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Optimization and modeling of the remote loading of luciferin into liposomes

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ABSTRACT

We carried out a mechanistic study to characterize and optimize the remote loading of luciferin into preformed liposomes of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine/1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPC/DPPG) 7:3 mixtures. The influence of the loading agent (acetate, propionate, butyrate), the metal counterion (Na^+, K^+, Ca^{+2}, Mg^{+2}), and the initial extra-liposomal amount of luciferin (n_L^{add}) on the luciferin Loading Efficiency (LE%) and luciferin-to-lipid weight ratio, i.e., Loading Capacity (LC), in the final formulation was determined. In addition, the effect of the loading process on the colloidal stability and phase behavior of the liposomes was monitored. Based on our experimental results, a theoretical model was developed to describe the course of luciferin remote loading. It was found that the highest luciferin loading was obtained with magnesium acetate. The use of longer aliphatic carboxylates or inorganic proton donors pronouncedly reduced luciferin loading, whereas the effect of the counterion was modest. The remote-loading process barely affected the colloidal stability and drug retention of the liposomes, albeit with moderate luciferin-induced membrane perturbations. The correlation between luciferin loading, expressed as LE% and LC, and n_L^{add} was established, and under our conditions the maximum LC was attained using an n_L^{add} of around 2.6 μmol. Higher amounts of luciferin tend to pronouncedly perturb the liposome stability and luciferin retention. Our theoretical model furnishes a fair quantitative description of the correlation between n_L^{add} and luciferin loading, and a membrane permeability coefficient for uncharged luciferin of 1×10^{-8} cm/s could be determined. We believe that our study will prove very useful to optimize the remote-loading strategies of moderately polar carboxylic acid drugs in general.

1. Introduction

Liposomes are artificial lipid aggregates that comprise a spherical bilayer arrangement of amphiphiles encasing an aqueous inner core. The potential use of liposomes in systemic drug delivery was highlighted back in the seventies (Gregoriadis et al., 1974), owing to their small colloidal size, controllable surface properties, large cargo capacity, biodegradability, and biocompatibility (Čeh et al., 1997). Liposomes can modulate the pharmacokinetic profile of the encapsulated drug as well as reduce the drug toxicity (Scheinberg et al., 2010). In addition, liposomal platforms can be further developed for active and selective drug delivery to the target site (Arouri et al., 2013; Park, 2014).

Because of the limited amount of lipids that can be administered systemically and in order to deliver the drug in clinical efficacious concentrations, it is essential that the liposomes are efficiently packed with the active drug compounds (Arouri et al., 2013). Whereas passive loading of water-soluble molecules often results in low encapsulation efficiencies, remote loading can provide a highly efficient alterative for drug encapsulation (Gubernator, 2011; Sur et al., 2014; Zucker et al., 2009). The remote loading process is based on the transport of molecules

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against their concentration gradient as the molecules are transported from the bulk solution into preformed liposomes (Cullis et al., 1991). The procedure can be used to remote load amphipathic weak acids with carboxylic acid functional groups or weak bases with amine functional groups (Nichols and Deamer, 1976; Zucker et al., 2009). Since most known drug compounds either contain acidic (20%) or basic (75%) functional groups (Barenholz, 2001; Joguparthi and Anderson, 2008; Kerns and Di, 2008), remote loading can prove very useful to achieve high drug-to-lipid ratios, high loading efficiencies, and improved retention of many available amphiphilic molecules.

So far, the two most extensively investigated remote loading methods rely on establishing either a transmembrane pH- or a transmembrane concentration-gradient. Other strategies also exist, like utilizing transmembrane valinomycin-dependent K^+ diffusion potentials (Mayer et al., 1985).

In the pH-gradient method, a low intra-liposomal pH and a high extra-liposomal pH are established to trap basic compounds containing amine functional groups (Cullis et al., 1991; Madden et al., 1990; Mayer et al., 1986), such as propranolol and doxorubicin. As the uncharged drug diffuses through the liposomal bilayer into the intra-liposomal interior it becomes protonated (charged) and therefor cannot diffuse back into the bulk. By an equivalent mechanism, carboxylic acids can be remotely loaded using a high intra-liposomal pH and a low extra-liposomal pH (Kheirolomoom et al., 2010).

