ENHANCEMENT OF LIPOSOMAL DRUG LOADING BY USING SUPERSATURATED DRUG SOLUTION

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Liposomes are used for systemic delivery of chemotherapy drugs to reduce their non-specific side effects. Liposomes can encapsulate hydrophilic and lipophilic drugs in the water compartment and the lipid membrane, respectively. However, typical drug loading capacity of liposomes by passive loading method is less than 1%. The low drug loading efficiency is problematic because it necessitates the use of a large amount of carrier materials that may cause undesirable biological effects. To increase drug loading in liposomes, we used supersaturated drug solutions with gemcitabine (GEM) and doxorubicin (Dox) as examples. The prepared liposomes showed higher drug loading compared with passive loading, maintained stability and provided sustained drug release for 48 hrs.

INTRODUCTION

Liposomes are phospholipid bilayer vesicles with aqueous cores, discovered by A. D. Bangham in 1960$^1$. Within a few years, a variety of enclosed phospholipid bilayer structures consisting of single bilayers, initially termed ‘bangosomes’ and later ‘liposomes,’ have been developed$^2$. Gregory Gregoriadis et al.$^3$-$^5$ have established the concept that liposomes could entrap drugs and serve as delivery systems. It has been also shown that the liposomes could change the in vivo biodistribution of the entrapped drug$^6$-$^7$. Many methods have been developed to produce large unilamellar liposomes with improved entrapment efficacy and homogeneity$^8$.

As a drug delivery system, liposomes can encapsulate both hydrophilic and hydrophobic compounds in the aqueous and lipid layers, respectively, and protect the entrapped drugs from decomposition, and help release the loaded drug at specific sites$^9$. Liposomes have gained a lot of interest as a drug delivery system because of their biocompatibility, biodegradability, increased efficiency, therapeutic index, low toxicity$^{10}$ and the potential for site-specific drug delivery to tumor tissues$^{11}$.

Doxorubicin (Dox) and gemcitabine (Gem) have been tested clinically for treatment of various cancer types such as breast cancer$^{12}$-$^{13}$ and hepatocellular carcinoma$^{14}$-$^{15}$. In addition, many studies tested the synergy between Dox and Gem in different types of cancer$^{16}$-$^{21}$. The main mechanism of action of Dox is the intercalation with DNA and disruption of topoisomerase-II-mediated DNA repair and generation of free radicals and their damage to cellular membranes.$^{22}$ Gem, is a prodrug, which needs to be activated by phosphorylation to a triphosphate form of Gem (2’,2’-difluoro-2’-deoxycytidine triphosphate; dFdCTP)$^{23}$. dFdCTP is incorporated into the end of elongating DNA, followed by one more
deoxynucleotide, whereupon DNA polymerases stop proceeding\textsuperscript{24}.

There is indeed need to produce liposomal Gem and Dox to increase the fraction of drug reaching tumor by EPR effect.

Low drug loading is problematic because it increases liposomal dose. Inefficient loading also increases the need for thorough removal of unentrapped drug\textsuperscript{25}. High drug loading is also needed for increasing the efficiency of chemotherapy as well as decreasing toxicity due to accumulation of drugs in tumor in preference to other organs as reported by Mayer\textit{ et al.}\textsuperscript{26}. They have compared LD\textsubscript{50} of liposomal Dox with different drug to lipid ratios (i.e., drug loading). The decrease of the drug to lipid ratio from 0.28:1 to 0.038:1 led to the decrease in LD\textsubscript{50} from 57 to 39 mg/kg and increase in the Dox accumulation in the heart by 1.8-fold. This increased toxicity results from the increase of drug leakage from the liposomes during the extended circulation.

In this study, we used supersaturated drug solution to increase the entrapment of the drug in the aqueous phase of the liposomes, with Gem and Dox as model drugs, and evaluated the physicochemical properties of the prepared liposomes.

**MATERIALS AND METHODS**

**Materials**

Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, N-(carboxylmethoxypolyethylene-glycol-2000)-1, 2-distearylsn-glycero-3-phospho-ethanolamine (DSPE-PEG\textsubscript{2000}) and NBD-cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Gemcitabine and Doxorubicin HCL were purchased from LC laboratories Woburn, MA, USA. All other materials, including solvents, were purchased from Sigma Aldrich (St. Louis, MO, USA).

