

SIMPLE AND FAST PROCEDURE TO INCORPORATE DOXORUBICINE IN SMALL UNILAMELLAR LIPOSOMES: EFFECTS ON LIPOSOME SIZE AND ZETA POTENTIAL

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ABSTRACT. This study aimed to find alternative, cheap and fast procedures to incorporate Doxorubicin in small unilamellar nanoliposomes made of Dipalmitoyl Phosphatidyl Choline and Cholesterol. It was compared the entrapment efficiency of doxorubicin in “home-made” liposomes (1) and in ImmunoSOME liposomes (2), a commercial, expensive formulation. It was obtained an entrapment efficiency of 27.6 % vs 12.1% for formulations (1) vs (2). The liposomes' size and zeta-potential of formulation (1) ranged from 116 and 120 nm, being smaller than in formulation (2). The zeta potential was negative for both formulations and showed a better stability and monodispersibility also for formulation (1). This fast procedure is convenient and assure efficient doxorubicin entrapment in the monodisperse nanoliposomes. Such simple formulations, easier to be obtained, can offer also good quality/price ratios and can be produced at larger scale, with higher incorporation efficiency for experimental *in vivo* studies which aims the targeted delivery of Doxorubicin to cancer tumors in animals.

Keywords: *Liposomes, Doxorubicin encapsulation, size and zeta potential*

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INTRODUCTION

Anthracyclines such as doxorubicin (DOXO), epirubicin and daunorubicin are among the most active cytotoxic agents, a highly potent chemotherapeutic agent for the treatment of a wide variety of solid tumors and hematological malignancies. Since the secondary effects associated with their chronic administration induce cardiomyopathy and congestive heart failure, new, less toxic formulations (pegylated or nonpegylated liposomal formulations) demonstrated the superiority of liposomal doxorubicin in comparison to conventional non-liposomal doxorubicin. Therefore, liposomal formulations with entrapped DOXO are currently the best-known alternatives to improve the index and spectrum of anticancer activity and decrease cardiotoxicity [1].

Liposomes are prepared, characterized and used since decades as potent drug delivery carriers, being largely characterized for their physico-chemical properties, highly dependent on their qualitative and quantitative phospholipid composition, pH, size [2-5]. Mostly used is Dipalmitoyl Phosphatidyl Choline (DPPC) in combination with other types of phospholipids and in different ratios with cholesterol, which confers stability and modulate membrane fluidity [6, 7]

It is well documented, that drug delivery using carriers like liposomes provides several advantages, e.g. increased solubility, improved tumor targeting, enhanced accumulation in tumor tissues and cells, decreased systemic toxicity and increased maximum tolerated dosage [8, 9].

Nowadays, new formulations containing doxorubicin encapsulated in liposomes are available and produced, at high prices, by some specialized companies. For example, Doxosome is a DOXO-entrapped liposomal suspension and Doxil, commercially known as Caelyx, is a Polyethyleneglycol-coated liposomal formulation including DOXO, used for the treatment of ovarian cancer, sarcoma and multiple myeloma. In spite of showing reduced DOXO-related toxicity, Doxil showed a lower clinical efficiency, since the PEGylated liposomal doxorubicin was sequestered in the cellular lysosomes, with a lower bioavailability of DOXO [10]. Another comparative study of the antitumor activity of free DOXO and Doxil in mouse lymphoma models concluded contrasting effects, e.g. free DOXO was more efficient by i.p administration while Doxil was superior by i.v. administration [11]. Another commercial formulation is ImmunoSOME, a surface reactive product without DOXO, which include PEG-conjugated liposomes linked to biotin. This product offers possibilities to link avidine-conjugated microbubble and target tumors by ultrasounds (US), recommending DOXO-liposomes as good nanotheranostic agents [12].

In this context, our experimental study aimed to find a fast and cheap procedure to incorporate DOXO in small unilamellar liposomes (SUV) as simple formulations made of Dipalmitoyl Phosphatidyl Choline (DPPC) and Cholesterol (CHOL) (1). Comparatively, ImmunoSOME, an expensive commercial PEGylated liposomal suspension was used to incorporate similar quantities of DOXO (2). The results were monitored by measuring the DOXO entrapment efficiency (EE), the liposomes' size and zeta-potentials, after DOXO incorporation in both formulations (1) and (2).

