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

Sara Zalba[1], Iñigo Navarro[2], Iñaki F. Trocóniz[1], Conchita Tros de Ilarduya[1] and María J. Garrido[1]

1.- Departament of Pharmacy and Pharmaceutical Technology; 2.- Department of Analytical Chemistry. University of Navarra. Spain.

Corresponding author:

María J. Garrido

Dept. Pharmacy and Pharmaceutical Technology

University of Navarra

Irunlarrea, 1

31008-Pamplona

Spain

Phone: +34948425600 (ext. 6529)

Fax: +34948425740

e-mail: mgarrido@unav.es

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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 to the lowest polydispersity index and the highest drug loading, 35%, compared to the other two lipids, suggesting an increase of the membrane stability. That stability was also evaluated according to the presence of cholesterol, the impact of the temperature, and the application of different cryoprotectans 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.

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 of 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, 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 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.

Material and methods

Materials

Oxaliplatin were 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-PEG2000) were purchased from Avanti polar lipids Inc. (Alabaster, Alabama, USA).

Methods

Oxaliplatin liposomes preparation

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

Film Method

Liposomes containing oxaliplatin were prepared employing the thin film hydratation method following the basic specifications described by Bangham et al [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.

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 above.

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 60º C for 30 min. Afterwards, it was cooled at 4º 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 (1mg/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 2,200 g for 30 min. After the ultrafiltration, both aliquots were collected, the liposomes and the ultra-filtered 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.

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].

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:

- **Drug release**

This study was carried out at two different temperatures, 4ºC used to storage the formulation, and 37 ºC used for *in-vitro* and *in-vivo* studies. Then, 100 µl of formulation mixed with 900 µl of complete cell medium was incubated at 37ºC in continuous shaking. Samples collected at different times: 0, 1, 4, 7 and 24 h, were ultrafiltered using the Amicon system (10,000 MWCO) to obtain the liposomes. The encapsulated and released oxaliplatin levels were quantified by atomic absorption spectrometry. In addition the parameters, particle size, PDI and Zeta potential, were also characterized in these samples.

- **Lyophilization assay**

Formulations were lyophilized following three different strategies: without cryoprotectant, with Trehalose (4:1,w/w sugar: lipid), or with L-arginine (4:1, w/w aminoacid: lipid). After the lyophilization process, the formulation was again characterized by determining size, PDI and Zeta potential.

Transmission electron microscopy (TEM)

Liposomes formulated without and with DSPE-PEG_{2000} were analyzed by TEM [32]. The measurements were carried out by means of a LIBRA-Zeiss 120 electron microscope operating at 80 kV, equipped with an electron spectrometer filtering out inelastic electrons for better imaging. 10 µl of the sample was incubated with OsO_4 1% during 30 minutes. 20 µl of the mixture was deposited over carbon-coated copper grids with 200 mesh during 60 seconds and dried. Negative contrast staining was done with 2% aqueous phosphotungstic acid solution. The samples were visualized 24 hours later. The same protocol was followed for the negative control corresponding to a sample without liposomes. Images were analyzed with iTEM Olympus Soft Imaging Solutions GmbH 5.1 software.

Antiproliferative activity in cultured cells

The human colorectal cancer lines HCT-116 and HT-29 (purchased from ATCC) were cultured in McCoy's Medium Modified, completed with Fetal Bovine Serum (10%) and Penicillin-Streptomicin (0.01%), at 37ºC in a humidified atmosphere containing 5% CO_2. All cells were used under sub-confluence condition.

Cells were seeded into 96-well microtiter plates at a density of 10×10^3 cells/well/200 µl of medium. After 24 h, cells were treated with several concentrations (ranged from 0.1 to 50µM) of free oxaliplatin, empty liposomes or oxaliplatin liposomes for 72 h. The

survival cells after each treatment were measured with the colorimetric Neutral Red Assay [33]. The optical density was read at 540 nm (Labsystems iEMS Reader MF).

***In vivo* study**

Twenty four female athymic nude mice weighing 20-25 g (aprox. 4 weeks old) were purchased from Harlan (Barcelona, Spain). Animals were housed in microisolator cages under positive-pressure ventilation and maintained in closed-shelf, laminar-flow racks to avoid contact with pathogens, odors or noises and kept under standard laboratory conditions. Sterilized food and water were available *ad libitum*.

