

# Effects of Lipid Composition and Preparation Conditions on Physical-Chemical Properties, Technological Parameters and *In Vitro* Biological Activity of Gemcitabine-Loaded Liposomes

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**Abstract:** The effects of lipid composition and preparation conditions on the physicochemical and technological properties of gemcitabine-loaded liposomes, as well as the *in vitro* anti-tumoral activity of various liposome formulations were investigated. Three liposome formulations were investigated: DPPC/Chol/Oleic acid (8:3:1 molar ratio, liposomes A), DPPC/Chol/DPPE (6:3:1 molar ratio, liposomes B) and DPPC/Chol/DSPE-MPEG (6:3:1 molar ratio, liposomes C). Multilamellar liposomes were prepared by using the TLE, FAT and DRV methods, while small unilamellar liposomes were obtained by extrusion through polycarbonate filters. Light scattering techniques were used to characterize liposome formulations. Loading capacity and release profiles of gemcitabine from various liposome formulations were also investigated. Caco-2 cells were used to evaluate *in vitro* the antitumoral activity of gemcitabine-loaded liposomes with respect to the free drug and also the intracellular drug uptake. Preparation methods and liposome lipid composition influenced both physicochemical parameters and drug delivery features. Liposomes with a size ranging from 200 nm to 7 µm were obtained. The gemcitabine entrapment was higher than that expected probably due to an interaction with the liposome lipid components. The following decreasing loading capacity order was observed: liposome B>liposome C>liposome A. Gemcitabine release from various liposome formulations is modulated by two different processes, i.e. desorption from and permeation through liposomal bilayers. MTT assay showed a greater cytotoxic effect of gemcitabine-loaded liposomes with respect to the free drug. The following decreasing anticancer activity order was observed between the various liposome formulations: liposome C>liposome A>liposome B. The increased anticancer activity is correlated to the ability of the colloidal carrier to increase the intracellular drug uptake. Due to the encouraging results and to the high liposome modularity various applications of potential therapeutic relevance can be envisaged for liposomes.

**Keywords:** Liposomes, Caco-2 cells, gemcitabine, MTT assay, drug release, *in vitro* anticancer activity, intracellular drug uptake.

## 1. INTRODUCTION

Current pharmacological therapy presents a number of problems related to body distribution and stability of drugs in the blood stream. This situation can modify the therapeutic index of drugs, by reducing the interaction with target sites and prompting side effects. A strategic approach to overcome these problems, at least in part, is based on the improvement of the selectivity and specificity of drugs by using advanced drug delivery systems [1,2]. In this context, liposomes are suitable drug carrier systems for therapeutic applications.

Liposomes have become suitable drug delivery devices for the treatment of various diseases, i.e. fungal, microbial and viral infections [3-5], tumors, enzymatic deficits and genetic pathologies [6-8]. The use of liposomes as drug carriers is mainly due to their versatility being able to encapsulate drugs with different physicochemical properties [9-11] and to modulate the biopharmaceutical features of these drugs. Liposome features are strictly related to chemical properties of the phospholipids used for their preparation. In fact, lipids can modify biodistribution, surface charge, permeability, and release and clearance of liposomal drug delivery [12,13].

Liposome versatility can be of particular interest for the therapeutic treatment of various cancer diseases where different requirements are to be fulfilled as a function of the cancer type. In particular, pH-sensitive liposomes containing

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unsaturated fatty acids, i.e. oleic acid or linoleic acid, can be used both to obtain fusogenic vesicles at low pH values ( $\leq 6.5$ ) [14,15] thus improving the intracellular drug entrance and increasing the percutaneous drug passage [16] when an anticancer topical treatment is possible [17]. While, the presence of polyethylene glycol moieties on the surface of liposomes provides long circulating properties [18], improved stability [19], drug defence from metabolic degradation/inactivation [20] and increased intracellular uptake [21,10].

Gemcitabine is a nucleotide analogue that exerts its activity through the inhibition of DNA synthesis [22] and it is active against different solid carcinomas [23,24] and is used in this paper as a model of a hydrophilic antitumoral drug. In a preliminary investigation, we reported that liposomes can improve *in vitro* the antitumoral activity of gemcitabine [7]. Therefore, in this paper we investigated the effects of lipid composition and preparation conditions on the physicochemical and technological properties of liposomes, as well as on the *in vitro* antitumoral activity of various gemcitabine-loaded liposomes. The cytotoxic effect of free or liposomally entrapped gemcitabine was assayed on a colon carcinoma cell line (Caco-2). The intracellular uptake of gemcitabine within Caco-2 cells was also investigated as a function of the lipid composition of liposomes.

## 2. MATERIALS AND METHODS

### 2.1. Materials

1,2-dipalmitoyl-sn-glycero-3-phosphocholine monohydrate (DPPC), N-(carbonyl-methoxypolyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-MPEG 2000) and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylserine (DPPS) were obtained from Genzyme (Suffolk, UK). Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS), penicillin (100 UI/ml) and streptomycin (100  $\mu$ g/ml) were obtained from GIBCO (Invitrogen Corporation, Giuliano Milanese (Mi), Italy). Phosphate buffer saline solution (PBS), tris-(hydroxymethyl)-aminomethane hydrochloride buffer (Tris-HCl buffer), sodium dodecyl sulphate (SDS), amphotericin B (250  $\mu$ g/ml), [4,5-dimethylthiazol-2-yl]-3,5-diphenyl-tetrazolium bromide (MTT) (TLC purity  $\geq 97.5$  %), cholesterol (Chol), oleic acid (AO), ammonium sulphate and dimethyl sulphoxide (HPLC analytical grade) were purchased from Sigma Chemicals Co. (St. Louis, USA). Double-distilled pyrogen-free water was purchased from Sifra S.p.A. (Verona, Italia). Sterile saline was the product of Frekenius Kabi Potenza S.r.l. (Verona, Italia). Gemcitabine (2,2'-

difluorodeoxycytidine) hydrochloride (HPLC purity  $>99\%$ ) was a gift of Eli-Lilly Italia S.p.A. (Sesto Fiorentino, Firenze, Italy) and was used without further purification. Colon carcinoma (Caco-2) cells were purchased from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertyni" (Brescia, Italy). BCA™ protein assay kit was purchased from Pierce (Rockford, IL, USA). All other materials used in this investigation were of analytical grade (Carlo Erba, Milan, Italy).