RESULTS AND DISCUSSION

Evaluation of DOXO entrapment efficiency

The influence of liposomal lipid composition on liposome size, zeta potential and liposome-induced dendritic cell maturation using a design of experiments approach was recently reported [13]. Another novel approach on drug delivery refers to the key-role of the lipid composition and nano-formulation of liposomal DOXO in relation to the biological evaluation of entrapped DOXO [14]. The antiproliferative long-circulating liposomes co-encapsulating DOXO and curcumin, through the use of a quality-by-design approach was also reported, recently. Six parameters, namely the phospholipid, curcumin and DOXO concentrations, the phospholipid: cholesterol molar ratios, the temperature during evaporation, buffer's pH and hydration steps were identified as potential risk factors for the quality of the final product. The influence of these variables was monitored by the particle size, zeta potential, drug loading and entrapment efficiency [15].

To intensify the accumulation of DOXO inside SUV vs ImmunoSOME liposomes, we tried either the classical procedure using only Hepes buffer at pH 7.4 (a) or a combination of Hepes buffer and gradients of ammonium sulfate to obtain a more 'active' loading of amphipathic weak bases into the aqueous compartment of liposomes (b), approach being applied previously to encapsulate anthracyclines inside the liposomes at very high efficiency [16]. The results were superior in our case using procedure (b) and are presented in Table 1 which includes the comparative values (mg of DOXO added to liposomal suspension (DOXO_{added}), DOXO found in the supernatant (DOXO_{out}), found in the pellet (DOXO_{in}, calculated as the difference DOXO_{added} - DOXO_{out} (column 4) or by direct determination after the dissolution of the pellet (DOXO_{in}* - column 5). The encapsulation efficiency (EE) was calculated as a ratio DOXO_{in}*/DOXO_{added}.

Table 1. The encapsulation efficiency (EE) of DOXO inside liposomes, taking into account the DOXO before and after encapsulation.
For sample abbreviations see the Experimental Section.

Sample	DOXO added (mg)	DOXOout (mg)	DOXOin (calculated) (mg)	DOXOin* (determined) (mg)	EE (%)
L1 (SUV)	1	0.73	0.27	0.276	27.60
L2 (SUV)	1.4	1.31	0.09	0.068	4.85
L3(ImmunoSOME)	1.4	1.28	0.12	0.110	7.85
L4(ImmunoSOME)	1	0.86	0.14	0.121	12.1
L5(ImmunoSOME)	1.4	1.24	0.16	0.125	8.92
L6 (SUV)	1.4	0.89	0.51	0.375	26.78

Significant differences were observed between the six samples: The SUV liposomes (L1 and L6) showed higher incorporation yields than ImmunoSOME. When sucrose was added, the incorporation decreased significantly (L2<L1 and L3<L5). No significant differences between EE values were observed when DOXO was added as 1 or 1.4 mg (see L1 vs L6), suggesting that the liposomal membrane structure is mainly responsible for the limitations of DOXO incorporation.

The more complex composition of ImmunoSOME membrane determined a limited incorporation of DOXO, at around 50% comparing to SUV liposomes (L4 vs L1).

Liposomes Size and Polydispersibility index

Fig. 1 shows the values of liposome sizes for samples L1 (SUV-DOXO liposomes), comparative to L3, L4, L5 (ImmunoSOME-DOXO liposomes).

The DOXO-SUV liposomes from samples L1 and L6 had similar sizes, of 116 and 120 nm, respectively. Also, their polydispersity index (PDI) is good (0.162-0.170), with values less than 0.2, suggesting a good monodispersion of liposomes. For samples containing DOXO- ImmunoSOME liposomes (L3, L4 and L5) the sizes ranged from 148 to 163 nm (larger size), and PDI values from 0.119 to 0.214 (still monodisperse).

