Liposomes for targeted drug delivery

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Abstract

MUC-1 aptamer-amphiphiles were used to functionalize DPPC liposomes to create nanoparticles. Various properties of the targeted liposomes drug delivery vehicles were examined including their drug-to-lipid ratio (DL), targeted ligand insertion efficiency (IE), and stability. The ratio of lipid and MUC-1 aptamer-amphiphile in the liposomes was varied to determine this ratio's influence on liposomal drug-to lipid ratio, insertion efficiency, and stability. We found that the insertion efficiency and drug-to-lipid ratio is unaffected by the presence of targeting ligand, with a maximum insertion efficiency of 85.4% for the targeted liposomes. The amount of drug loaded into targeted and non-targeted liposomes ranged from 0.10 mg per 1 mg lipids to 0.22 mg per 1 mg lipid. The liposomal leakage was found to be small over the course of 4 days for both targeted and non-targeted liposomal formulations, but there was a 50% increase in leakage in the presence of targeting ligand. The liposomal leakage was lower than 10% for 37°C and 5% in 4°C.

1. Introduction:

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths) in 2008[1]. Although potent anti-cancer drugs have been discovered, they cannot effectively treat the cancer due to their poor solubility, tissue damage on extravasation, and poor biodistribution[2]. Drug delivery vehicles, such as liposomes, have been developed over the past decades to maximize the therapeutic effects of drugs and to minimize the negative side effects[3]. In aqueous solutions, lipids self-assemble to form bilayer vesicle structures called liposomes, with hydrophilic head groups forming the inner and outer layers of the lipid bilayer and hydrophobic tails between the headgroup layers. Liposomes can be used as drug delivery vehicles due to their high encapsulation efficiency of drug within their aqueous cores and their sustained release behavior[4]. Research shows that by adding targeting ligands to the surface of liposomes the vesicles, upon accumulating in tumor regions, can specifically target the receptor of choice on the cell surface, a process called active targeting[5].

The drug/lipid ratio should be as high as possible to maximize the payload of drug reaching the tumor but should not compromise the liposome's stability[6]. Liposome stability is defined as their capability to retain the structural integrity of the lipid bilayer and prevent leakage of their aqueous contents[7]. For applications of liposomes where specific delivery of liposome-associated drug to solid tumors is desired, liposomes must substantially retain their contents while in the circulation [8]. The stability of drug-loaded liposomes over time is an important concern in pharmaceutical formulations[6].

The Goal of this report is to study the properties of targeted liposomes as targeted drug delivery vehicles by analyzing their drug-to-lipid ratio (DL), targeted ligand insertion efficiency (IE), and stability. MUC-1 aptamer-amphiphile (Amph) was used as the targeting ligands to functionalize liposomes and allow them to actively target cells over-expressing the Muc-1 glycoprotein. The ratio of lipids (DPPC and cholesterol) and MUC-1 aptamer-amphiphile in the liposomes was varied to determine their influence on liposomal drug-to lipid ratio, insertion efficiency, and stability.

2. Materials and methods

2.1. Materials

Cholesterol, DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and DPPE-PEG_{2000} (a headgroup modified DPPC lipid conjugated to a 2000MW PEG) lipids were purchased from Avati. MUC-1 aptamer-amphiphiles were synthesized from MUC-1 aptamer headgroups and

$(C16)_2$-Glu-$(CH_2)_2$ tails. 2 mM Calcein in TBE (Tris/Borate/EDTA) buffer with pH 7.4 was used as loaded drug. TE buffer (10mM Trid/1mM EDTA) with pH 8.0 was used to solubilize DNA.

2.2. Methods

2.2.1. Liposomal Preparation and characterization

The liposomes were prepared by the lipid film hydration method[9] with DPPC, DPPE-PEG_{2000}, and cholesterol (mole ratio of 6:0.5:3.5) first dissolved in chloroform and added to a round bottom flask and then dried using argon gas to form a lipid film. The lipid film was then hydrated with the 2mM Calcein solution and vortexed vigorously. The resulting liposomes were freeze-thawed[10] and extruded through 100 nm pore size nucleopore polycarbonate membranes (21 times) using the hand held extruder (Avanti) to obtain uniform vesicles of approximately 100 nm in size. The liposomes were then loaded into a dialysis membrane and the membrane placed in a beaker containing TBE buffer at room temperature for 24 hours to dialyze away the unencapsulated Calcein. The lipid concentration of dialyzed liposomes was determined using a phosphorus assay.

