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A simple passive equilibration method for loading carboplatin into pre-formed liposomes incubated with ethanol as a temperature dependent permeability enhancer

Moe Wehbe [a,b,*], Armaan Malhotra [a], Malathi Anantha [a], Jeroen Roosendaal [a,c], Ada W.Y. Leung [a,d], David Plackett [b], Katarina Edwards [e], Roger Gilabert-Oriol [a], Marcel B. Bally [a,b,d,f]

[a] Experimental Therapeutics, British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada.
[b] Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, BC V6T 1Z3, Canada.
[c] Department of Pharmaceutics, Section of Biopharmacy and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Utrecht University, PO Box 80082, 3508 TB Utrecht, The Netherlands.
[d] Department of Pathology and Laboratory Medicine, University of British Columbia, 2211 Wesbrook Mall, Vancouver, BC V6T 2B5, Canada.
[e] Department of Chemistry, University of Uppsala, 3 Husargatan (B7), Uppsala, Sweden
[f] Center for Drug Research and Development, Vancouver, BC V6T 1Z4, Canada

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ABSTRACT

A passive equilibration method which relies on addition of candidate drugs to pre-formed liposomes is described as an alternative method for preparing liposome encapsulated drugs. The method is simple, rapid and applicable to liposomes prepared with high (45 mol%) or low (<20 mol%) levels of cholesterol. Passive equilibration is performed in 4-steps: (i) formation of liposomes, (ii) addition of the candidate drug to the liposomes in combination with a permeability enhancing agent, (iii) incubation at a temperature that facilitates diffusion of the added compound across the lipid bilayer, and (iv) quenching the enhanced membrane permeability by reduction in temperature and/or removal of the permeabilization enhancer. The method is fully exemplified here using ethanol as the permeabilization enhancer and carboplatin (CBDCA) as the drug candidate. It is demonstrated that ethanol can be added to liposomes prepared with 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and Cholesterol (Chol) (55:45 mol ratio) in amounts up to 30% (v/v) with no change in liposome size, even when incubated at temperatures > 60 °C. Super-saturated solutions of CBDCA (40 mg/mL) can be prepared at 70 °C and these are stable in the presence of ethanol even when the temperature is reduced to <30 °C. *maximum* CBDCA encapsulation is achieved within 1 h after the CBDCA solution is added to pre-formed DSPC/Chol liposomes in the presence of 30% (v/v) ethanol at 60 °C. When the pre-formed liposomes are mixed with ethanol (30% v/v) at or below 40 °C, the encapsulation efficiency is reduced by an order of magnitude. The method was also applied to liposomes prepared from other compositions include a cholesterol free formulations (containing 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000] (DSPE-PEG_{2000})) and a low Chol (<20 mol%) formulations prepared with the distearoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) DSPG)). The cytotoxic activity of CBDCA was unaffected when prepared in this manner and two of the resultant formulations exhibited good stability *in vitro* and *in vivo*. The cytotoxic activity of CBDCA was unaffected when prepared in this manner and the resultant formulations exhibited good stability *in vitro* and *in vivo*. Pharmacokinetics studies in CD-1 mice indicated that the resulting formulations increased the circulation half life of the associated CBDCA significantly ($AUC_{0-24\ h}$ of CBDCA = 0.016 μg·hr/mL; $AUC_{0\text{-}24h}$ of the DSPC/Chol CBDCA formulation = 1014.0 μg·hr/mL and $AUC_{0\text{-}24h}$ of the DSPC/DSPG/Chol CBDCA formulation = 583.96 μg·hr/mL). Preliminary efficacy studies in Rag-2M mice with established subcutaneous H1975 and U-251 tumors suggest that the therapeutic activity of CBDCA is improved when administered in liposomal formulations. The encapsulation method described here has not been disclosed previously and will have broad applications to drugs that would normally be encapsulated during liposome manufacturing.

1. Introduction

Although rarely discussed in academic settings, the ability to prepare liposomal formulations at a scale sufficient for commercialization was

* Corresponding author at: Experimental Therapeutics, British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada.
E-mail address: mwehbe@bccrc.ca (M. Wehbe).

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key to the successful development and FDA approval of products like MyoCet® [1,2] and Doxil® [3] (liposomal doxorubicin formulations), Marqibo® [4,5] (a liposomal vincristine formulation) and others [6]. The manufacturing successes of these formulations were achieved, in part, through the development of efficient drug loading methods (>98% efficient) that facilitated the formation of products exhibiting high drug-to-lipid ratios and resulted in minimal unencapsulated drug loss [7]. Perhaps most importantly, the loading process (i.e. the step required to put the drug inside the liposome) was separated from the liposome manufacturing processes. Simple and scalable methods for manufacturing liposomes are in place based on the well-established extrusion methodology [8,9] or emerging new methods relying on microfluidics [10]. These methods require the use of large mixing containers, low pressure pumps and/or some amount of continuous processing. When a cytotoxic drug is incorporated into these methods, it carries some risks to those manufacturing the product. When using remote loading, selected drugs with appropriate chemical attributes can be added to pre-formed liposomes. The added drug diffuses across the liposomal membrane, where a change in chemical properties results in the drug being better retained. Using pH gradient loading methods as an example, drugs exhibiting protonizable amine functions (e.g. doxorubicin, vincristine) can be added to pre-formed liposomes prepared with an internal pH that is acidic and an external pH that is neutral [11–13]. Provided that the pKa of the drug is close to the external pH, a significant portion of the drug exists in the neutral form which is membrane permeable. Once inside the liposome, however, the drug becomes protonated and charged. The charged form of the drug is not membrane permeable and is trapped [14–17]. While versatile and efficient, this "remote loading" method is only applicable to drugs that have protonizable amine functions [12,13].