### 2.2. Preparation of liposome formulations

Various liposomal formulations were obtained by carrying out the following preparation methods: TLE (thin layer evaporation), FAT-MLVs (frozen and thawed multilamellar vesicles), DRVVs (dehydrated-rehydrated vesicles) and SUVET (small unilamellar vesicle by extrusion technique). Three different lipid mixtures were used for the liposome formulations (Table 1).

#### 2.2.1. TLE Method

Liposome formulations were prepared in a round-bottomed flask by dissolving 20 mg of the different lipid mixtures with 2 ml of chloroform-methanol (3:1 v/v). A thin layer lipid film was obtained by evaporating the organic solvent with a Heidolph-Laborota Digital 4010 rotary evaporator under a slow nitrogen flux. Any trace of residual organic solvent was removed over night at 30 °C by using a Büchi T51 glass oven drier connected to a high-vacuum pump. Lipid films were mixed and hydrated with 100  $\mu$ l of an isotonic solution of gemcitabine hydrochloride (50 mg/ml). Multilamellar liposomes were achieved by submitting the lipid/aqueous phase mixtures to three alternate cycles (3 min each) of warming at 50 °C (thermostated water bath) and vortexing at 700 rpm.

#### 2.2.2. FAT-MLVs Method

To achieve a homogeneous distribution of solutes and to improve gemcitabine loading within vesicles, MLVs obtained with the TLE method were submitted to ten cycles of freezing in liquid nitrogen at -180 °C and thawing in a water bath at 40 °C. The liposome suspensions obtained were kept at room temperature for 3 h to anneal the liposomal structure.

#### 2.2.3. DRV Method

Two different procedures were carried out in the DRV method. In the first procedure, empty liposomes obtained with the TLE method by using double-distilled water were freeze-dried with an Edwards Modulyo lyophilizer connected to an Edwards high vacuum pump mod. 8 (Edwards

**Table 1. Lipid Composition of Various Liposome Formulations**

Formulation	Lipid Composition (molar ratio)				
	DPPC	Chol	DSPE-MPEG	DPPS	OA
Liposome A	8	3	—	—	1
Liposome B	6	3	—	1	—
Liposome C	6	3	1	—	—

Scientific Instrument, Ringoes, NJ, USA) for 24 h. Freeze-dried lipids were rehydrated with 1 ml of a solution made up of 100  $\mu$ l of an isotonic solution of gemcitabine hydrochloride (50 mg/ml) and 900  $\mu$ l of sterile isotonic saline solution under continuous stirring (1000 rpm) at room temperature.

In the second procedure, gemcitabine containing liposomes obtained by the FAT-MLV method were dehydrated as reported above and then were rehydrated with double-distilled water (1 ml).

#### 2.2.4. pH Gradient Method

A pH gradient method [25,26] was carried out to improve the liposome drug entrapment. An acid environment has to be created in the intra-liposomal aqueous compartments. Briefly, multilamellar liposomes were prepared following the TLE method by hydrating lipid films with a 250 mM ammonium sulphate solution (1 ml). MLVs were submitted to the FAT-MLV method. The untrapped ammonium sulphate solution was then removed by centrifugation at 14000  $\times$  g at 4  $^{\circ}$ C for 1 h using a Beckman Avanti<sup>TM</sup> 30 centrifuge equipped with an F1202 fixed angle rotor (Beckman Coulter Inc., Fullerton, CA). The pellet was resuspended in an isotonic solution (1 ml) of gemcitabine hydrochloride (50 mg/ml) and kept at room temperature for 3h. Liposomes thus obtained were submitted to DRV and SUVET techniques.

#### 2.2.5. SUVET Method

Liposome sizing was carried out by extruding vesicle suspensions through two stacked 400 nm, 200 nm and then 100 nm pore size polycarbonate filters (Whatman Inc., Clifton, NJ, USA) (ten passages each) [27,28]. A stainless steel extrusion device (Lipex Biomembranes, Northern Lipids Inc., Vancouver, Canada) equipped with a 10 ml water-jacket "thermobarrel" connected to a GR 150 thermostat system (Grant Instruments Ltd, Cambridge, UK), was used for the extrusion. The working pressure was 425 kPa for 400 nm, 850 kPa for 200 nm and 1700 kPa for 100 nm pore size filters. Unilamellar liposome suspensions were obtained.

### 2.3. Liposome Loading Capacity

The determination of the loading capacity of various liposome formulations was carried out by removing the untrapped gemcitabine by means of a Beckman Avanti<sup>TM</sup> 30 Centrifuge (20000  $\times$  g for 1 h at 4  $^{\circ}$ C). The unloaded gemcitabine in the supernatant was determined spectrophotometrically at  $\lambda_{\max}$  268.8 nm by using a Perkin Elmer Lambda 20 UV-Vis spectrophotometer using Perkin Elmer UV WinLab<sup>TM</sup> 2.8 acquisition software (Perkin-Elmer GmbH Uberlingen, Germany). The following gemcitabine calibration curve was used:

$$\text{Eq. 1} \quad y = 0.6958 \times 10^{-3} + 0.3971x$$

where  $y$  is the absorbance at 268.8 nm and  $x$  is the drug concentration ( $\mu$ M),  $r^2$  value was 0.9993. The amount of drug encapsulated is expressed both as encapsulation yield ( $EY$ ) and encapsulation capacity ( $EC$ ).  $EY$  values were calculated using the following equation:

$$\text{Eq. 2} \quad EY = [(D_t - D_u)/D_t] \times 100$$

where  $D_t$  is the total amount of the drug used for liposome preparation and  $D_u$  is the amount of untrapped drug.