2.2.2. Insertion and characterization of targeted ligand

Liposomes samples containing 3 μmol total lipid were added to 1.5 ml centrifuge tubes and named as NoAmph, LowAmph and HighAmph groups. The NoAmph samples received no amphiphile, while the LowAmph samples received 0.02 mol% amphiphile and the HighAmph samples received 0.2 mol% amphiphile. The centrifuge tubes were placed in a 45°C water bath for 12 hours as a post insertion reaction, where the amphiphiles incorporated in the liposome bilayers. Fast protein liquid chromatography (FPLC) was used to purify liposomes by removing un-inserted amphiphiles. The concentrations of lipids in the purified liposome samples were determined using the phosphorus assay. The inserted amphiphiles were extracted using an ethanol precipitation technique. Extracted amphiphiles were then dissolved in TE buffer and quantified by reading their absorbance at 260 nm. The insertion efficiency (IE) of the amphiphiles into the liposomes is defined as:

$$IE = \frac{amount\ of\ inserted\ Amph}{amount\ of\ added\ Amph} \times 100\%$$

2.2.3. Liposomal Drug-to-lipid ratio

The Calcein encapsulating liposomes were broken apart using TBE buffer + 10% Triton solution. Fluorescent readings (Excitation: 490nm, Emission: 520nm, gain 135) was performed to determine the concentration of encapsulated Calcein. The lipid concentration was tested using phosphorous assay. The liposome's drug-lipid ratio (DL) was calculated using the equation:

$$DL = \frac{Mass\ of\ Drug\ (Calcein)}{Mass\ of\ Lipid}$$

2.2.4. Targeted Liposomal Stability

Small amounts of liposomes loaded with Calcein and previously purified were broken apart and diluted using TBE buffer + 10% Triton solution and fluorescent readings made (Excitation: 490nm, Emission: 520nm, gain 135) to determine the initial concentration of Calcein in each liposome sample. The rest of the purified liposomes were loaded into dialysis membranes. The membranes were placed in beakers containing TBE buffer and put in a 37°C incubator or a 4°C refrigerator. Any Calcein that leaked out of the liposomes would travel across the membrane and into the TBE buffer. Thus, fluorescent readings of the TBE buffer were performed over the next 96 hours to determine the concentration of the dye that leaked out of the liposomes. Higher fluorescence in TBE buffer would mean more Calcein had leaked. The stability of liposomes was indicated using %Calcein leakage, which is defined as:

$$\%\text{Calcein leakage} = \frac{C_b}{C_m} \times \frac{V_b}{V_m} \times 100\%$$

where C_b is the leaked Calcein concentration in beaker, C_m is the Calcein concentration in the liposomes within the dialysis membrane at the beginning of the experiment. V_b is the volume of TBE buffer in the beaker, and V_m is the volume of liposome solution in the dialysis membrane at the beginning of the experiment. The experiment was repeated with varying temperature (4°C and 37°C) and the percentage of targeted ligands on the liposomes.

3. Result and Discussion

3.1. Insertion Efficiency studies

The insertion efficiency of six groups of targeted liposomes was tested. The amounts of amphiphile added to the liposome samples and the amounts of amphiphile that inserted into the liposome samples are shown in table 2.1.1. The maximum insertion efficiency was found to be 85.4%.

Table 3.1.1 Insertion efficiency

Amount of Added Amph (nmol)	Amount of Inserted Amph	IE (%)
1.60	1.04	65.00
2.00	0	0.00
2.00	0.68	33.8
8.00	6.83	85.40
10.00	3.97	39.73
10.00	7.49	74.88

3.2. Drug-lipid Ratio Studies

A correlation curve was generated to show the correlation between Calcein concentrations and their fluorescent readings (Excitation: 490nm, Emission: 520nm, gain 135) and is shown in figure 3.2.1.

