first pass through the lipid membrane. This requires compounds to go through both polar and nonpolar regions of the amphipathic lipids, which can be thermodynamically unfavorable. In order to increase the diffusion of molecules, and therefore the probability that small molecules enter the core, we incubated the liposomes at the T_m. The increased temperature increases both the random movement of small molecules in solution, and the disorder of lipids in the membrane. Liposomes were incubated for one hour at 55 ^{O}C with DXR (Fig. 1b), a chemotherapeutic that has been well studied for its use with liposomes. As a control, liposomes were also incubated with DXR for one hour at 4 ^{O}C. This low temperature causes the lipids to stay in the gel phase, and retards movement of molecules across membrane. We then resuspended both samples back to initial concentrations in PBS and used sequential repetitions of filter centrifugation to remove the non-encapsulated molecules[8].

B. Delivery of Therapeutics to Cancer Cells.

Liposomal DXR is a well-studied example of a chemotherapeutic that is able to passively target tumor cells in vivo. Furthermore, it is well equipped for studying

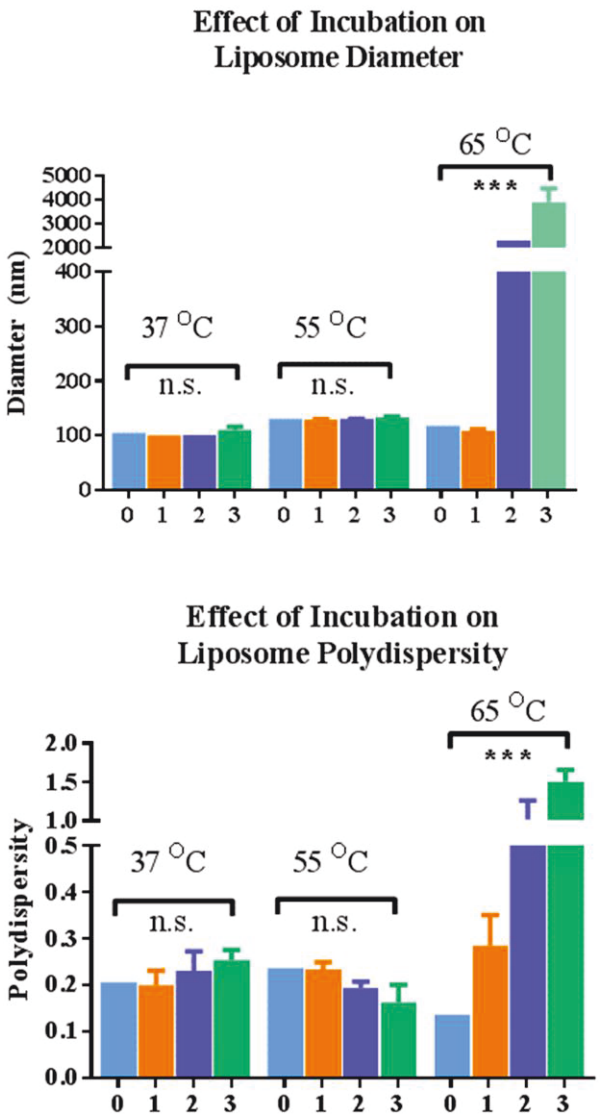

Figure 2: Change in Liposome Populations after incubation. Liposomes were incubated for up to three hours and then analyzed using DLS. The diameters of the liposomes did not change significantly ($p>0.05$) at either 37 ^{O}C or 55 ^{O}C. However at 65 ^{O}C, once the liposome diameter sharply increases after the first hour. The same pattern is seen in the polydispersity of the liposomes.

encapsulation and delivery because it is highly fluorescent with an excitation and emission of 480nm and 560nm, respectively. Furthermore, DXR is extremely polar with a logP value of -1.67[9], suggesting that while it will have limited permeability but high stability once inside the core. After liposomes were loaded via incubation with DXR, we introduced the purified liposomal suspension to MDA-MB-231 cells. Overtime, the DXR diffuses from the liposome core into the surrounding media. Once taken up by the cells, the drug intercalates within the DNA of cancer cells, inhibiting replication and biosynthesis of macromolecules. Therefore, presence of DXR can be denoted by an intense fluorescence at the cell nucleus. After incubating for two days, we remove all dead cells from the well plate and analyzed the fluorescence of

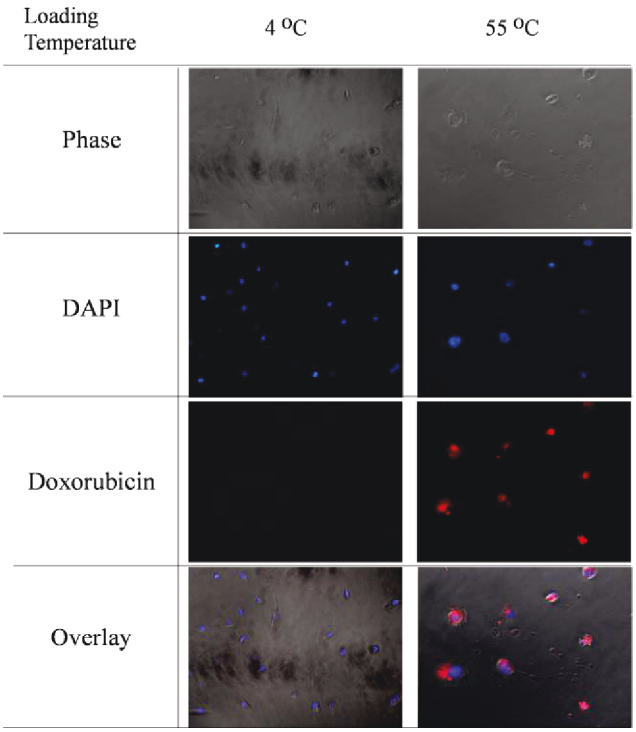

Figure 3: Delivery of Doxorubicin to Cells. Liposomes were incubated with DXR for one hour at either 4 ^{O}C or 55 ^{O}C. At 55 ^{O}C, the membrane is more fluid and the drug can diffuse in, however diffusion is limited at 4 ^{O}C. Following equilibration, liposomes are introduced to cell cultures and allowed to interact for 2 days. Cells incubated with the 55 ^{O}C treated liposomes show an increased fluorescence as compared to 4 ^{O}C samples.

cells (Fig. 3). Liposomes incubated at 55 ^{O}C demonstrate an intense fluorescence at the nucleus, as expected, while cells incubated with DXR at 4 ^{O}C do not. This is due to the decreased diffusion and inability of DXR to pass the membrane at 4 ^{O}C, and it is therefore removed during the filtration process.

IV. Conclusions

Encapsulations strategies are a critical aspect of the development of nano-vehicles for POC applications. Yet, despite the importance, they are very underdeveloped. Current high yield protocols only work for charged and weakly basic drugs, and thus are not universally applicable, while the universal approaches are terribly inefficient and wasteful. Here we show the use of passive equilibration to encapsulate and chemotherapeutic, DXR, and deliver it to cancer cells in vitro. By first concentrating our liposomes, we are able to minimize the amount of drug used while maintaining high efficiency. Because this method uses diffusion as the driving force for encapsulation, we foresee this approach working with many other molecules such as proteins and drug conjugates.

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