Whereas,  $EC$  values were calculated using the following equation:

$$\text{Eq. 3} \quad EC = [D_e]/[D_a] \times [L]$$

where  $[D_e]$  is the concentration of the encapsulated drug,  $[D_a]$  is the concentration of the drug added during liposome preparation and  $[L]$  is the total concentration of lipids used for liposome preparation [29]. Concentrations are expressed as  $\mu$ moles/ $\mu$ l.

Gemcitabine loading capacity was also evaluated by gel permeation chromatography. The instrument used was an Äkta Prime Plus (Amersham Biosciences, Uppsala, Sweden) equipped with a Sephadex G25 column (Amersham Biosciences, Uppsala, Sweden). Sterile saline solution filtered through 0.2  $\mu$ m pore membranes was used as the eluent with a flux of 0.2 ml/min.

### 2.4. Physicochemical Characterization of Liposomes

Mean size of multilamellar liposomes were evaluated by using an optical instrument (TurbiscanLab, Formulacion, France) which moved up and down along a flat-bottomed cylindrical cell [30]. An amount of liposome suspension (5 ml) was placed in a cylindrical glass tube and then analyzed. The detection head was made up of a pulsed near-infrared light source ( $\lambda = 850$  nm) and two synchronous transmission (T) and backscattering (BS) detectors. The detection head scanned the full height of the sample ( $\sim 50$  mm), acquiring T and BS data at intervals of 40  $\mu$ m every minute for (3 h). Backscattering photons were detected at 135 $^{\circ}$  from the incident beam.

Mean size, size distribution and zeta potential of reduced-size liposomes were measured by using photon correlation spectroscopy (Zetamaster, Malvern Instruments Ltd., Sparing Lane South, Worcester Shine, England). The instrument was equipped with a 4.5 mW laser diode operating at 670 nm as a light source. Scattered photons were detected at 90 $^{\circ}$ . Thirty measurements were carried out for each sample. Liposome mean size and polydispersity index values were achieved by applying a third-order cumulant fitting autocorrelation function. The instrumental parameters were set as follow: real refractive index 1.59, imaginary refractive index 0.0, medium refractive index 1.330, medium viscosity 1.0 mPa $\cdot$ s and medium dielectric constant 80.4. Quartz cuvettes were used for the analysis. Multiscattering phenomena were avoided by a suitable dilution with the liposome dispersion medium filtered through 200 nm pore size membranes (Whatman Inc., Clifton, NJ, USA).

Zeta potential values were determined with a Zetamaster particle electrophoresis analyzer equipped with a 5 mW He-Ne laser operating at 633 nm. Also in this case, a suitable dilution of liposomes was carried out. A Smoluchowsky constant ( $K_a$ ) with a nominal value of 1.5 was used to calculate the zeta potential value from the electrophoretic mobility.

### 2.5. Caco-2 Cell Culture

The cytotoxic effect of free and liposomally entrapped gemcitabine was assayed on Caco-2 cells. Cells were transferred from cryovials into 100 mm plastic culture dishes and

incubated (5% CO<sub>2</sub>) for seven days at 37 °C using DMEM supplemented with 5 ml penicillin (100 UI/ml), streptomycin (100 µg/ml), 5 ml amphotericin B (250 µg/ml) and 50 ml FBS. After three days of incubation, the culture medium was replaced with fresh medium (8 ml). The 7<sup>th</sup> day cells were treated with trypsin-EDTA solution (200 µl) and the content was divided into four culture dishes and incubated up to a confluence of 70 %. Cells were then treated with trypsin-EDTA solution (200 µl) and transferred into 10 ml plastic centrifuge tubes containing culture medium (4 ml). Tubes were centrifuged with a Megafuge 1.0 (Heraeus Sepatech Centrifuge) at 1200 rpm for 10 min at 24 °C. Pellets were resuspended in 10 ml of culture medium and 5×10<sup>3</sup> cells/100 µl were seeded into 96-well tissue culture plates and then subjected to the MTT test.

## 2.6. Caco-2 Cellular Viability

The MTT test was used to evaluate the cellular viability so as to determine the cytotoxic effect of free and liposomally entrapped gemcitabine on Caco-2 cells. Cell viability was evaluated by determining the amount of the coloured formazan crystals formed during the biological test. Cells were incubated (5 % CO<sub>2</sub>) for 24 h at 37 °C, thus allowing cell adhesion to the culture plates. Cells were then treated (100 µl) with different concentrations of free or liposomally entrapped gemcitabine. Cells were incubated for 48 h. After incubation, 10 µl of MTT (5 mg/ml dissolved in PBS solution) were added to each well and incubated for 3 h. Supernatants of wells were removed after 3 h and 200 µl of a dimethyl sulfoxide/ethanol solution (1:1 v/v) were added to dissolve the coloured formazan crystals. 96-well plates were gently shaken at 230 rpm (IKA® KS 130 Control, IKA® WERKE GMBH & Co, Staufen, Germany) for 20 min. Absorbance of various samples was measured with an ELISA microplate reader (Labsystems mod. Multiskan MS, Midland, ON, Canada) at 570 nm in absorbance and 670 nm in emission. The percentage of cell viability was calculated according to the following equation:

$$\text{Eq. 4} \quad \text{cell viability} = \text{Abs}_T / \text{Abs}_C \times 100$$

where *Abs<sub>T</sub>* represented the absorbance of treated cells and *Abs<sub>C</sub>* the absorbance of control (untreated) cells. Cell viability was the mean of nine different investigations ± standard deviation.

## 2.7. Intracellular Uptake of Gemcitabine in Caco-2 cells

Caco-2 were seeded (2.1×10<sup>6</sup> cells/ml) in 12 wells plastic culture dishes and incubated with free or liposomally entrapped gemcitabine at a final drug concentration of 10 µM. At different times, Caco-2 cells were washed twice with 1 ml of PBS, scraped from the wells, transferred into 10 ml plastic centrifuge tubes and centrifuged with a Megafuge 1.0 (Heraeus Sepatech Centrifuge) at 1200 rpm for 10 min at 22 °C. Supernatants were withdrawn and the pellets were resuspended in double-distilled water (1 ml). Cells were disrupted by sonication (SONOPOLUS GM 70, Bandelin Electronic, Berlin, Germany) at 50 cycle/s for 3 min and the cellular proteic components were separated using a pre-heated (80 °C) denaturing buffer made up of 1M pH 8 Tris-HCl buffer (700 µl), SDS 2% v/v (157 µl) and a protease inhibitor buffer (134 µl). The intracellular protein concentration was deter-

mined using a BCA™ Protein Assay Kit according to the manufacturer's instructions. Drug intracellular uptake in Caco-2 cells was determined by HPLC.

