International Journal of Pharmaceutics 524 (2017) 279–289

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

The effect of thermosensitive liposomal formulations on loading and release of high molecular weight biomolecules

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ARTICLE INFO

Article history:
Received 24 January 2017
Received in revised form 28 March 2017
Accepted 31 March 2017
Available online 2 April 2017

Keywords:
Thermosensitive liposomes
Protein encapsulation
Macromolecular drug delivery
Lysozyme
BDNF

ABSTRACT

Thermosensitive liposomes are clinically-relevant nanocarriers which have been used to deliver chemotherapeutic agents to tumors in combination with local hyperthermia. However, the encapsulation and release of macromolecular therapeutic agents (proteins, nucleic acids, bioactive polymers) is often hindered by their instability during the liposome formation as well as by the low encapsulation efficiency.

The objective of this study was to investigate the influence of the thermosensitive liposomal formulation on the encapsulation and release of low and high molecular weight hydrophilic drugs, in order to identify the key parameters to control during nanocarrier design, depending on the specific drug delivery application.

Thermosensitive liposomes with different formulations were prepared through the combinations of different lipids, including dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), cholesterol (Chol), 1-palmitoyl-2-hydroxy-sn-*glycero*-3-phosphocholine (P-Lyso-PC), and the PEGylated lipid distearoyl-sn-*glycero*-3-phosphoethanolamine-*N*-methoxy(PEG)-2000 (DSPE-PEG2000). The thin film hydration method was used for liposome preparation and loading of different water soluble molecules. The encapsulation efficiency and release profiles were investigated for a low molecular weight compound such as carboxyfluorescein (CF), proteins (albumin), and hydrophilic polymers which do not interact with the lipid bilayer, such as a linear dextran and a poly(ethylene glycol)-based star polymer. An optimised liposomal formulation [DPPC/P-lyso-PC/DSPE-PEG2000 90/10/4 (mol/mol) (LTSL)] was chosen for further application in encapsulating therapeutic proteins, such as lysozyme and the brain-derived neurotrophic factor (BDNF), which are recognized as drug carriers and potential therapeutic agents for kidney diseases and neurological disorders.

1. Introduction

Liposomes represent the first nanocarriers for drug delivery which made the transition from bench to clinical application, and they are now an established technology platform with considerable clinical acceptance and with major impact on many biomedical areas(Allen and Cullis, 2013; Sercombe et al., 2015). These phospholipid vesicles consist of at least one lipid bilayer enclosing a discrete aqueous domain. Hydrophobic compounds can be inserted into the lipid membrane, whereas hydrophilic molecules can be entrapped in the aqueous core, and this characteristic enables a variety of different drugs, including hydrophilic and hydrophobic, low and high molecular weight molecules, to be encapsulated and later released at the targeted site(Akbarzadeh et al., 2013; Sercombe et al., 2015).

Liposome surfaces can be easily functionalised with an appropriate ligand for targeted delivery and also decorated with a biocompatible protein-repellent polymer, such as polyethylene glycol (PEG), to significantly improve in-serum stability by reducing opsonization and clearance by the mononuclear phagocytic system (Hwang et al., 2012; Immordino et al., 2006; Pattni et al., 2015).

Although liposomes are a good candidate for *in vivo* delivery of high molecular weight compounds such as protein/peptide drugs and nucleic acids, the encapsulation of these therapeutic molecules is often hindered by their instability during the

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http://dx.doi.org/10.1016/j.ijpharm.2017.03.090
0378-5173/

liposome formation as well as by the low encapsulation efficiencies (Xu et al., 2012).

The film hydration method followed by extrusion seems to be the preferred choice for encapsulating these large molecules in liposomes (Azeem et al., 2009), since other processes based on the reverse-phase evaporation and detergent depletion methods use organic solvents/detergents which can denature proteins and are difficult to remove (Hwang et al., 2012; Xu et al., 2012). However the process of film hydration and extrusion requires a temperature above the gel-liquid crystal transition temperature (T_m) (Dua et al., 2012; Zhou et al., 2012), i.e. a temperature above which the lipid bilayer is soft enough to hydrate, envelope to form a vesicle, and passively encapsulate the payload in suspension (Dua et al., 2012; Zhou et al., 2012). For several lipid formulations, T_m is too high to preserve protein stability and avoid denaturation (Li et al., 2015a).

This limitation can be overcome by using thermosensitive liposomes, obtained by mixing lipids with low T_m (Al-Ahmady and Kostarelos, 2016; Kneidl et al., 2014). The low transition temperature made these liposomes a promising tool for targeted and triggered drug delivery to cells, tissues, organs in combination with local hyperthermia or high-intensity focused ultrasound (Grüll and Langereis, 2012; Schroeder et al., 2009). At body temperature (below T_m) the lipid membrane presents low permeability for hydrophilic compounds, whereas at the targeted area where there is a local increase of temperature (above T_m), the drug is released either by an enhanced permeability of the membrane or by its rupture (Kneidl et al., 2014).

The low encapsulation efficiency of macromolecules in liposomes is also a critical aspect to take into account during nanocarrier design (Xu et al., 2012). It generally depends on several parameters, including the phospholipid concentrations and composition, buffer pH and ionic strength, colloidal size (hydrodynamic diameter) of the payload, the size of the liposome (including the size of the aqueous interior) and the interaction between the molecule and the lipid bilayer (Colletier et al., 2002; Hwang et al., 2012; Zhao and Lu, 2009).