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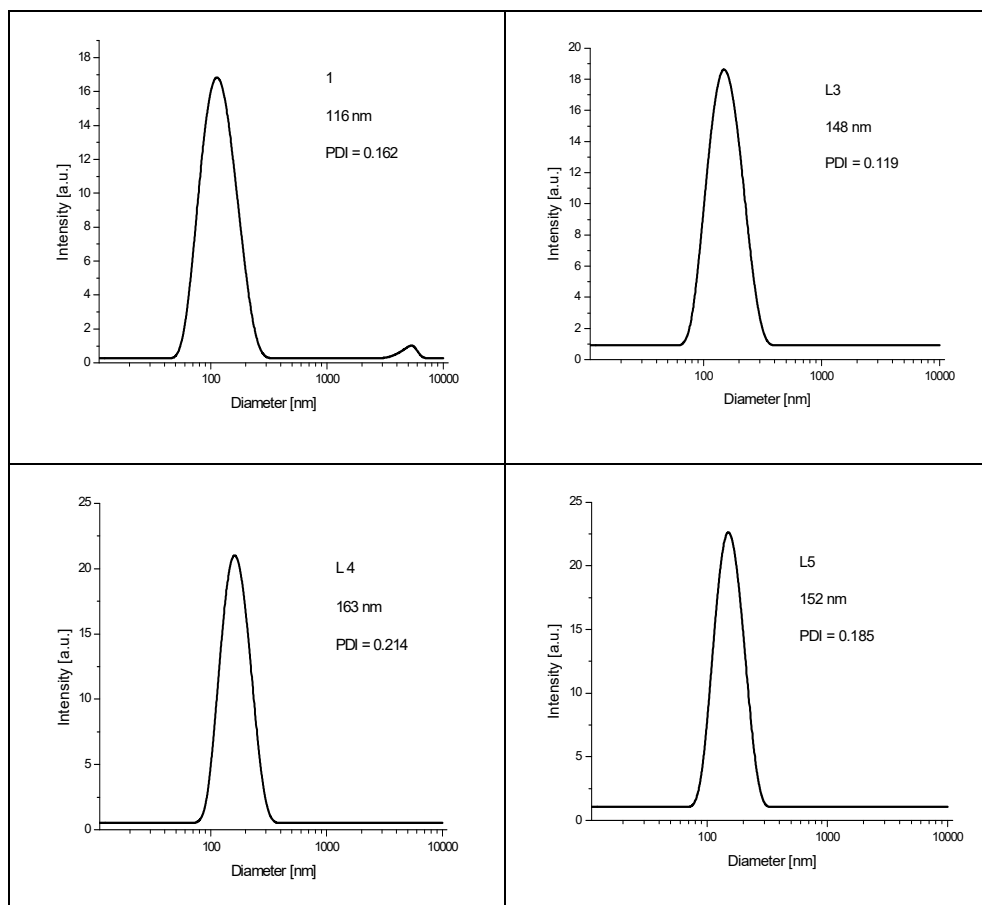


Figure 1. The liposome size for samples L1 (SUV-DOXO liposomes), comparative to L3,L4, L5 (ImmunoSOME-DOXO liposomes)

Zeta-potential of DOXO liposomes

The measurements of zeta potential of the six samples (L1 and L6 containing DOXO-SUV liposomes and L3-L5 containing DOXO-ImmunoSOME liposomes) showed negative values in all cases, as shown in Fig.2.

The zeta potential of the SUV-DOXO and ImmunoSOME-DOXO liposomes was negative (ranging from -26 to -48mV), due to their charge and pH of the buffer, The higher values were noticed for L1 (-48 mV) suggesting the best stability, followed by L2 and L6 (-30 and -26mV, respectively). The samples L3-L5 corresponding to DOXO-ImmunoSOME liposomes had closer values, from -27 to -29 mV.