The HPLC apparatus was a Hewlett Packard 1050 instrument with a 20 µl loop. The analytical column (5 µM, 250 by 4 mm i.d.) was a reverse-phase C18 Lichrospher® 100 (Hewlett-Packard, Milan, Italy) equipped with a RP-18 Lichrospher® 100 guard column (5 µM, 4 by 4 mm i.d.). Chromatographic separation was carried out at room temperature. A nitrile acetate/water (2:98 v/v) mixture was used as the mobile phase, that was filtered through 0.22 µm pore size nylon membranes (Whatman Inc., Clifton, NJ, USA) before use. The flow rate was 1 ml/min. Chromatographic determination was carried out at 272 nm. A 2 % (w/v) zinc sulphate solution (1.4 ml) in a methanol/water (30:70 v/v) mixture, containing 50 µg/ml of caffeine as internal standard, was added to each sample. This mixture was vortex-mixed for 5 min, centrifuged at 6000 rpm for 15 min with a Megafuge 1.0, the supernatant filtered through a 0.22 µm pore size nylon membrane and then submitted to HPLC analysis. The gemcitabine recovery from Caco-2 cells spiked with a known amount of the drug was 99.67 ± 0.98 % (S.D.). Untreated Caco-2 cells were used as control samples and no interference in the HPLC chromatogram due to cellular components was observed.

## 2.8. Statistical Analysis

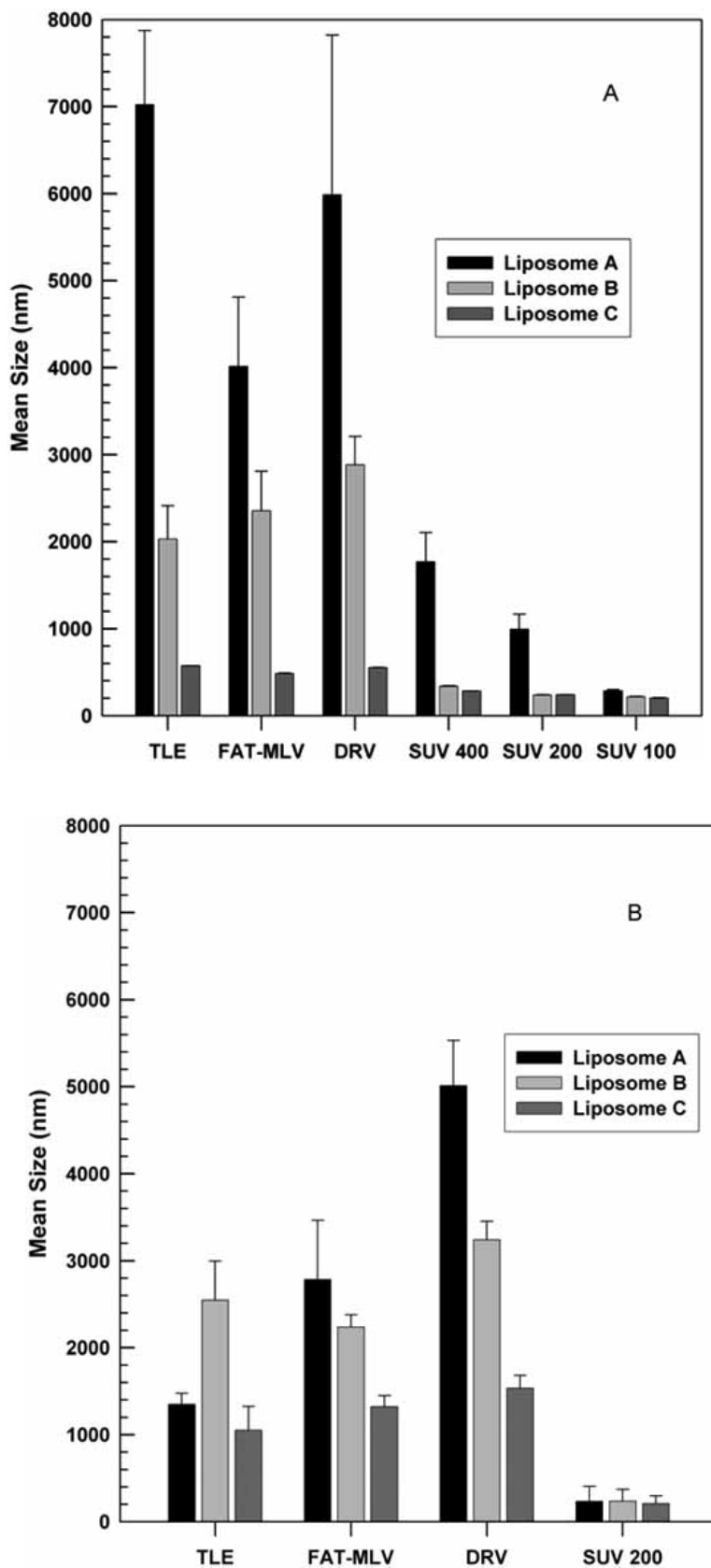
One-way ANOVA was used for statistical analysis of the various experiments. A posteriori Bonferroni t-test was carried out to check the ANOVA test. A *p* value <0.05 was considered statistically significant. Values are reported as the average ± standard deviation.

## 3. RESULTS AND DISCUSSION

### 3.1. Physicochemical Characterization of Liposomes

Different lipid compositions could modulate both technological and biopharmaceutical parameters of colloidal vesicles thus influencing the application of liposomes as drug delivery systems. For this reason, three different liposomal formulations were prepared and investigated as potential colloidal carriers for gemcitabine.