In the concentration-gradient method, a high intra-liposomal concentration of an appropriate organic salt can drive the accumulation of the drug inside the liposomes, which is coupled to the countertransport of the membrane-permeant state of the encapsulated ion pair. For instance, basic drugs, like bupivacaine, quinidine, and doxorubicin, can be remotely loaded using an ammonium sulfate gradient (Chen et al., 2010; Lasic et al., 1992; Zucker et al., 2009). At alkaline pH, ammonium sulfate will dissociate into sulfate anions and ammonium cations that will further produce ammonia and protons. Ammonia can freely exit the liposomes down its concentration gradient (membrane permeability coefficient, $P = 0.13$ cm/s), whereas the sulfate counter anion has a much lower membrane permeability ($P < 10^{-12}$ cm/s) and therefore will remain inside the liposomes (Clerc and Barenholz, 1995). The process will decrease the intra-liposomal pH, thereby allowing for the trapping of basic drug compounds inside the liposomes in a fashion similar to the pH-gradient method (Clerc and Barenholz, 1995). Acidic drugs, like diclofenac, glucocorticoid prodrugs, and methylprednisolone succinate, can also be remotely loaded using an acetate gradient of different salts (Avnir et al., 2007; Hwang et al., 1999; Zucker et al., 2009), since acetic acid (AH) can diffuse freely through the liposomal membrane ($P = 6.6 \times 10^{-3}$ cm/s (Walter and Gutknecht, 1984)), which will increase the intra-liposomal pH allowing for trapping of carboxylic acid compounds. The remote loading of weak acids and bases will maintain electroneutrality in both intra-liposomal and extra-liposomal compartments (Clerc and Barenholz, 1995). The concentration-gradient method appears to be more efficient, especially for drugs with limited water solubility (Hwang et al., 1999).

The drug surrogate and molecular reporter (*S*)-2-(6-hydroxybenzo[*d*]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (luciferin) is a readily water soluble compound. As illustrated in Fig. 1, luciferin (L) can be remotely loaded into liposomes using a carboxylic acid gradient, which will drive the transport of the protonated (uncharged) form of luciferin (LH_2) through the lipid membrane leading to an accumulation of luciferin inside the liposomes (Armarego and Chai, 2013; Cern et al., 2012; Zucker et al., 2009). Unlike doxorubicin, the salts of which can form low-solubility gel-like or fibrous-like bundles inside the liposome (e.g., sulfate and citrate salts) (Lasic et al., 1992; Li et al., 1998; Madden et al., 1990), luciferin molecules do not easily form precipitates.

Despite the advantages of drug remote-loading methods, only few attempts have been made to understand the effect of the loading conditions, which could allow for process optimization as well as prediction of the content and properties of the final formulation (Zucker et al., 2009). In the present mechanistic study, we investigated the process of luciferin remote-loading into preformed liposomes as well as the effects of the loading agent (acetate, propionate, butyrate), the metal counterion, and the

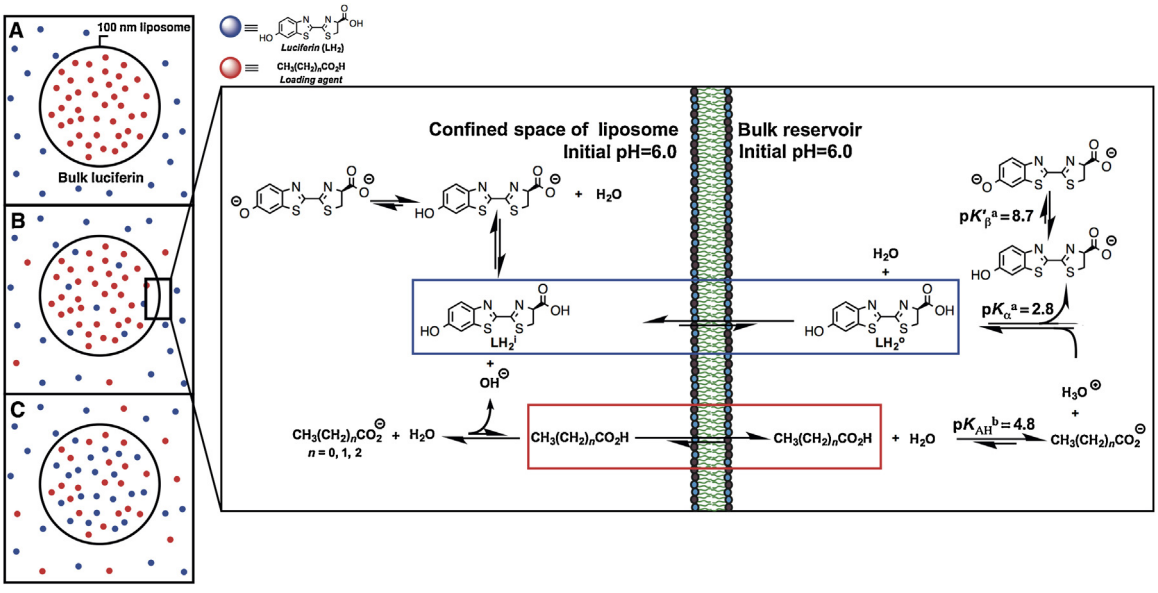

Fig. 1. Schematic illustration of the remote loading of luciferin (L) into liposomes using a carboxylate/carboxylic acid gradient. (A) Initially, the loading agent (e.g., acetic acid) is encapsulated inside the liposomes. The untrapped loading agent in the bulk is exchanged for potassium sulfate by size-exclusion column chromatography and L is added to the bulk solution. At this stage, the uncharged L (i.e., LH_2) diffuses down its own concentration gradient into the liposomes. (B) The stage at which the L concentration is equal inside and outside the liposomes. (C) L is being transported against its own concentration gradient driven by the efflux of the carboxylic acid loading agent (e.g., acetic acid) down its own concentration gradient.