To induce the tumor, 100 μl of PBS containing 1.5 x 10^6 HT-29 cells were subcutaneously injected into the right flank of the mice. Tumor growth expressed as volume (V) was calculated by $V = 4/3\ \pi\ (A^2B/2)$, where A and B correspond to the smallest and the largest diameter, respectively [34]. One week after cells injection and when the volume of the tumor was around 200 mm^3, animals were randomly divided into four groups with 6 animals per group: Group I, control (PBS), Group II, animals treated with empty liposomes at corresponding dose of oxaliplatin, Group III, free oxaliplatin (5 mg/kg) and Group IV, oxaliplatin encapsulated in liposomes (5 mg/kg). The dose of liposomes was calculated based on the μg of oxaliplatin encapsulated per mg of lipid, avoiding a concentration higher than 1.25 mg of lipids in each injection. All animals received 2 consecutives cycles of treatment with 5 days apart. In each of them, two doses of 2.5 mg/kg every 48 h were administered. At the end of the study, animals were sacrificed by cervical dislocation. The tumor was removed to quantify the oxaliplatin levels. This organ was weighted and homogenized with nitric acid 0.1 N overnight (100 mg tissue/1mL acid) and diluted with 5mL of deonized water. Then, the samples were measured by atomic absorption spectrometry. Toxicity was also evaluated by measuring the body weight at the same time that the tumor.

The protocol of the study was approved by the Animal Experimentation Committee of the University of Navarra and is in accordance with the applicable European guidelines.

Statistical analysis

The results were expressed as the mean ± standard deviation (SD). The statistical study was performed using SPSS, version 15.0 for windows. All data were analyzed with the non-parametric Kruskall-Wallis test followed by the Mann-Whitney U test. The level of significance was set at $P < 0.05$.

Results

Characterization of PEG-coated liposomes of Oxaliplatin

Film Method

The results found with FM are shown in Table 1. The particle size was similar to two formulations as it was expected, due to the use of the extrusion technique with the same type of membrane. The Zeta potential was negative for PC, due to the combination of PEG and positive for DOTAP, a typical cationic lipid. In relation to the EE, the percentages were similar between both lipids, although it was slightly higher for PC than for cationic lipid ($P>0.05$). However, this value was higher than those reported by other authors [35-38].

Reverse Phase Evaporation Method

REV is the most common technique used to encapsulate platinum derivatives. Table 1 shows the results found for liposomes of oxaliplatin, where all parameters were very similar for both lipids, except the Zeta potential, as it was expected. The EE for drug was slightly lower for DOTAP, but this difference was not significant ($P>0.05$), suggesting that the method ruled in the same way with independence of the ionic charge of the lipid.

Heating Method Modified

This method has been modified adding citrate and Hepes buffers to reach a gradient of pH. This change was because the amount of oxaliplatin incorporated into liposomes without difference of pH between inside-outside, was very low, 6% approximately. However, when the oxaliplatin was in a solution of pH 7.8, the encapsulation rate was higher than 20%. Although the size and Zeta potential obtained in these formulations, were similar to those observed with the other two methods, and the EE was lower ($P<0.05$) (Table 1).

No differences were observed between liposomes with vs. without oxaliplatin in each of the methods (data not shown).

The three methods can be considered for manufacturing liposomes of oxaliplatin. However the REV was one of the most complex due to the number of steps. In fact, several initial conditions of oxaliplatin/lipid ratio were tested to increase the loading, but this value did not increase when the amount of oxaliplatin was higher than 4 mg/mL. In addition, the liposome suspension showed quite high polydispersity in terms of size. The reason could be that to obtain homogeneous population of liposomes the extrusion technique could be applied with two types of membranes before the evaporation step.

On the other hand, although HM has the advantage to produce liposomes without volatile organic solvents to dissolve the lipids, the EE for oxaliplatin was the lowest compared to FM or REV. The main step is the incorporation of the drug into the liposomes previously formulated by heating at temperatures not lower than the transition temperature (Tc) of the lipids. This Tc was around 60º C, which is not high enough to modify the stability of the platinum molecule.