Our objective in the present study was to investigate the influence of the thermosensitive liposomal formulation on the encapsulation and release of low and high molecular weight hydrophilic drugs, in order to identify the key parameters to control during nanocarrier design, depending on the specific drug delivery application.

Thermosensitive liposomes with different formulations were prepared through the combinations of different lipids, including dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), cholesterol (Chol), 1-palmitoyl-2-hydroxy-sn-*glycero*-3-phosphocholine (P-Lyso-PC), and the PEGylated lipid distearoyl-sn-*glycero*-3-phosphoethanolamine-*N*-methoxy(PEG)-2000 (DSPE-PEG2000). Different water soluble molecules were selected as model drug for encapsulation into the hydrophilic core of liposomes, and a thin film hydration method combined with the freeze-thaw cycling technique was used for the preparation of these drug- loaded nanocarriers. The loading and release of the low molecular weight carboxyfluorescein (CF) was compared with a protein (albumin), and hydrophilic polymers which are assumed not to interact with the lipid bilayer, such as a linear dextran (Andrieux et al., 1998) and a PEGylated star polymer (Schulz et al., 2012).

The encapsulation efficiency and drug release profile were investigated, and an optimised liposomal formulation was chosen for further application in encapsulating therapeutic proteins, such as lysozyme and the brain-derived neurotrophic factor (BDNF), which are recognized as drug carriers or potential therapeutic agents for kidney diseases (Li et al., 2015b; Sava, 1996; Zhou et al., 2014) and neurological disorders (Nagahara and Tuszynski, 2011).

2. Materials and methods

2.1. Materials

1,2-distearoyl-*sn-glycero*-3-phosphocholine (DSPC), 1,2-dipalmitoyl-*sn-glycero*-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-*sn-glycero*-3-phosphocholine (P-Lyso-PC), Cholesterol (Chol), 1,2-distearoyl-*sn-glycero*-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-mPEG(2000)) were purchased form Avanti Polar Lipids (Alabaster, USA). Albumin-fluorescein isothiocyanate conjugate (FITC-albumin), lysozyme from chicken egg white, fluorescein isothiocyanate isomer I (FITC, ≥90% (HPLC)), 5(6)-Carboxyfluorescein (CF), tetramethylrhodamine isothiocyanate–Dextran (TRITC-dextran, number average mol wt Mn = 40000 Da), sepharose CL-4B, HEPES, Triton X-100, Dichloromethane (DCM), Methanol (MeOH), sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, Tris(hydroxymethyl)aminomethane (Tris base), hydrochloric acid were purchased from Sigma-Aldrich (Milan, Italy). Synthesis and characterization of Rhodamine-labelled PEG star polymer (Rho-A4PEG5), Z-average size 6.8 nm, and FITC conjugation of lysozyme were reported in the Supporting information. Brain Derived Neurotrophic Factor (BDNF, human recombinant) was purchased form QED Bioscience Inc. (Histo Line, Milano, Italy).

Deionized (DI) water was obtained from Milli-Q® water purification system (Merck Millipore).

2.2. Buffers

PBS buffer (10 mM, pH = 7.4) was prepared as follow: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO4, 0.24 g of KH_2PO4 were dissolved in 800 mL of deionized (DI) water, and the pH was adjusted to 7.4 with 1 M HCl, then DI water was added to a total volume of 1 L.

HBS buffer (20 mM HEPES, 150 mM NaCl, pH = 7.4) was prepared as follows: 4.766 g HEPES and 8.766 g NaCl was dissolved in 500 mL of DI water, and the pH was adjusted to 7.4 with 1 M NaOH, then DI water was added to a total volume of 1 L.

Tris-HCl buffer (10 mM, 0.9% NaCl, pH = 8.0) was prepared as follows: 1.211 g Tris base and 9 g NaCl was dissolved in 500 mL of DI water, and the pH was adjusted to 8 with 1 M HCl, then DI water was added to a total volume of 1 L.

2.3. Preparation of liposomes with different payloads

Liposomes with different formulations [lipid molar ratio NTSL: DSPC/Chol/DSPE-PEG2000 = 65/30/5; TSL1: DPPC/DSPE-PEG2000 = 95:5; TSL2: DPPC/DSPC/DSPE-PEG2000 = 80/15/5; TTSL: DPPC/DSPC/Chol/DSPE-PEG2000 = 50/25/15/3; LTSL: DPPC/P-Lyso-PC/DSPE-PEG2000 = 90/10/4 (Table 1)] were prepared by the lipid film hydration and extrusion method. In brief, respective lipids were dissolved in DCM/MeOH mixed solutions (9:1 v/v) in a round-bottomed flask, and the solvent was evaporated under vacuum in a rotary evaporator at 25 °C until 20 μmol of a thin lipid film was formed. The film was further dried under vacuum over night to make sure that all the solvent was evaporated. Depending on the payload to be encapsulated, CF (100 mM), FITC-albumin (2 mg/mL), TRITC-Dextran (2 mg/mL), Rho-A4PEG5 (2 mg/mL), FITC-lysozyme (0.2 mg/mL), BDNF (0.2 mg/mL) solutions were prepared by dissolving the compounds in 1 mL HBS buffer (or PBS buffer in case of lysozyme and BDNF). The dry film was hydrated with the above aqueous solution at either 60 °C (for NTSL) or 50 °C to obtain a resulting lipid concentration of 20 mM. Afterwards, the multilamellar vesicles were subjected to 6 cycles of freeze-thawing and sized by repeated extrusion through polycarbonate membranes of well-defined pore size (0.1 μm).