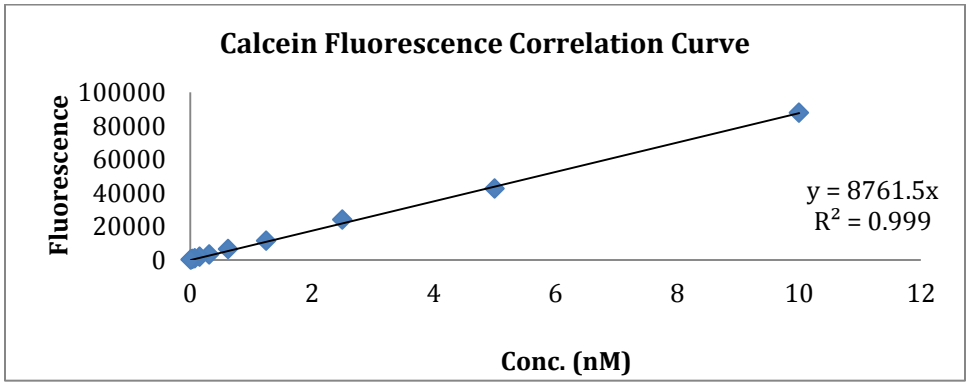

Figure 3.2.1. Calcein Correlation curve

The fluorescence of the liposome samples was compared to the correlation curve to determine the total amount of Calcein within the liposome samples and a phosphorous assay was used to determine the total amount of lipids within the liposome sample. The mass of the lipids and Calcein of the different formulations, as well as the drug-to-lipid ratio in 1L purified liposome samples are shown in table 3.2.1.

Table 3.2.1. Lipid and Calcein Mass in Purified Liposomes

Sample Type	Mass of lipid (mg)	Mass of Calcein (mg)	DL(w/w)
NoAmph	975.4	18.68	0.019
NoAmph	1146.3	18.68	0.016
LowAmph	1189.0	24.90	0.021
LowAmph	1231.8	18.68	0.015
HighAmph	1139.2	24.90	0.022
HighAmph	1303.0	12.45	0.010

Table 3.2.1. shows that the Drug-lipid ratio of targeted liposomes are not affected by the presence of the amphiphiles. With this assumption, the drug-lipid ratio of liposomes is obtained by plotting the mass of Calcein versus the mass of lipid (figure 3.2.1.).

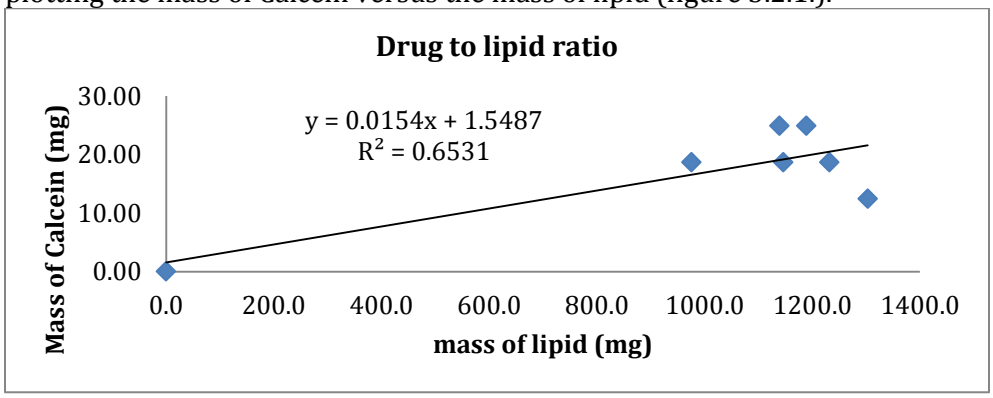

Figure 3.2.1. Correlation of Calcein concentration and lipid concentration.

The slope of the fitting line indicated that the average drug-to-lipid ratio was 0.015:1.

3.3. Liposome Stability Studies

The amount of Calcein leakage from NoAmph, LowAmph, HighAmph samples over 96 hours at 37°C or a 4°C was found to be less than 10%. The leakage data from three experiments (n = 3) were averaged and shown in figure 3.3.1 and figure 3.3.2. Error bars indicated the standard error ranged from ±0.2 to ±0.8.