Based on the results found in Table 1, the FM appeared to be the simplest method to prepare liposomes with an adequate size, PDI and EE of oxaliplatin. Since PC and DOTAP were used as two basic lipids for selecting the method, another semisynthetic hydrogenated lipid, HSPC, was included. This lipid was of interest because is one of the main components of several marketed liposomal formulations. Lipids with different degree of saturation in their aliphatic chain seem to provide a higher stabilization effect to the liposomal membrane. The physicochemical parameters of this formulation were similar to those obtained for the liposomes formulated with PC. Thus, the particle size for this formulation was 115.6 ± 2.0 nm, with a Zeta potential of -18.4 ± 3.9 mV, the EE was 34.23 ± 2.9 % and a loading of 68.5 ± 4.2 μg /mg lipid. Moreover, the PDI was lower than the PC formulation, 0.034 ± 0.01 vs. 0.161 ± 0.02, suggesting that HSPC contributed to increase the stabilization of the membrane. Therefore HSPC-DSPE-PEG_{2000} liposomes were selected for the next studies.

Stability assay

Previous studies showed that at 4 ºC the formulations were stable in relation to size, Zeta potential and EE, at least for one month. The impact of several factors such as the inclusion of Chol, the temperature, different solutions for the incubation and freeze-drying processes, was also investigated to evaluate the retention of oxaliplatin into the liposomes. Table 2 shows that at 37ºC the inclusion of the sterol increased the stability of the membrane, although the difference in the retention of oxaliplatin between both formulations, with vs. without Chol, was only 10%. However, the drug release in the culture cell medium was slower for liposomes with Chol, suggesting that this release takes time, because Chol is able to stabilize the lipid bilayers. Therefore, a depot effect in tumor area could be achieved using this PEGylated liposomal formulation. This effect represents an advantage to oxaliplatin because, the drug would be more stable in

the blood circulation and could be released slowly at the tumor site. In addition, the PDI had a higher value for liposomes without Chol. This observation is compatible with an aggregation of the particles.

Since the structural integrity of the liposomes for long period of time is one of the objectives to optimize the formulation, the effect of the dehydration/reconstitution process was assayed in this work. Two different cryoprotectants, Trehalose and L-arginine were used to prevent the thermodynamic instability evaluated by the changes in the size and Zeta potential. L-arginine was included with the thought of the possible problems of diabetic patients. The results listed in Table 3, showed that the presence in the formulation of Chol together with Trehalose or L-arginine, was the best combination to obtain a stable formulation. Both cryoprotectans displayed similar behaviour, supporting the fact that both could be used.

Transmission electron microscopy (TEM)

Figure 1 shows that HSPC:Chol of liposomes were small vesicles with a concentric interior space. In the case of liposomes associated with DSPE-PEG_{2000}, a white coated film was observed in the surface [32].

Cytotoxic studies in colon cancer cell lines

The two cell lines were sensitive for oxaliplatin with IC_{50} values between 9.2-2.8 μM for the free drug. Oxaliplatin-loaded liposomes showed a reduced cytotoxicity. This effect was observed for the three types of lipids used in the formulation. Table 4 lists the IC_{50} values found for all treatments in both cell lines, HT-29 and HCT-116. HCT-116 was more sensitive to oxaliplatin, free and encapsulated, than HT-29. The cytotoxic effect was higher in both cell lines for free than for the encapsulated oxaliplatin in anionic liposomes, PC LP and HSPC LP. However in the case of the cationic liposomes, the value of the IC_{50} was very similar to the value for the free drug. This difference

could be explained by the effect of the empty DOTAP liposomes. They were able to reduce the proliferative effect to 30 % compared to the control group. PC and HSPC liposomes without oxaliplatin did not affect the cell proliferation.

***In vivo* study**

The formulation, [HSPC:Chol:DSPE-PEG_{2000}]-oxaliplatin liposomes, was intravenously administered to HT-29 tumor bearing mice. The dose was selected based on previous results found in our group (data not shown) and on the dose reported by Abu-Lila et al [35-37], although the cell lines were not the same. Figure 2 shows that PEGylated liposomes suppressed tumor growth more efficiently than free oxaliplatin. This inhibition reached statistical significance ($P<0.05$) during the second cycle. This enhanced anti-tumor activity of the liposomes is in line with those results reported by several authors for different antitumor drugs encapsulated in PEGylated liposomes. The levels of oxaliplatin found in the tumor at the end of the study, were three times higher for encapsulated drug in comparison with free drug: 560 ± 200 vs. 190 ± 101 ng/ mg tissue, respectively. In addition, this dose schedule was compatible with a low toxicity for both treatments (Figure 3). So, the encapsulation of oxaliplatin exhibited an improvement in the therapeutic effect of the drug.