**Liposomes preparation**

Liposomes were prepared in the following procedures. A mixture of DPPC, cholesterol and DSPE-PEG\textsubscript{2000} at a weight ratio of 6:3:1 (20 mg in total) was dissolved in 3 mL of mixture of chloroform and methanol (3:1 vol ratio). The organic solvent was removed using a rotary evaporator and thin lipid film was obtained. Sonicat on of hydrated lipid film was performed using an ionic water bath for 15 min and extruded through polycarbonate membranes with a pore size of 400 nm and 200 nm, sequentially, using a mini-extruder (Avanti Polar lipid, Inc., AL.)

A thin lipid film was obtained by removing the solvents with a rotary evaporator at 45°C and hydrated according to the procedures detailed below. The hydrated lipid film was sonicated in a sonic water bath for 15 min and extruded through polycarbonate membranes with a pore size of 400 nm and 200 nm, sequentially, using a mini-extruder. The drug-loaded liposomes were washed with deionized (DI) water 3 times by centrifugation using a Beckman Optima XL-I ultracentrifuge at 135,700 relative centrifugal force (rcf) at 4°C.

**Passive loading:** The lipid film was hydrated with 1 mL of 5 mg/mL Gem or Dox solution and stirred in a rotary evaporator for 45 min at 45°C, extruded and washed. The Gem-loaded liposomes prepared by this method is called L\textsubscript{P}G and L\textsubscript{P}D.

**Small volume loading (indirect loading with supersaturated drug solution)**\textsuperscript{27}: The formed thin lipid film was hydrated with 1.2 mL of phosphate-buffered saline (PBS, pH 7.4). The hydrated film was bath sonicated for 10 min, extruded and collected by centrifugation at 305, 400 rcf. The liposomal pellet was mixed with 0.1 mL of DI water containing 5 mg of Gem (or Dox by vortexing which above the solubility limit\textsuperscript{25} and 20 mg/ml for Gem and Dox respectively, and followed by overnight incubation at 60°C. The prepared liposomes are called L\textsubscript{S}G and L\textsubscript{S}D.

**Preparation of labeled liposomes:** Fluorescently labeled liposomes were prepared by the same method applied for small volume loading with replacement of cholesterol by 25 NBD-cholesterol and called L\textsuperscript{*} liposomes.

**Liposome characterization**

**Measuring the size and zeta potential**

The size-average (z-average) and zeta potential of each liposomal formulation were measured by a Malvern Zetasizer Nano ZS90, as dispersed in DI water (z-average) or in 1 mM phosphate buffer (pH 7.4) (zeta potential).
To estimate the loss of injection mass after injection, 1 mL of LpG, LpD, LsG and LsD suspension equivalent to 25 µg/mL Gem/Dox was passed through a 28G needle. The optical density of each suspension at 600 nm was measured before and after the passage by a SpectraMax M3 reader (Molecular Devices, CA, USA).

TEM imaging of liposomes

The liposomes were photographed by the Tecnai F20 transmission electron microscope (FEI, Hillsboro, OR, USA) after negative staining with 1% uranyl acetate.

Drug loading efficiency

The prepared liposomes were lyophilized, weighed, dispersed in 1 mL of acetonitrile to release the entrapped drug and bath-sonicated in cold water for 2 hrs. The liposomal suspension was diluted with an equal volume of DI water and centrifuged at 14000 rpm for 20 min to obtain a clear supernatant. The supernatant was analyzed by high-pressure liquid chromatography (HPLC). HPLC analysis was performed by the Agilent 1100 system, (Agilent Technologies, Palo Alto, CA), equipped with a C18 column (25 cm × 4.6 mm, particle size 5 µm) (Supelco, St. Louis, MO.). Gem was eluted with a 90:10 mixture of water and acetonitrile at a flow rate of 1 mL/min and detected at 269 nm. Dox was eluted with a 70:30 mixture of water and acetonitrile with 0.1% trifluoroacetic acid at a flow rate of 0.8 mL/min and detected at 490 nm. The drug loading efficiency (%) is defined as \( \frac{W_D}{W_L} \times 100 \), where \( W_D \) is the amount of drug detected and \( W_L \) the total amount of the liposomes analyzed.

Stability of the liposomes

The prepared liposomes are evaluated for their stability upon storage at 4°C. Particle size, zeta potential and drug content were monitored monthly for 3 months.