Mean sized, size distribution and zeta potential values are physicochemical parameters that have to be modulated as a function of the proposed application for a certain liposomal system. As reported in Fig. (1), mean size of liposomes was influenced by both the lipid composition and the preparation method. TLE, FAT and DRV techniques achieved the formation of multilamellar liposomes characterized by a mean size ranging from 0.55 µm to 7 µm and a polydispersity index ranging from 0.4 to 1 (Table 2), thus showing the presence of a very heterogeneous size distribution. The greatest mean size and polydispersity values were obtained for liposomes A and B, which had in their composition the presence of OA and DPPS, respectively. OA is able to increase the liposome bilayer fluidity [30,31,15] thus behaving as an edge activator, while DPPS can influence the packing of the lipid bilayer structure as a function of the environment and hence it is able to provide fusogenic properties [32,33]. Therefore, bilayer fluidity and fusogenic features may promote the for-



**Fig. (1).** Mean size of various liposome formulations prepared by different methods both in the absence (panel A) and in the presence (panel B) of gencitabine. Samples were diluted to achieve the most suitable optical density for light-scattering analysis. Each value represents the mean  $\pm$  standard deviation of at least three different experiments in triplicate.

**Table 2.** Size Distribution Expressed as Polydispersity Index of the Various Liposomal Formulations Prepared by Means of Different Methods Both in the Absence and in the Presence of Gemcitabine<sup>a</sup>

Formulation	Preparation method					
	TLE	FAT-MLV	DRV	SUV <sup>b</sup>		
				400	200	100
without gemcitabine						
Liposome A	0.7±0.2	0.8±0.2	1.0±0.0	0.3±0.1	0.5±0.2	0.4±0.1
Liposome B	0.9±0.1	0.9±0.1	0.9±0.1	0.2±0.1	0.2±0.1	0.2±0.1
Liposome C	0.4±0.1	0.4±0.1	0.4±0.1	0.1±0.0	0.1±0.0	0.1±0.0
with gemcitabine						
Liposome A	1.0±0.0	1.0±0.0	0.8±0.2	—	0.6±0.2	—
Liposome B	1.0±0.0	1.0±0.0	1.0±0.0	—	0.3±0.1	—
Liposome C	0.6±0.1	0.7±0.1	1.0±0.0	—	0.1±0.0	—

<sup>a</sup>The sample was diluted to achieve the most suitable optical density for light-scattering analysis and the experimental data of colloidal formulations were carried out at 20°C. The values represent the mean value ± standard deviation of at least three different experiments in triplicate.

<sup>b</sup>Multilamellar vesicles extruded through 400 nm, 200 nm and 100 nm pore size filters.

mation of multivesicular aggregates, that justify both the wide size distribution and the greater values of mean size. Smaller mean size (0.55 μm) and a reduced size distribution (0.4 polydispersity index) were observed for liposome C, which are characterized by a rigid bilayer structure and a colloidal stability due to the presence of both Chol and DSPE-MPEG.

Size reduction is often required for some specific applications of liposomes, i.e. systemic administration [34]; for this reason, various liposome formulations were submitted to an extrusion process through polycarbonate membranes to reduce liposome mean size and to obtain a homogeneous size colloidal suspension. As shown in Fig. (1), the extrusion of multilamellar liposomes A through polycarbonate membranes of 400 nm and 200 nm did not efficaciously reduce the mean sizes. This finding was probably due to the presence of OA that, as already specified, was able to increase the deformation of liposomal vesicles during their passage through the membrane pores rather than the bilayer collapse and reorganization in the vesicle with a mean size similar to pore size.

An effective reduction of mean sizes (Fig. 1) and polydispersity index (Table 2) following the passage through 400 nm pore size membranes was observed for liposomes B and C. These two parameters were further reduced following extrusion through 200 nm and 100 nm pore size membranes (Fig. 1 and Table 2).

To verify the influence of the drug on the mean size and polydispersity index of the three liposome formulations, light scattering analysis was also carried out in the presence of gemcitabine. As shown in Fig. (1), the presence of gemcitabine was able to significantly influence the mean size of liposomes A, particularly the multilamellar ones. In these cases, the lower mean size with respect to empty liposomes may be due to the strong interaction between gemcitabine and the negative polar head groups of the lipids as demon-

strated by DSC data reported in a previous paper [35]. The interaction of gemcitabine with OA may lead to a reduction of the bilayer fluidity, thus hampering the formation of vesicle aggregates. Also the extrusion of gemcitabine-loaded liposomes is much more effective in size reduction with respect to empty liposomes, due to the increased bilayer rigidity.

In the case of liposomes B and C, the presence of gemcitabine did not significantly influence the mean size and polydispersity index values with respect to empty liposomes (Fig. 1 and Table 2).

Zeta potential of colloidal vesicles is an important parameter to investigate in drug delivery systems. In fact, the dynamic electrophoresis mobility could modulate biopharmaceutical aspects of the colloidal carrier [36]. Namely, the surface charge of liposomes can influence blood circulation times, the opsonization process and hence the reticuloendothelial system uptake, as well as the interaction with biological compartments [37]. Experimental data reported in Table 3 show that zeta potential values were influenced by lipid composition of liposomes and not by the preparation methods. Zeta potential values of about -5 mV and -38.5 mV were observed for liposomes A and B, respectively.

Liposome C showed zeta potential values approaching zero. In this case, zeta potential values were much lower as an absolute value than that expected [38] due to the presence of polyethylene glycol moieties that shielded the DPPC polar head group.

The presence of gemcitabine poorly influenced the surface charge of the various liposome formulations (Table 3).