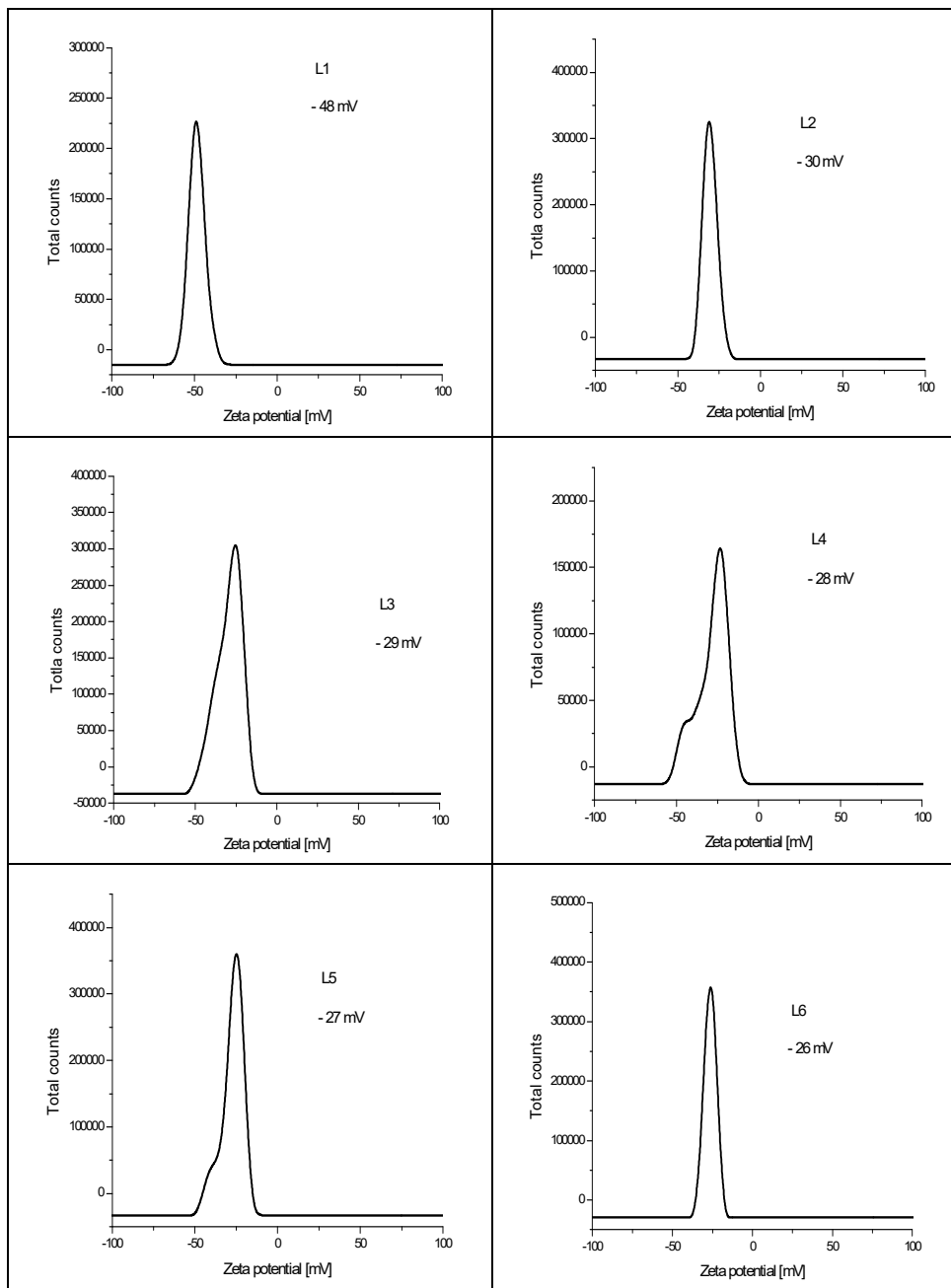


Figure 2. The values of zeta potential for the six samples (L1 to L6).
For abbreviations see the Experimental Section Table 2.

The significance of the zeta potential is related to the short- and long-term stability of emulsions and liposomal suspensions. Zeta potential becomes more negative when pH increases and is a measure of magnitude of charges on liposomal vesicles. Recently such assumptions were demonstrated for DPPC and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) liposomes investigated in 1 mM NaCl (pH 6.2) and phosphate buffer (pH 8.1) in relation to the liposome size, polydispersity index, and zeta potentials [17]. The higher the value of the zeta potential is (positive or negative), the more stable the colloid dispersion is. Normally, a value of > 30 mV indicates good stability [17]. Our data are in agreement with recent reports which demonstrated the superiority of liposomal doxorubicin in comparison to conventional non-liposomal doxorubicin [18], especially in SUV formulations with high zeta potentials.

Considering these data, sample L1 (SUV-DOXO liposomes) showed best stability and dispersibility. The ImmunoSOME-DOXO samples had inferior properties comparing to SUV-DOXO.

CONCLUSION

The results of this study show that simple and fast procedures can be applied to incorporate DOXO in “home-made” SUV-unilamellar nanoliposomes containing cheap and simple lipid composition. We have shown that entrapment efficiency of DOXO into SUV-liposomes (L1) was higher than into ImmunoSOME liposomes (L4), having values of 27.6% vs 12.1% respectively, when the same initial DOXO quantity was added.

The addition of sucrose decreased significantly the entrapment efficiency, but no significant differences.

Meanwhile, no significant differences between the entrapment efficiency were observed when DOXO was added as 1 or 1.4 mg, suggesting that the liposomal membrane structure is mainly responsible for the limitations of DOXO incorporation.

The more complex composition of ImmunoSOME membrane may explain the lower incorporation rate of DOXO, at around 50% comparing to SUV liposomes. This is an expensive commercial liposomal formulation, using the transmembrane ammonium ion gradient procedure and its complex structure seems to hinder the DOXO incorporation.