Discussion

In this work PEGylated liposomes of oxaliplatin have been developed using different methods. FM and REV were selected as the most common methods used to prepare conventional liposomes [4-5]. The HM was chosen as one of the novel methods, introduced in the last years. The absence of volatile organic solvents in this last method is the main characteristic. It represents a potential benefit in relation to the toxicity exerted by these components in *in vivo* [25]. In addition two types of lipids have been used to evaluate their influence in the physicochemical characteristics of the

formulations obtained for each type of method. The results showed that the lipid did not have any influence in the studied characteristics. However, in the case of the REV method, the particle size was slightly higher than in the FM and HM, even when all methods were associated to the extrusion technique with the same type of polycarbonate membrane. This difference could be explained by the fact that, REV is one of the most complex methods compared to FM and HM. For example, the main step is the formation of a viscous gel which is responsible of the spontaneous formation of liposomes dispersion [24]. The characteristics of these liposomes depend on the mixture (lipid-water) in the emulsion and the time for the evaporation, among other steps. This leads to a higher variability in the final formulations than with the other two methods, which are methodologically simpler.

In the case of the HM, the encapsulation of oxaliplatin dissolved in glucose 5%, was extremely low (aprox. 6%). However, when a gradient of pH was reached between inside (pHi 4) and outside (pHo 7.8) of the liposomal formulation, the efficiency of encapsulation achieved levels of 20%. This result is in the same order as those values reported by other authors for oxaliplatin using the REV method [35-38]. In general, the transbilayer transport of weak acids and weak bases is more efficient in the presence of a pH gradient, but little or none encapsulation is observed in the absence of this gradient [30], as it was demonstrated for doxorubicine.

Finally, the FM associated with the extrusion technique allowed the formulation of an homogenous population of liposomes following very simple steps. Although some liposomal formulations with other antitumor drugs have been developed using this method, in the case of oxaliplatin most of them have been formulated with REV. The main advantage of this method is the encapsulation rate which can be up to 50%. Nevertheless, a significant difference is found in relation to the particle size and EE of

oxaliplatin depending on the author. For example, with REV and neutral lipids, Abu Lila et al [35-37] have reported liposomes with a particle size of 200 nm and an EE of 20 %, higher than the EE found by Suzuki et al [38]. In the last year, Yang et al [39] have described the methodology to develop PEG-liposomes of oxaliplatin with a particle size of 150 nm, and an EE of 40%. Note that this value has not been described how it was calculated.

In this work, the particle size was reduced to 115.3 ± 3.5 nm obtaining an EE of 34.2 ± 2.9 %. This loading drug was calculated as Abu Lila and coworkers have described. Although several concentrations of oxaliplatin (2, 4 and 5 mg/mL) were assayed, the loading did not change between 4 and 5 mg/mL.

On the other hand, different techniques have been found in the literature for the removal of the non-encapsulated drug: the dialysis technique against 5% of dextrose [35-37] or the ultrafiltration [39]. In our study the applied ultrafiltration technique showed that the 99.72% of the free drug added to empty liposomes, was in the ultrafiltered solution justifying its use.

The three methods have similar behaviour for neutral and cationic lipids, as it was previously reported by Abu-Lila et al. [40]. This data shows that each method to encapsulate a specific drug, had a similar behaviour with a non-dependence of the superficial charge of the lipid.

Taking into account that the formulations obtained with the three methods were very similar, the FM was selected to study several factors that influence the stability of the formulations. One of them is the use of semisynthetic lipid such as HSPC, a component of many marketed liposomal formulations such as, Doxil or Caelyx for doxorubicin (HSPC/Chol/DSPE-PEG2000); Ara-c (HSPC/Chol/DSPE-PEG2000); Lurtotecan (HSPC/Chol); Ambisome (HSPC/Chol/DSPG) [5, 41, 42]. Although the

physicochemical characteristics of HSPC liposomes were similar to those found for PC, the PDI was lower and the EE slightly higher. The degree of saturation of the aliphatic chain of the lipid confers a more dynamic structure of the membrane. This property should represent an advantage for entrapping more of the drugs with a low permeability across the cellular membranes, as in the case with oxaliplatin [7].

Finally, HSPC-LP was selected to study other factors that influence the stability of the formulation. These factors were the temperature at 37 ºC, the medium of the liposomes incubation, and the presence of Chol in the membrane. In the case of the incorporation of Chol, this factor did not influence significantly the amount of oxaliplatin released from the formulation. After 24 h of incubation at 37ºC, both types of formulations with and without sterol, release oxaliplatin with a difference of 10%. This difference in drug concentration suggests a minimum impact in the effect. Nevertheless, the most important aspect in the *in-vivo* activity is the time-release of the drug. Therefore, this point could be a limitation for the formulation for further studies. In this work, the retention time of oxaliplatin into the formulation assayed in culture cell medium was slightly higher than the value found by Abu Lila et al. [37] in plasma. Plasma does not have the same compositions of culture cell medium, but its complexity suggests that the behaviour of the formulation could be similar to plasma.