In-vitro release

Liposomes equivalent to 115 µg/mL of Gem and 250 µg/mL of Dox were placed in a Float-A-Lyzer G2 dialysis device (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) with a molecular weight cut-off of 100 kDa. The device was incubated in 20 mL of PBS (pH 7.4) at 37°C with constant agitation. At predetermined time points, 0.3 mL of the release medium was sampled and replaced with 0.3 mL of fresh buffer. The sampled buffer was filtered with a syringe filter (0.45 µm pore size) and analyzed for drug content by HPLC.

Confocal imaging of labeled liposomes

Huh7 cells were seeded in a 35 mm glass-bottomed dish (Mat Tek Corp., Ashland, MA) at a density of 100,000 cells per dish. At 70% confluence, the cells were treated with fluorescently labeled liposomes L* for 1 h. or 3 h. At each time point, the cells were rinsed twice with PBS. After nuclei staining with Hoechst 33342 (5 µg/mL) for 10 min, the cells were rinsed again with PBS and imaged in medium by a Nikon-AIR confocal microscope (Nikon America Inc., Melville, NY).

RESULTS AND DISCUSSION

Liposomal drug loading

Liposomes prepared by passive loading (Gem for LpG and Dox for LpD) showed a negligible drug loading of 0.14 and 0.5wt%, respectively. However, when loaded indirectly with supersaturated solutions of Gem and Dox, the drug loading of Gem and Dox increased to 3.8 wt and 4.2% respectively as shown in Table 1. The efficient drug loading by the latter is explained by the high concentration gradient between the external medium and the aqueous core. The supersaturated drug is provided from the outside of the liposomes, keeping the concentration gradient across the membrane at the maximum throughout the loading process. The drug loading continues until the concentration gradient reaches zero.

<p>| Table 1: Physical properties of prepared liposomes. |</p>
<table>
<thead>
<tr>
<th>Formula</th>
<th>Passive loading</th>
<th>Small volume loading</th>
<th>Loading efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LpG</td>
<td>+</td>
<td>0.14 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>LpD</td>
<td>+</td>
<td>0.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>LsG</td>
<td>+</td>
<td>3.8 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>LsD</td>
<td>+</td>
<td>4.2 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± standard deviations of 3 tests of a representative batch. Drug loading efficiency: mass of loaded drug/mass of liposomes.
Particle size and Zeta potential

The average size of liposomes measured by the DLS were 210-220 nm with an exception of L\text{blank} which did not have any drug inside, the measured size was 142.2 nm (Table 2). The zeta potential was consistently negative irrespective of the loaded drug, due to the presence of cholesterol and DSPE-PEG\textsubscript{2000}. The optical density of L\text{S}GorL\text{S}Ddid not significantly change by needle passage, but that of L\text{p}GorL\text{p}Ddid, suggesting the loss of liposomes (Table 3). This difference is due to low loading in L\text{p}GorL\text{p}D which requires higher amount of liposomal nanoparticles to get the same drug concentration of ofL\text{S}G or L\text{S}D. The higher amount of nanoparticles increased the loss during needle passage.

Table 2: Particle size and zeta potential of prepared liposomes.

<table>
<thead>
<tr>
<th>Formula</th>
<th>NP size (d, nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LpG</td>
<td>181 ± 30</td>
<td>0.10 ± 0.1</td>
<td>-29.7 ± 6.4</td>
</tr>
<tr>
<td>LpD</td>
<td>200 ± 10</td>
<td>0.20 ± 0.08</td>
<td>-28.8 ± 4.3</td>
</tr>
<tr>
<td>LsG</td>
<td>171 ± 15</td>
<td>0.08 ± 0.02</td>
<td>-28.2 ± 5.5</td>
</tr>
<tr>
<td>LsD</td>
<td>188 ± 9</td>
<td>0.10 ± 0.04</td>
<td>-30.2 ± 0.2</td>
</tr>
<tr>
<td>L\text{blank}</td>
<td>142 ± 9.4</td>
<td>0.66 ± 0.13</td>
<td>-26.1 ± 1.1</td>
</tr>
</tbody>
</table>

In-vitro drug release

L\text{S}D and L\text{S}G released 45% and 65% of the loaded drug over 48 hrs for respectively. However, LpD and LpG showed burst release and released almost 100% of its content in 3 hrs. This burst release may be attributed to the presence of the drug in the hydrophobic lipid layer which is entrapped in liposomal membrane during preparation so it is released upon contact of release media. In contrast, with L\text{S}D and L\text{S}G the drug was entrapped in the aqueous core phase due to passage of drug with the concentration gradient form high to low concentration and faced the lipid bilayer which acts as diffusion barrier (Fig. 1).

