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Note

Development of lysolipid-based thermosensitive liposomes for delivery of high molecular weight proteins

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ABSTRACT

The purpose of the present investigation was to determine the feasibility of using lysolipid-based thermal sensitive liposomes (145 nm) for high molecular weight molecule delivery. Fluorescein isothiocyanate conjugate-albumin was used as a model drug (MW 66 kDa). Thermal sensitive liposomes, which encapsulated the protein were prepared using a passive encapsulation methodology involving freeze-thawing cycles followed by extrusion. *In vitro* release studies at various temperatures indicated rapid release behaviour of the encapsulated protein at 42 and 44.5 °C but a good stability at 37.5 °C. The current findings suggest that lysolipid-based thermal sensitive liposomes can be used to deliver high molecular weight molecules.

Unilamellar liposomes are artificial bilayer vesicles composed mainly of a mixture of phospholipids that possess a hydrophilic interior (Drummond et al., 1999). Using bilayer structures as a barrier, it has been shown that hydrophilic drugs can be encapsulated into the interior of liposomes to prevent undesirable *in vivo* exposure (Allen and Chonn, 1987). Since the first application of liposomes as a potential therapeutic delivery system (Bangham, 1978), extensive formulation studies have been carried out to grant liposomes the ability to deliver encapsulated therapeutic agents to targeted areas, with minimum premature release (Allen et al., 1991; Charrois and Allen, 2003). Thermally sensitive liposomes enable a liposome-based drug delivery system that is capable of releasing encapsulated drug in response to elevation of temperature to the mild-hyperthermia range, whilst preventing premature leakage of encapsulated drug at 37.5 °C (Banno et al., 2010).

Lysolipids are a type of phospholipid that contain only one, rather than two, hydrocarbon chains. Such lipids, when mixed with normal phospholipids can lower the phase transition temperature of the lipid bilayer to the mild hyperthermia range. Thus lysolipid incorporated thermal sensitive liposomes (LTSL) are the most widely studied and applied formulation among various types of thermally sensitive liposomes. Upon heating, liquid–solid boundaries start to form in the lipid bilayer and lysolipids have been reported to create and stabilize hydrophilic water pores along these highly compressible liquid–solid boundaries, which significantly enhances the permeability of liposome (Needham et al., 2001). ThermoDox® is a doxorubicin-encapsulated LTSL that consists of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC),1,2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[methoxy(polyethylene, glycol)-2000 (PEG.DSPE.2000) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) in 10:4:90 molar ratio (Poon and Borys, 2009). This commercialized formulation has been reported to completely release the encapsulated doxorubicin within 30 s at 42 °C (Mills and Needham, 2005).

However, the application of LTSL has so far been restricted to low MW therapeutic agents, such as doxorubicin for cancer therapy. The aim of this note is to demonstrate the potential of using LTSL to carry and release high MW molecules, such as proteins. Fluorescein isothiocyanate conjugate-albumin (FITC-BSA) was used as a model protein-based drug. The liposome membrane formulation used in this study is similar to that of ThermoDox®. This will not only expand the potential applications of LTSL, but also provide indications of the maximum size and/or the stability of water pores that lysolipids are able to form upon heating.

FITC-BSA encapsulated LTSL liposomes were prepared using a passive encapsulation methodology followed by extrusion. Generally, stock lipid chloroform solutions were mixed (DPPC, MSPC and PEG.DSPE.2000 at 90:10:4 molar ratio) and solvent was then removed using a rotary evaporator to form a dried thin layer. The dried lipid film was hydrated using FITC-BSA (60 mg/ml) containing

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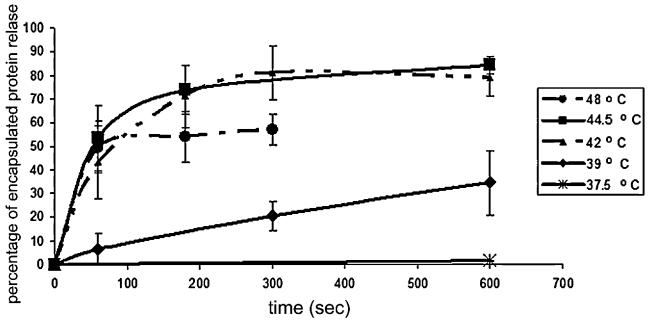

Fig. 1. Release behaviour of FITC-BSC encapsulated LTSL at 48, 44.5, 42, 39 and 37.5 °C respectively ($n = 3$).

HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7). 5 freeze-thawing cycles were applied during the hydration process, with the temperature during the thawing step being carefully selected (50 °C) in order to avoid irreversible conformational alteration to the secondary structure of the protein (Griebenow and Klibanov, 1995). The liposome formulation was then extruded through a 100 nm filter to reduce the size of the liposomes. FITC-BSA encapsulating LTSL were then separated from any unencapsulated protein on a Sepharose CL-2B column (0.8 cm × 10 cm) equilibrated with HEPES buffer (Barenholz et al., 1977). The encapsulation efficiency was determined by comparing the fluorescent intensity difference prior to and after the lysis of liposome using aqueous 1 v/v% Triton X-100 (emission wavelength at 520 nm excitation wavelength at 490 nm).

The thermal sensitivity of FITC-BSA encapsulated LTSL was determined by observing the release behaviour of encapsulated protein at various temperatures (Mills and Needham, 2006). HEPES buffer (5 ml) in a plastic tube was heated to a desired temperature using water bath. Liposome solution (500 μl) was then introduced into the temperature equilibrated buffer solution and taken out at preset time points. The sample was then quickly transferred into an ice bath to prevent subsequent release. The temperature of all samples was allowed to equilibrate with room temperature (20 ± 2 °C). The fluorescent intensities of samples were then measured to determine the release behaviour of encapsulated protein molecules (emission wavelength at 520 nm, excitation wavelength at 490 nm).

The particle size of liposome was measured using photon correlation spectroscopy and found to be 145 nm. The lipid to protein ratio of the liposome (weight to weight ratio) was 156:1. 72% of protein molecules were found to be firmly associated with the lipid membrane (i.e. in the form of transmembrane protein). The rest of the protein molecules were encapsulated in the interior core of liposomes. Little change in protein conformation (i.e. proportion of α helix, β sheet and random coil) is expected during the thawing (i.e. 50 °C for 20 min) and freeze process based on the stability studies reported by others (e.g. Griebenow and Klibanov, 1995; Lin and Koenig, 1976).

The release behaviour of FITC-BSA encapsulated LTSL at various temperatures are shown in Fig. 1. As can be seen, the liposomes demonstrated not only impressive stability at 37.5 °C, but also a rapid release behaviour at 42 and 44.5 °C.

In order to determine the necessity of the incorporation of lysolipid, the release behaviour of FITC-BSA encapsulated liposome without the incorporation of MSPC (DPPC and DSPE.PEG.2000 at 90:10 molar ratio) was investigated. Release study indicated that lysolipid-free vesicles were unable to release encapsulated protein at either 42 or 48 °C (data not shown).

It is interesting to note that the nature of the high molecular weight molecules might have an important influence on their release rate. Preliminary experiments using dextran (MW 40 kDa) indicated a much slower release at the phase transition temperature of the vesicle (i.e. 42 °C). This may be related to the different conformations of dextran and albumin.

In summary, our results have demonstrated that lysolipid based thermally sensitive liposomes are able to release high MW hydrophilic protein molecules upon temperature rise. In the near future, optimization of the formulation will be carried out to accelerate its release rate in a mild-hyperthermia temperature range. The preparation procedure will also be optimized in order to enhance the encapsulation efficiency of the protein.

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