European Journal of Pharmaceutics and Biopharmaceutics 81 (2012) 273–280

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Application of different methods to formulate PEG-liposomes of oxaliplatin: Evaluation *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 28 September 2011
Accepted in revised form 10 February 2012
Available online 18 February 2012

Keywords:
Film Method
Reverse-Phase Evaporation method
Heating Method
PEG-coated liposomes
Oxaliplatin
In vitro and *in vivo* study

ABSTRACT

In this work, the Film Method (FM), Reverse-Phase Evaporation (REV), and the Heating Method (HM) were applied to prepare PEG-coated liposomes of oxaliplatin with natural neutral and cationic lipids, respectively. The formulations developed with the three methods, showed similar physicochemical characteristics, except in the loading of oxaliplatin, which was statistically lower ($P < 0.05$) using the HM.

The incorporation of a semi-synthetic lipid in the formulation developed by FM, provided liposomes with a particle size of 115 nm associated with the lowest polydispersity index and the highest drug loading, 35%, compared with the other two lipids, suggesting an increase in the membrane stability. That stability was also evaluated according to the presence of cholesterol, the impact of the temperature, and the application of different cryoprotectants during the lyophilization. The results indicated long-term stability of the developed formulation, because after its intravenous *in vivo* administration to HT-29 tumor bearing mice was able to induce an inhibition of tumor growth statistically higher ($P < 0.05$) than the inhibition caused by the free drug.

In conclusion, the FM was the simplest method in comparison with REV and HM to develop *in vivo* stable and efficient PEG-coated liposomes of oxaliplatin with a loading higher than those reported for REV.

1. Introduction

Liposomes are considered as efficient carriers for drugs, vaccines, nutrients, diagnostics, and other biomolecules [1–6]. This is due to some advantages, such as the ability to incorporate water and lipid soluble agents, high versatility in terms of fluidity of liposomal membrane, size and superficial charge [7]. The new generation of liposomes by the insertion of polyethylene glycol (PEG)-derivatized phospholipids into liposomal membrane leads to obtain sterically stabilized liposomes [3,8–10]. The main characteristics of these liposomes are the decrease in their clearance [11,12] and their increased accumulation in affected organ sites [13,14]. Therefore, this system is able to alter the pharmacokinetics and biodistribution of the encapsulated drug [15]. In this way, oxaliplatin is a third generation of platinum (Pt), antitumor drug used as a first-line chemotherapy for metastatic colorectal cancer [16–19]. This Pt derivative shows higher tolerability of adverse effects than cisplatin or carboplatin [20–22]. However, its efficacy is relatively low due to its pharmacokinetics properties, such as high irreversible binding to plasmatic and tissue proteins and erythrocytes, among other components. For this reason, the encapsulation of oxaliplatin represents a strategy to overcome these limitations, delivering in a selective manner the drug into the tumor.

On the other hand, the methods used to prepare liposomes have a significant impact in some physicochemical characteristics such as size or efficiency of encapsulation of the agent. In this way, Film Method (FM) [23] and Reverse-Phase Evaporation (REV) method [24] have been selected by several authors as two conventional methods to prepare liposomes. However, in the last years, other methods have been described in literature, and one of them is the Heating Method (HM) [25]. This new method is characterized by the absence of organic solvent for the solubilization of lipids, representing an advantage in terms of toxicity. In general, all methods have advantages and disadvantages.

FM is characterized by the fact that it can be used for all different types of lipid combinations and it is very easy to perform. The

Abbreviations: PC, Phosphatidylcholine; HSPC, soy hydrogenated L-α-phosphatidylcholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DSPE-PEG200, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]; Chol, cholesterol; PDI, polydispersion index; EE, encapsulation efficacy; LP, liposome; REV, Reverse-Phase Evaporation; FM, Film Method; HM, Heating Method; RT, room temperature; TC, transition temperature; FBS, Fetal Bovine Serum; ED, encapsulated drug; pH_i, internal pH; pH_o, outside pH; 5-FU, 5-fluorouracil.

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doi:10.1016/j.ejpb.2012.02.007

main step is the hydration of the lipids, and the acceptable encapsulation rates that can be obtained [26]. For REV, the main step is in the oil/water emulsion, which is diluted with further aqueous phase for liposomes formation. This method is very popular due to a high encapsulation rate, up to 50%; however, the problem is the remaining solvent and the high polydispersity index (PDI) in the particle size. In both cases, to formulate a homogeneous population of liposomes regarding the particle size, it needs the application of a homegenization technique. Finally, the HM has not been widely applied, because few examples are only reported in the literature with 5-FU and DNA [27–29].

Taking into account that most of the publications about liposomes of oxaliplatin have used REV method, the aim of this work is the development of PEG-coated liposomes of oxaliplatin using different methods and lipids. It is also evaluated the stability of the formulation under different conditions. In addition, the cytotoxicity and antitumor effects, respectively, were assayed in *in vitro* and *in vivo* models with colorectal cancer cell lines.