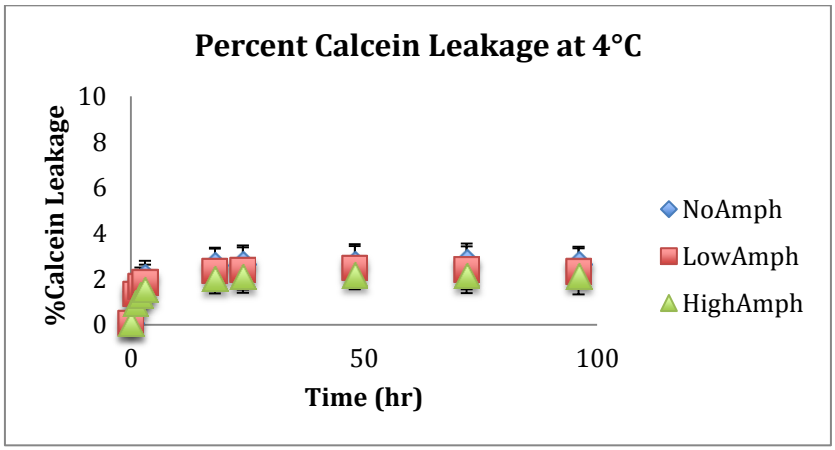

Figure 3.3.1. Percent Calcein leakage at 4 °C

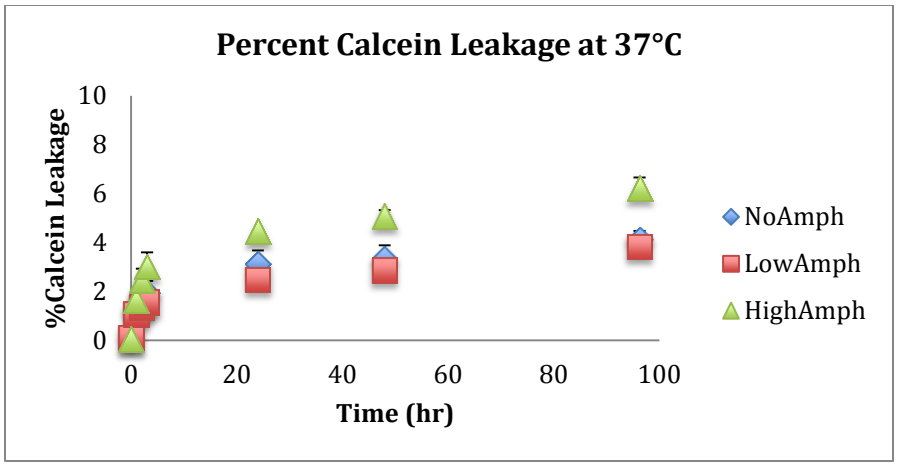

Figure 3.3.2. Percent Calcein leakage at 37 °C

The Calcein percent leakage increased rapidly in the first three hours and decelerates in the next 4 days. The rapid increase in percent Calcein leakage might caused by some of the Calcein had already leaked out of the liposomes between the time the FPLC happened and the liposomes were added to the dialysis membrane. This free Calcein could quickly diffuse out of the membrane, while the rest of the Calcein had to get through the lipid bilayer as well as the membrane. For 4°C samples, the release rate was very small after 3 hours while the release rate slightly increased in 37 °C samples.

4. Discussion

4.1. Insertion efficiency:

The inserted targeting ligand increased with the amount of the ligand added during the post insertion reaction. In this experiment, the maximum insertion efficiency of Calcein targeted liposomes was (85 ± 9)%.