Table 3: % Optical density after syringe transfer.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>LpG</th>
<th>LpD</th>
<th>LsG</th>
<th>LsD</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Optical density</td>
<td>90% ± 0.5</td>
<td>89% ± 0.5</td>
<td>95% ± 1.0</td>
<td>99% ± 0.3</td>
</tr>
</tbody>
</table>

Stability of liposomes

The prepared liposomes showed good stability upon storage as indicated by insignificant change in particle size, zeta potential or drug content (Table 4).
Cellular uptake of liposomal drugs vs. free drug counterparts

Confocal microscope imaging clearly showed cellular uptake of the liposomes. Liposomal signals appeared near the nuclei and increased with time indicating that the liposomes were taken up and released their content inside the cells (Fig. 3).

Table 4: Stability of prepared liposomes.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Storage period (Months)</th>
<th>Initial size (nm)</th>
<th>Initial Zeta potential</th>
<th>Size after storage at 4°C (nm)</th>
<th>Zeta potential (mv)</th>
<th>% Drug Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>LsG</td>
<td>1</td>
<td>171 ± 15</td>
<td>-28.2 ± 5.5</td>
<td>181 ± 8</td>
<td>-30.1 ± 0.0</td>
<td>99.8 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>182 ± 6</td>
<td>-28.2 ± 1.0</td>
<td>98.5 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>181 ± 7</td>
<td>-29.5 ± 4.5</td>
<td>99.0 ± 1.0</td>
</tr>
<tr>
<td>LsD</td>
<td>1</td>
<td>188 ± 9</td>
<td>-30.2 ± 2.0</td>
<td>223 ± 2</td>
<td>-29.8 ± 1.5</td>
<td>99.1 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td>208 ± 6</td>
<td>-28.6 ± 2.0</td>
<td>99.0 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>235 ± 1</td>
<td>-29.0 ± 1.5</td>
<td>98.2 ± 2.3</td>
</tr>
</tbody>
</table>

Data are presented as the averages ± standard deviations of 3 independently and identically prepared batches.

Fig. 3: Confocal microscopic images with 20x zoom of Huh7 cells incubated with 25-NBD cholesterol labeled L* a) for 1 h, b) for 3 hs and c) Z-slice showing the liposomes beside the nuclei indicating that the liposomes were uptaken by the cells. Scale bars: 50 μm.
Conclusion

The use of supersaturated drug solution helped increase drug loading compared with passive loading. The prepared liposomes showed sustained drug release for 48 hrs and were stable on the shelf for 3 months. Confocal images showed that the liposomes were taken up by the cells and released its contents in the cells.

REFERENCES


تحسين معدل تحميل الدوائي للليبوزمات عن طريق استخدام سوائل دوائية مشبعة

حسن تامام 3 , جيلان عبد الرازق عبد العلي 1 , سيد إبراهيم عبد الرحمن 1
على عبد الظهر عبد الرحمن 1 - يوون يو 2

قسم الصيدلة الصناعية ، كلية الصيدلة ، جامعة أسوان ، أسوان ، مصر
قسم الصيدلة الصناعية والفيزيائية ، كلية الصيدلة ، جامعة بوردو ، فرنسا
كلية الهندسة الطبية الحيوية ، جامعة بوردو ، فرنسا

الجسيمات النانوية هي أنظمة دوائية متطورة لتوصيل العلاج الكيميائي لعلاج السرطان. تستطيع الجسيمات النانوية زيادة كمية الدواء التي تصل إلى الورم عن طريق زيادة النفاذية والاحتفاظ بالدواء. كما أنها تستطيع التغلب على المشاكل المرتبطة بالعلاج الكيميائي مثل مشاكل الندوان، والسمية، ومقاومة الورم للعلاج. وباستخدام سوائل دوائية مشبعة أمكن تحسين التحميل الدوائي للليبوزمات مقارنه بباقي الطرق التقليدية. ولقد أعطت هذه الليبوزمات نتائج دوائية لمدة 48 ساعة.