### 3.2. Liposomal Drug Delivery Characterization

A very important parameter to be evaluated for a liposomal system is the loading capacity. Table 4 shows that lipid composition and preparation method can influence the en-

**Table 3. Zeta Potential Values (mV) of Various Liposome Formulations Prepared with Different Methods Both in the Absence and in the Presence of Gemcitabine<sup>a</sup>**

Preparation method	Formulation		
	Liposome A	Liposome B	Liposome C
Vesicles without gemcitabine			
TLE	-5.0 ± 0.3	-38.5 ± 1.8	-1.4 ± 0.6
FAT-MLV	-4.3 ± 3.7	-36.0 ± 2.8	-0.8 ± 0.6
DRV	-4.9 ± 2.4	-38.2 ± 4.1	-0.5 ± 0.3
SUV 400 nm	-5.4 ± 0.5	-36.2 ± 3.8	-0.6 ± 0.2
SUV 200 nm	-5.6 ± 1.5	-36.8 ± 3.7	-1.7 ± 0.4
SUV 100 nm	-4.2 ± 0.5	-37.9 ± 3.2	-1.0 ± 0.5
Vesicles with gemcitabine			
TLE	-15.8 ± 2.2	-39.9 ± 1.9	-2.6 ± 1.3
FAT	-12.7 ± 1.1	-35.4 ± 1.7	-3.1 ± 0.2
DRV	-9.9 ± 1.7	-34.2 ± 1.3	-2.3 ± 0.8
SUV 200 nm	-10.3 ± 2.1	-38.1 ± 2.3	-2.5 ± 1.2

<sup>a</sup>Values represent the average of at least three different experiments in triplicate ± standard deviation.

trapping efficiency of liposome formulations. Various liposome suspensions prepared with the TLE method showed EC values  $\geq 14$  ( $\mu\text{l}/\mu\text{mol}$ ), which were much higher than expected by using the TLE procedure [39]. These findings can be explained not only by a simple drug entrapment within the liposomal aqueous compartments but also by an interaction of gemcitabine with the negatively charged polar head group of phospholipids, as demonstrated in a previous paper [35]. Therefore, in the case of gemcitabine a significant contribution to liposome drug loading comes from the drug adsorption with liposomal bilayer.

The FAT method (Table 4) allowed an increase of the gemcitabine loading capacity of various liposomes with respect to the TLE method.

A further improvement of the loading capacity was achieved by submitting various liposome formulations, produced by the FAT method, to the DRV method (Table 4). In this case, the dehydration and re-hydration process could improve not only the amount of drug entrapped in the liposome aqueous compartments but also the interaction between the gemcitabine and liposome bilayer [40].

To evaluate whether the drug addition step during the preparation process can have a significant role influencing encapsulation parameters, unloaded FAT liposomes were dehydrated and then rehydrated with a gemcitabine aqueous solution. As reported in Table 4, the DRV liposomal encapsulation of gemcitabine is higher if the drug is added at the beginning of liposomes preparation, i.e. during the lipid film formation, and not at the end of the process. This find could be due to the more suitable gemcitabine-lipid interaction when the drug is added at the beginning.

Due to the presence of a protonable amino group in the gemcitabine molecule, we investigated whether the application of an acidic pH gradient between the inner liposomal aqueous compartment and the external medium could further improve the amount of drug entrapped in the vesicular colloidal carrier. According to observations for other protonable drugs [41], the presence of an acidic pH gradient allowed a significant improvement of the liposome loading capacity, i.e. an EY value of ~94 % was achieved for liposome C.

No significant difference (data not reported) in the loading capacity was observed after extrusion of various liposome formulations (SUVET method) through polycarbonate filters, thus showing no significant leakage of the entrapped gemcitabine.

As concerns the lipid composition, the following loading capacity increasing order was observed: liposome A < liposome C < liposome B. This result was probably due to the presence of DPPS in liposome B, which could efficaciously interact with gemcitabine, thus allowing an encapsulation yield of ~89 % for DRV liposomes (Table 4). In a previous work, to improve the gemcitabine loading capacity within liposomes, the preparation of gemcitabine lipophilic prodrugs was also proposed with the aim to obtain the protection of the drug from plasma catabolism [42]. In particular, gemcitabine has been linked to a long fatty acyl chain through the amino group and then encapsulated in a pegylated liposomal formulation, thus obtaining a great stability both *in vitro* and *in vivo*. In addition, gemcitabine lipophilic prodrugs demonstrated an higher cytotoxicity (between two- and seven fold) than the free drug.

The release of gemcitabine from various liposome formulations was investigated for a suitable use of liposomes as a

**Table 4. Encapsulation Capacity of Various Liposome Formulations Prepared with Different Preparation Methods<sup>a</sup>**

Preparation method	Formulation					
	Liposome A		Liposome B		Liposome C	
	EC <sup>b</sup>	EY <sup>c</sup>	EC <sup>b</sup>	EY <sup>c</sup>	EC <sup>b</sup>	EY <sup>c</sup>
TLE	13.9±0.8	46.1±1.2	20.1±0.4	63.3±1.5	19.3±0.3	48.4±1.5
FAT-MLV	14.5±0.3	49.2±1.1	22.6±0.5	68.3±2.0	21.4±0.7	52.1±1.1
DRV	16.1±0.6	52.1±1.3	29.1±0.7	89.2±1.1	25.8±0.4	70.2±1.3
DRV <sub>drug</sub> <sup>d</sup>	13.8±0.4	44.5±1.0	27.6±0.8	85.1±1.6	23.7±0.8	67.5±1.0

<sup>a</sup>Values represent the average of at least five different experiments ± standard deviation.

<sup>b</sup>Encapsulation capacity is expressed as ml/mmol and is calculated according to Eq. 3.

<sup>c</sup>Encapsulation yield is expressed as the percentage of the starting drug that becomes liposomally entrapped. It is calculated according to Eq. 2.

<sup>d</sup>DRV rehydrated with a gemcitabine solution (50 mg/ml).

drug delivery system. Experimental findings showed that preparation method and lipid composition also influenced the release profiles of gemcitabine (Fig. 2). Among the liposomes prepared by the DRV method, only the liposomal formulations that showed the highest encapsulation parameters (i.e. drug added at the beginning of the procedure) were assayed for drug release.