In Ishiba's experiment[11], ligands (Sheep IgG) coupled to the terminus of polyethylene glycol (PEG) in micelles formed from PEG-lipid derivatives (mPEG2000-DSPE) were transferred into pre-formed, drug-containing liposomes from the micelles. Most of the IgG coupled to the preformed micelles and approximately 50% of the targeted ligands were inserted into liposomes after 1 hour for insertion reaction at 60 °C. In Moreira's experiment, Antagonist G-targeted liposomes (PLG) were prepared and loaded with doxorubicin. The hexapeptide antagonist G was covalently coupled via a thioether bond to the terminus of polyethylene glycol (PEG) in micelles formed from maleimide-derivatized poly(ethylene glycol) distearoylphosphatidylethanolamine followed by transfer into preformed liposomes during a one-step incubation. The coupling efficiency was approximately 60%. Under these circumstances, 2–6% of antagonist G-PEG-DSPE in the micellar conjugates mixture was inserted into liposomes, which lead to a 36% maximum insertion efficiency[12].

The comparison with Ishiba and Moreira's experiments suggests that our post-insertion technique at 45 °C leads a high insertion efficiency. Longer post insertion reaction time (12hr) may also contribute to the high insertion efficiency.

4.2. Drug-to-lipid ratio

Calcein, a fluorescent molecule, was used as a model drug and loaded into liposomes. The amount of Calcein loaded into liposomes ranged from 0.10 mg per 1 mg lipids to 0.22 mg per 1 mg lipid. The targeting ligand concentration on the surface of the liposomes did not influence the drug-

lipid ratio (Table 2.2.1.).

In Patel's experiment[13], tacrolimus (FK-506) was entrapped in the liposomes. The liposomes were also prepared using the thing film hydration method. Their amount of drug loaded into vesicles ranged from 4.4mg drug per 115mg lipid to 8.2 mg drug per 140 mg of lipid. In Chiu's experiment[14], doxorubicin was encapsulated into two thermosensitive liposome formulations which were composed of DPPC/MSPC/DSPE-PEG2000 (90/10/4 mole ratio) or DPPC/DSPE-PEG2000 (95/5 mole ratio). When doxorubicin was encapsulated via a pH gradient for both thermosensitive liposome formulations, the drug-lipid ratio reached a maximum of 0.05 mg drug to 1 mg lipid. In contrast, the final maximum drug to lipid ratio achieved through manganese complexation was 0.2 mg to 1 mg.

The comparison with Patel and Chiu's experiments shows that our drug to lipid ratio is in a reasonable range. One way to raise the drug to lipid ratio is to increase the molar concentration of Calcein solution in the hydration film step.

4.3. Leakage

The addition of targeted ligands might be expected to cause membrane defects resulting in leakage[15]. We studied the leakage at 4 °C and 37 °C with No Amph, Low Amph, and High Amph mol percentage by inserting amphiphiles into the liposome bilayers. At 37 °C, it was observed that 50% increase in leakage in the presence of targeting ligand. The leakage in this experiment is lower than 10% for 37 °C and 5% in 4 °C. The leakage is therefore very small in our experiment, indicating the liposomes were stable at both 4 °C and 37 °C storage environments over the course of 4 days.

In Uster's experiment[15], MPEG1900-DSPE was used as a targeting ligand and inserted into liposomes made with HSPC/CH/DPPG/DTPA-DSPE (52.4/45.4/2.0/ 0.2, molar ratio). Less than 8% dye leakage occurred after 5 hours at 60 °C in the presence of targeting ligand, only a one third increase of leakage in its absence. In Moreira's experiment[12], 1 hour incubation at 60 °C of DXR-loaded liposomes containing 9 mol% of mPEG-DSPE resulted in 60% drug leakage. Ishida *et al.* [11]observed that little DXR leakage occurred when DXR-loaded liposomes (similar to those in this study) were heated at 60°C for 6 hours, either alone or in the presence of IgG-PEG-DSPE micelles.

5. Conclusion

The ratio of lipid and MUC-1 aptamer-amphiphile in the liposomes was varied and the influence on liposomal drug-to lipid ratio, insertion efficiency, and stability was measured. Both insertion efficiency and drug-to-lipid ratio is not affected by the presence of the targeting ligand. The experiments showed that high insertion efficiency (85%) of MUC-1 aptamer-amphiphile in liposomes. The liposomal leakage was negligible in our experiment. The encapsulation efficiency was low compared with the literature. Further studies on increase encapsulation efficiency will be performed.

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