Then, the Chol exerted its function as a stabilizing agent of the liposomal membrane, which was reflected in the lower PDI compared to the PDI of formulations without Chol. Therefore, the final formulation using the mixture HSPC:Chol:DSPE-PEG_{2000} was selected to assay its *in-vivo* activity.

The results found with the lyophilisation technique represent a promising strategy to provide a stable formulation for a long period of time. Sugars have been reported to act as protective agents during the dehydration/reconstitution of liposomes by preventing

vesicle fusion and enhancing the retention of the encapsulated compounds within the liposomes [43-46]. Because amino acids exhibite similar lypoprotective effect as sugars, L-arginine was assayed regarding the problems for diabetic patients [47]. The application of amino acids or sugars as potential cryoprotectants did not show significant differences, although in both cases, the presence of Chol lead to a marked stability of liposomes during the freeze-drying process. This effect has been previously reported by Popova and Hincha (2007) [48]. They have reported an interaction between phospholipids and sugars due to the presence of Chol. This sterol could increase the lipid space leading the sugars to interact with lipid head-groups. Nevertheless, more studies are required to optimize the use of cryoprotectants in the lyophilisation of liposomes, because the EE of the reconstituted liposomes decreased in 6.1 ± 2.9 %.

The *in-vitro* antitumor activity showed that HCT-116 had a higher sensibility to oxaliplatin compared to HT-29. This result was supported by Kalimutho et al. [49], because they have reported that the status of p-53, wild-type in HCT-116 and mutated in HT-29, could be involved in this phenomenon explaining that difference. In this study, the free oxaliplatin led to a better antiproliferative effect than the encapsulated (Table 4). These results are according to the results reported by several authors regarding the IC_{50} for free drug vs. liposomal formulation [50]. In the case of the cationic formulation, its effect could be explained by the additional cytotoxicity found for empty liposomes discarding these liposomes for further studies. Additionally, the *in- vivo* toxicity of DOTAP liposomes have been reported in the literature [51, 52].

On the other hand, the *in-vivo* study carried out with the HSPC:Chol:DSPE-PEG_{2000} liposomes, showed an efficient antitumor activity in the murine tumor-xenograft model, reflecting the stability and ability of the formulation to reach the tumor area. This result suggests that PEG-coated liposomes could act as a depot of oxaliplatin in the tumor

area, delaying its RES uptake due to the presence of the PEG in the surface of liposomes [13-15]. The antitumor activity for this formulation was more evident *in-vivo* than *in-vitro*, which is according to the results found by other authors [38]. Therefore, PEG-coated liposomes of oxaliplatin developed by the FM, the simplest method, provided a potent antitumor activity compared to the free drug.

In our knowledge, this is the first study where PEG-coated liposomes of oxaliplatin have been developed using several methods, FM, REV and HM to compare the impact of them in the physicochemical parameters of the formulations, including the efficiency of encapsulation. Moreover, the effect of the inclusion of a semi-synthetic lipid and the Chol led to obtain a formulation stable during the incubation at high temperature and the lyophilisation process.

Finally, the *in-vivo* antitumor efficiency was characterized by a reduction followed by a stabilization of the tumor. This effect, together with the oxaliplatin levels found in this organ, suggest a long-time stability of the formulation.

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Legends to figures.

Figure 1. Transmission electron microscope photograph. Left panel shows non-pegylated liposomes (HSPC:Chol (2:1)), whereas right panel shows pegylated-liposomes (HSPC-Chol-PEG_{2000} (2:1:0.2)). Bars represent 100 nm. The negative control is in the low panel.

Figure 2. Time profiles of the tumor growth after two cycles of treatments with free and encapsulated oxaliplatin. Each symbol represent the mean of six animals and the bars the corresponding standard deviation. Oxal-LP, liposomes of oxaliplatin; Oxal-free, oxaliplatin in solution and Empty-LP, liposomes without oxaliplatin. (*P<0.05)

Figure 3. Body weight changes in mice treated with PBS (control), empty liposomes, oxaliplatin liposomes and free drug. The results represent the mean ± SD. (*P <0.05)

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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