As shown in Fig. (2), a burst effect was observed in the gemcitabine release profiles of all liposome formulations during the first 30 min. This finding was probably due to a rapid desorption of gemcitabine from external liposomal bilayers. The highest burst effect was observed for liposome A, which was more permeable to gemcitabine than the other liposome formulations, due to the presence of OA [15].

As reported in Table 5, a noticeable and significant ( $p < 0.005$ ) difference in the release profiles of gemcitabine was mainly observed at the level of the burst effect (0-30 min release), which showed the following decreasing order: liposome A > liposome C > liposome B. This order was mostly determined by the presence of two factors, i) the strength of the gemcitabine-liposomal lipid interaction, i.e. the stronger the interaction the less the desorption and hence the burst effect, ii) the fluidity of the bilayer, i.e. the more fluid the bilayer the greater and more rapid the drug leakage from the outer liposomal aqueous compartments.

In the slow release phase (30 min-48 h), there was not a noticeable difference between the various liposome formulations and a permeation order similar to the previous one was observed. As evidenced in Table 5 the main contribution to the total amount of drug released comes from the 0-30 min release phase and only a minor drug amount is released in the 30 min-48 h phase. This finding can be due to a slow trans-bilayer permeation kinetic according to previously reported DSC experiments [40].

Also the preparation method influenced the gemcitabine release from liposomes (Fig. 2). According to what has been stated for gemcitabine entrapment within liposomes, the DRV procedure was able to favour the drug adsorption. This behaviour was reflected in the drug release thus showing the following decreasing order: DRV > FAT > TLE (Fig. 2). For all liposome formulations, a significant difference ( $p < 0.05$ )

between various preparation methods (Table 5) was observed in the first release phase. Multilamellar liposomes prepared by TLE, FAT and DRV showed no noticeable difference in the second phase of drug release, where similar release profiles were observed (Fig. 2). Only the TLE method (for liposome A) showed a significant difference in the second release phase with respect to FAT and DRV, by showing a greater gemcitabine leakage (Fig. 2 and Table 5). This result may be due to the not homogeneous distribution of the entrapped drug, which is confined mainly at the level of the outer aqueous compartments of liposomes, and to the bilayer fluidity. In any case, a complete release of 100 % was not achieved from the different liposomes up to 48 h.

The application of a pH gradient to increase the liposome loading capacity was also able to influence the gemcitabine release (Fig. 3). A significant ( $p < 0.001$ ) reduction of the drug release was observed for liposome A, while liposomes B and C showed only a slight reduction of the released drug. It is noteworthy that for all the three liposome formulations a reduction of the release burst effect was achieved by the pH gradient method. These findings were probably due to the fact that this method favored the drug entrapment within the liposomal aqueous compartments and led to a reduction of the lipid bilayer absorption. Furthermore, the formation of the sulphate salt could reduce the drug bilayer permeability.

As expected, the size reduction by extrusion of these liposome formulations elicited an increase of the gemcitabine release with respect to multilamellar liposomal systems (Fig. 3). In fact, in case of unilamellar liposomal systems only a bilayer has to be permeated.

### 3.3. *In Vitro* Evaluation of Anticancer Activity

Biological efficacy of gemcitabine-loaded liposomes was tested on Caco-2 cells by using the *in vitro* MTT assay. The cytotoxic activity of free and liposomally entrapped gemcitabine was evaluated as a function of the drug concentration following 48 h incubation. Liposomes prepared with the pH gradient method and submitted to extrusion through 200 nm pore size polycarbonate membranes were used for *in vitro* experiments of cell vitality due to their improved colloidal properties and enhanced drug loading capacity.