Considering the liposomes' size and the zeta potential, SUV-DOXO liposomes showed best stability and dispersibility and is recommended to be obtained at larger scale by this simple, cheap and fast procedure.

Finally, we consider this simple and fast procedure to prepare SUV-DOXO liposomes can be upgraded, in order to obtain higher quantities of this liposomal suspension. Such simple formulations with high entrapment efficiency of DOXO may offer good quality/price ratios and can be produced at larger scale for experimental studies *in vivo*, for a targeted delivery to cancer tumors in small animals.

EXPERIMENTAL SECTION

Chemicals, reagents and materials

1,2-dipalmitoyl sn-glycero-3-phosphatidylcholine (DPPC, MW=734), Cholesterol (Roth puritate 99%, (CHO L, MW=386), Doxorubicin clorhydrate (DOXO) 2 mg/ml saline solution, ImmunoSOME (5 ml, Encapsula Nanosciences) a mixture of HSPC:CHOL:DSPE-PEG: DSPE-PEG-Biotin (55:40:4:1) representing 5.96 mg lipids/ml in 100 nm liposomes. Buffer HEPES 10mM (pH 7.4), buffer Ammonium Sulphate pH 5.5 (Sigma-Aldrich).

Instrumentation

BENACOR rotavapor with automatic regulation of vacuum, temperature and rotation, ultrasonic generator UP200St-G (Hielscher Ultrasonics GmbH) 200W, 26kHz with an S26d2 sonotrode of 2 mm and ultrasonic bath, thermostate with recirculated water, extrusion system of LiposoFast-50, criocentrifuge Hettich 460R, Eppendorf centrifuge (max. 15000 rpm), Cell Media mixer. The spectrophotometric measurements were made on a Perkin Elmer UV WinLab Spectrophotometer (200-700 nm at 1 nm/min).

Preparation of Unilamellar liposomes

To a mixture of 375 mg lecithin and 125 mg cholesterol, in a molar ratio 61.5:38.5 it was added a solvent mixture of chloroform:methanol 2:1 (v:v), in a glass round bottom flask of 50 ml. The solvent was evaporated under vacuum in the Rotavapor at 43°C, for 30 min. The lipid layer was then dehydrated in an exicator containing anhydrous Calcium chloride, during 48 hrs. The lipid layer was suspended in 20 ml (NH₄)₂ SO₄ 300 mM, pH 5.5, and strongly vortexed. The liposome suspension was taken from the flask and centrifuged at 4°C, 4600 rpm, 20 min. The upper, opalescent phase containing multilamellar liposomes was retained and extruded successively through

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polycarbonate filters of 400 and 100 nm, using the Lipofast system. The extruded suspension contained small unilamellar liposomes (SUV), whose size was determined, as mentioned below.

Incorporation of DOXO in SUV and ImmunoSOME liposomes

The incorporation of DOXO in unilamellar liposomes was realized by the transmembrane ammonium ion gradient procedure, as follows: under mix in the ultrasonic bath at 65°C, 30 min, to a volume of 0.3 ml liposome suspensions (SUV or ImmunoSOME) there were added 0.5 or 0.7ml DOXO and 0.5 ml Hepes 10 mM pH 7.4. as mentioned in Table 2. The mixture was maintained 10 min at 4°C in the refrigerator, then a volume of 0.8 Ammonium sulphate 300 mM pH 5.5 was added to the suspension, kept 10 min at room temperature and centrifuged at 15000 rpm, 10 min. The supernatant was separated and the DOXOout (the non-incorporated DOXO) was determined by UV-Vis spectrometry. The pellet was washed with Hepes buffer two times and then re-suspended in 0.7 ml Hepes pH 7.4, mixed for 15 min at 45°C.

Fig. 3 reflect the differences between the two types of liposomes used for DOXO entrapment, as well the mechanisms behind the DOXO loading using a transmembrane ammonium ion gradient in the unilamellar liposomes.