For drugs that cannot be loaded remotely, the alternative has been passive encapsulation where the compound of interest is added to dried lipid films or lipids solubilized in compatible solvents such as ethanol. The drug solution is processed with the lipids to prepare liposomes which, as indicated above, carry inherent risks. Further, the trapping efficiency of these methods can be very poor and is limited by the aqueous trapped volume of the liposome, the liposome size and the liposomal lipid concentration. Therefore, the loading efficiencies are low assuming the selected compound does not interact with the lipids used to prepare the liposome [18]. Additionally, because of issues related to non-equilibrium solute distribution, when using methods involving hydration of dried lipids, the concentration of solute in the aqueous space inside liposomes can be substantially lower than the concentration in the external solution [19,20]. Since there is substantial waste of often expensive drugs, this method can be uneconomical [21].

The studies reported here describe a process referred to as passive equilibration. The purpose of this system is to examine if drugs could be efficiently loaded into liposomes using simple equilibration approaches. This method relies on adding candidate drugs of interest to the outside of pre-formed liposomes in the presence of a permeability enhancer added in amounts that do not impact liposome structure (size) but do increase liposomal lipid bilayer permeability in a temperature-dependent manner. This allows for a safer, more economical, alternative method of drug loading which creates liposomal products with higher trapping effiencies. The method should be suitable for selected class 3 solvents deemed by the FDA to be less toxic and negative in genotoxicity studies [22] and is exemplified here using ethanol. It should be noted that others have reported on the use of ethanol for liposome manufacturing [23,24] and ethanol-injection methods have been applied in large-scale production [25] as well as in novel microfluidic approaches [10]. In these examples, ethanol is added during the manufacturing process rather than after the preparation of liposomes. Further, we have previously reported that low amounts of ethanol can be added to cholesterol-free liposomes exhibiting a transmembrane pH gradient in order to enhance the rate of remote loading for drugs that have protonizable amine functions [26]. This previous study suggested that ethanol could be used to selectively enhance liposomal lipid bilayer permeability as the amount of added ethanol did not impact the transmembrane pH gradient but did affect the ability of the added compound to cross the lipid bilayer in response to the gradient.

The method described here was developed using carboplatin (CBDCA), a drug that exhibits limited aqueous solubility (~10 mg/mL) at room temperature [27] and has been prepared previously as a liposomal formulation using passive encapsulation methods [28]. The Log P of CBDCA is −0.46, suggesting very low permeability across lipid membranes [29]. The solubility of CBDCA can be increased by heating at 70 °C to >40 mg/mL, however when incubated at room temperature the drug will precipitate from solution to achieve a solution concentration of ~10 mg/mL within 1.5 h (unpublished observation). This can be accelerated by cooling the sample in an ice bath. We examined liposomes prepared from DSPC/Chol (55:45 mol ratio) to assess ethanol's influence on a stable formulation commonly used in the field. DSPC/DSPG/Chol (70:20:10 mol ratio) liposomes were also studied to assess the effect of Chol content on ethanol-induced destabilization of the liposomes. We demonstrate that for DSPC/Chol (55:45 mol ratio) and DSPC/DSPG/Chol (70:20:10 mol ratio) liposomes, ethanol can be added without affecting liposome size, even when incubated at 70 °C. This effect has not been noted before and was exploited to allow for drug loading in liposomes, wherein the increase in lipid membrane permeability induced by temperature and ethanol can be rapidly decreased by simply decreasing temperature and/or removing ethanol. The compositions selected for these studies where designed to establish proof-of-principle. It is important to emphasize that the method could be applied to other lipid compositions, however the loading temperature and the type and concentration of the permeability enhancer used will need to be empirically determined. In the presence of ethanol, CBDCA was able to equilibrate across the DSPC/Chol lipid bilayer when incubated at ≥70 °C, but not at temperatures ≤ 40 °C. The trapping efficiency of CBDCA was 2-fold greater when compared to passive encapsulation methods where CBDCA solutions were used to hydrate DSPC/Chol mixtures prior to manufacturing.

2. Materials and methods

2.1. Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), Cholesterol (Chol), 1,2-distearoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (DSPG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) were purchased from Avanti Polar Lipids (Alabaster, AL) and ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) from PerkinElmer Life Sciences (Boston, MA). Pico-Fluor 40 scintillation cocktail was purchased from PerkinElmer Life Sciences (Woodbridge, ON, Canada). CBDCA powder was purchased from Biorbyt (San Francisco, CA) or as ready to inject product (Hospira). Sucrose, HEPES, Sephadex G-50, and all other chemicals (Reagent grade) were purchased from Sigma Aldrich (Oakville, ON, Canada) unless otherwise stated.

2.2. Tissue culture

In vitro studies were completed using U-251 MG glioblastoma multiforme cells and *in vivo* studies were performed with U-251 MG and H1975 non-small cell lung cancer cells. H1975 cells were maintained at 37 °C and 5% CO_2 in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 2 mM L-glutamine (Gibco) while U-251 MG cells were cultured with DMEM supplemented with 10% FBS and 2 mM L-glutamine. Both U-251 MG glioblastoma cells (formerly known as U-373 MG) and H1975 cells were originally obtained from American Type Culture Collection (Manassas, VA). Our lab has recently purchased the U-251 MG from Sigma-Aldrich (product number 09063001). A microsatellite analysis was performed in order to