2. Material and methods

2.1. Materials

Oxaliplatin was purchased from Sigma (Barcelona, Spain). Phosphatidylcholine (PC), cholesterol (Chol), soy hydrogenated L-α-phosphatidylcholine (HSPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG 2000) were purchased from Avanti polar lipids Inc. (Alabaster, Alabama, USA).

2.2. Methods

2.2.1. Oxaliplatin liposomes preparation

Three different methods were carried out to develop oxaliplatin-loaded liposomes.

2.2.2. Film Method

Liposomes containing oxaliplatin were prepared employing the thin film hydratation method following the basic specifications described by Bangham and Lea [23]. Briefly, lipids were dissolved in chloroform forming a mixture. The organic solvent was then removed by rotary evaporation under reduced pressure (Büchi-R144, Switzerland) at room temperature (RT) to obtain a film on the wall of the flask. The dry lipid film was hydrated with a solution of oxaliplatin dissolved in glucose 5% (2 mg/ml). The dispersion of the lipid was facilitated by mechanical shaking in an ultrasound bath for 1 min. To control the particle diameter, the emulsion was extruded through a polycarbonate membrane (Mini-Struder Set, Avanti Polar Lipids Inc (Albaster, Alabama, USA)) with a pore size of 100 nm.

2.2.3. Reverse-Phase Evaporation method

This method, described by Szoka and Papahadjopoulos [24], is used to prepare liposomes with a large internal aqueous space. Lipids solubilized in a mixture of chloroform:diethyl ether (1:2, v/v) were added to the aqueous phase containing oxaliplatin (4 mg/mL) dissolved in glucose 5%, in a ratio 3:1 (v/v) between organic and aqueous phase. The mixture was sonicated at RT for 5 min and placed on the rotary evaporator to remove the organic solvent under reduced pressure (200 mm Hg). At this point, the material forms a viscous gel, which becomes an aqueous suspension by shaking in a vortex. The liposomes were extruded following the method described earlier.

2.2.4. Modified Heating Method

In the last technique, the Heating Method [25] was combined with a gradient of pH [30]. The lipids were hydrated in a citrate solution (pH 4) and mixed in a bath-ultrasound for 1 min. The mixture was extruded as it has been described in the previous methods, and the excess of citrate was removed by ultrafiltration (Amicon with a cut-off membrane of 10,000 MWCO membrane, Millipore, Billerica, USA). The incorporation of oxaliplatin (2 mg/mL) dissolved in glucose 5% was achieved by adding the drug solution together with Hepes solution (pH 7.8). This mixture was heated at the corresponding lipids transition temperature 60 °C for 30 min. Afterward, it was cooled at 4 °C.

In all methods, the amount of non-encapsulated oxaliplatin was removed from the formulation by ultrafiltration using the Amicon devices (10,000 MWCO) The final formulation was washed, at least two times, with 3 ml of PBS and ultrafiltered again. To evaluate the efficiency of this method, a constant concentration of free oxaliplatin (1 mg/mL) was added to empty liposomes. This mixture was shaking for 30 min at room temperature, and it was ultrafiltered using the Amicon system (10,000 MWCO) at 2200 g for 30 min. After the ultrafiltration, both aliquots were collected, the liposomes and the ultrafiltered solution, to measure the levels of oxaliplatin by the atomic absorption spectrometry technique. Liposomes without oxaliplatin, empty formulation, were prepared following the same procedure but adding glucose 5%.

These methods were carried out with two different types of lipids neutral, such as PC and cationic, DOTAP, in order to study the influence of them in the physicochemical characteristics of the liposomes developed, and in the efficiency of encapsulation (EE) of oxaliplatin.

2.3. Characterization of liposomes

The particle size, polydispersity index (PDI), and Zeta potential of liposomes were analyzed by laser diffractometry using a Zetasizer Nano-Z (Malvern Instruments, UK). Formulations were diluted 1:100 (v/v) in deonized water in order to ensure a convenient scattered intensity on the detector.

The oxaliplatin encapsulation was measured by atomic absorption spectrometry using a validated method. Then, the EE expressed in percentage (%) was calculated by dividing the drug to lipid ratio recovered after ultrafiltration in the final formulation by the initial amount of oxaliplatin and lipid.

The phospholipid concentration was quantified following the Zöllner and Kirsch method [31].

2.4. Stability of liposome formulations

Stability is a critical factor that must be considered during formulation design and development. Physical or colloidal stability based on size distribution under storage conditions as well as in a biological medium must be considered. Based on the results found during liposome formulation, HSPC liposomes developed with FM were selected to characterize the stability of the liposomes formulated without and with cholesterol [HSPC:Chol:DSPE-PEG_{2000}]. Chol was used at 40% in the lipid mixture.

In addition, other different approaches were followed to complete this study:

2.5. Drug release

This study was carried out at two different temperatures, 4 °C used to storage the formulation and 37 °C used for *in vitro* and *in vivo* studies. Then, 100 μl of formulation mixed with 900 μl of complete cell medium was incubated at 37 °C in continuous shaking. Samples collected at different times: 0, 1, 4, 7, and 24 h were