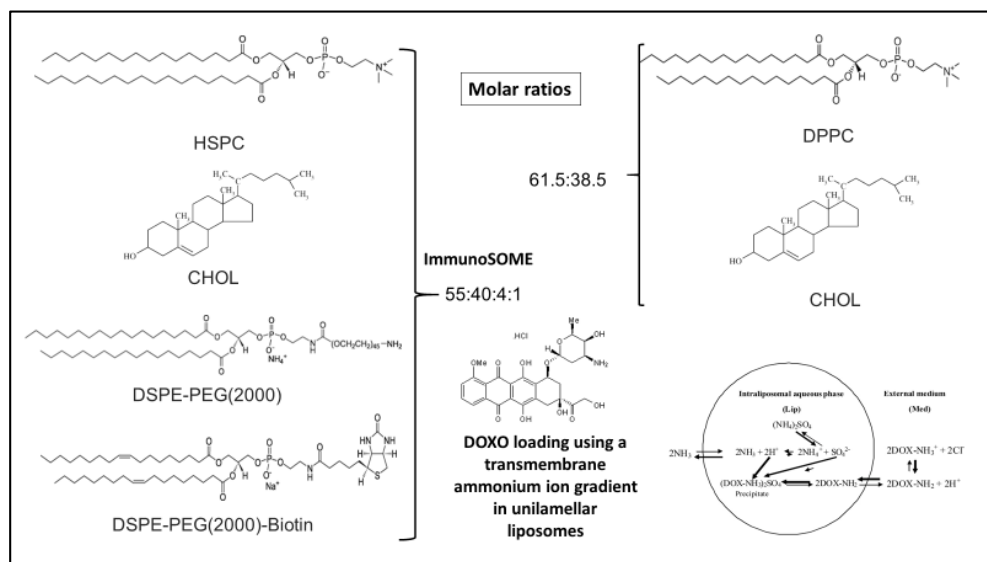


Figure 3. The composition of the SUV and ImmunoSOME liposomes used for DOXO entrapment and the mechanisms behind the DOXO loading using a transmembrane ammonium ion gradient.

Table 2 represents the volumes of liposome suspensions (SUV and ImmunoSOME) and Hepes buffer, with or without addition of sucrose, used to incorporate DOXO (volumes of 0.5 or 0.7 ml representing 1 and 1.4 mg, respectively).

Table 2. Incorporation of DOXO (using 0.5 or 0.7 ml from a solution of 2 mg/ml) into SUV or ImmunoSOME liposomes, in 0.5 ml Hepes buffer 10 mM (pH 7.4), with or without sucrose 10%.

Abbreviation	Liposome type and volume	DOXO (ml)	+/- sucrose 10% (ml)
L1	0.3 ml SUV	0.5	-
L2	0.3 ml SUV	0.7	2 ml sucrose
L3	0.3 ml ImmunoSOME	0.7	2 ml sucrose
L4	0.3 ml ImmunoSOME	0.5	-
L5	0.3 ml ImmunoSOME	0.7	-
L6	0.3 ml SUV	0.7	-

After the incorporation procedure, a volume of 0.5 ml from each final suspension was taken to determine the liposomes size and zeta potential > concomitantly, to 0.2 ml suspension, a volume of 0.1 ml methanol and 0.8 ml Hepes buffer was added and the absorption intensity was recorded at 480 nm, in order to evaluate the DOXO incorporated in liposomes (DOXOin). In parallel, the empty liposomes were dissolved, and considered as blank.

Evaluation of DOXO entrapped in SUV or ImmunoSOME liposomes

First, a calibration curve was built, using different dilutions of the initial DOXO solution (2 mg/ml), the curve factor ($f = c/A_{480nm}$) having the value $f=0.133$.

The formula used to calculate the quantity of DOXOin and DOXOout was the following:

$$\text{DOXO (mg)} = f \times \text{dilution factor} \times \text{total volume of the solution collected (from supernatant or dissolved liposomes containing DOXO)}.$$

Evaluation of liposomes' size and zeta potential

The liposome hydrodynamic diameter and polydispersity index (PDI) were determined at 25 °C by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS-90 (Malvern Instruments) with a He-Ne laser operating at a wavelength of 633 nm and an avalanche photodiode detector. The zeta

potential of the liposomes was determined also at 25 °C by the laser Doppler microelectrophoresis technique using the same Malvern equipment. Before measurements, the samples were diluted 5 fold with ultrapure water.

ACKNOWLEDGMENTS

This study was supported by the Grant nr. 4945/12/2016 funded by the University of Medicine and Pharmacy "Iuliu Hațieganu" Cluj-Napoca. The authors would like to thank Prof. Dr. Simion Aștilean and Dr. Monica Potara (Interdisciplinary Research Institute in Bio-Nano-Sciences, University "Babeș-Bolyai") for their kind support and help related to the measurements of liposomes' size and zeta potential.

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