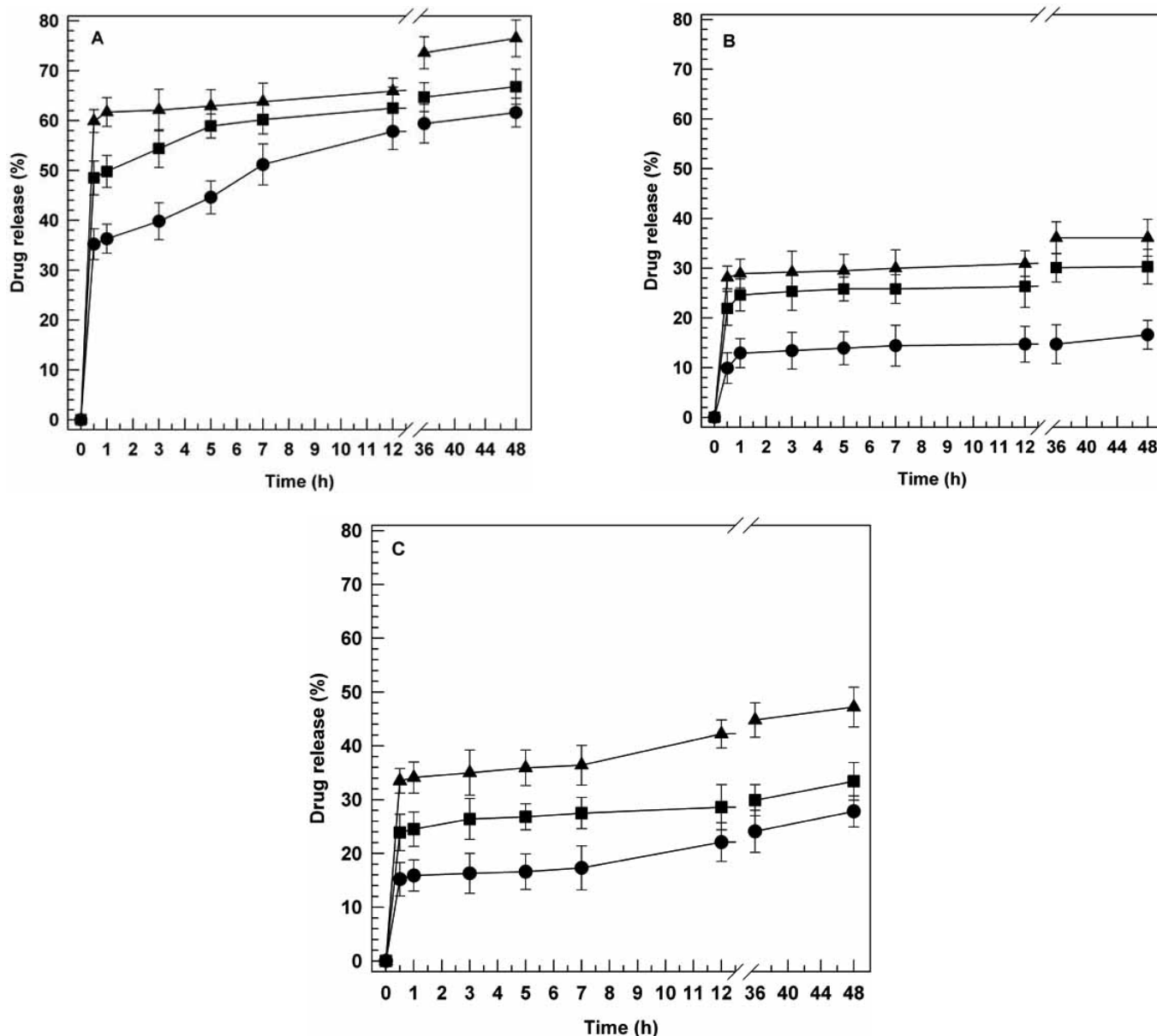


Fig. (2). Release profiles of gemcitabine from liposome A (panel A), liposome B (panel B) and liposome C (panel C) prepared by different methods. Keys: ●, liposomes prepared using the TLE method; ■, liposomes prepared using the FAT-MLVs method; ▲, liposomes prepared using the DRV procedure. Experiments were carried out at room temperature. Values represent the average of five different experiments ± standard deviation.

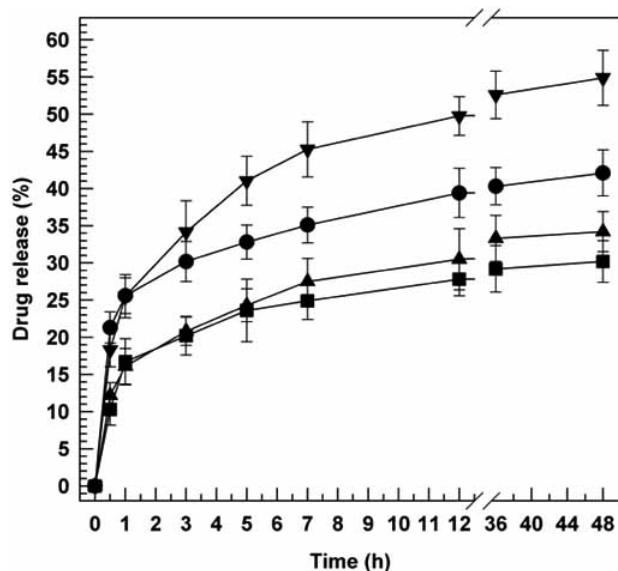
Table 5. Parameters of the Gemcitabine Release Profiles from the Three Liposome Formulations Prepared with Different Methods<sup>a</sup>

Preparation method	Percentage of release		
	0-30 min	30 min-48 h	Total release
Liposome A			
TLE	35.2 ± 1.1	26.4 ± 1.3	61.6 ± 2.4
FAT	48.5 ± 1.3	18.3 ± 1.3	66.8 ± 2.6
DRV	59.9 ± 0.7	16.6 ± 1.0	76.5 ± 1.7

(Table 5) contd...

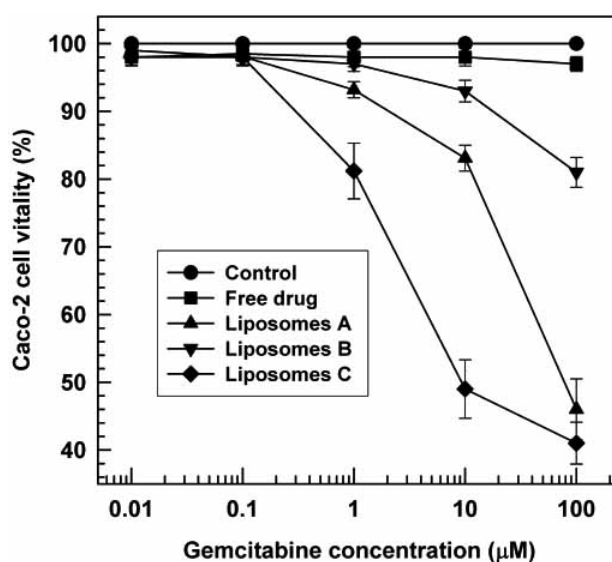
Preparation method	Percentage of release		
	0-30 min	30 min-48 h	Total release
Liposome B			
TLE	9.9 ± 1.5	6.7 ± 1.8	16.6 ± 3.3
FAT	21.9 ± 1.3	8.4 ± 1.5	30.3 ± 2.8
DRV	28.1 ± 1.0	8.0 ± 1.5	36.1 ± 2.5
Liposome C			
TLE	15.2 ± 2.0	12.6 ± 2.2	27.8 ± 4.2
FAT	23.9 ± 2.1	9.5 ± 2.0	33.4 ± 4.1
DRV	33.5 ± 1.1	13.7 ± 1.9	47.2 ± 2.9

<sup>a</sup>Values represent the average of at five different experiments ± standard deviation.



**Fig. (3).** Release profiles of gemcitabine from liposome A (●), liposome B (■) and liposome C (▲) prepared by the pH gradient method and then extruded through 200 nm pore size polycarbonate membranes (liposome C, ▼). Experiments were carried out at room temperature. Values represent the average of five different experiments ± standard deviation.

Empty liposomes were used to evaluate possible toxic effects of the carrier on Caco-2 cells. As shown in Fig. (4), no cytotoxic effect was elicited, at the concentration investigated in this paper, by vesicular carriers on Caco-2 cells, thus proving that liposomes are in some ways safe and that in case of drug-loaded liposomes the cytotoxic effect was due to the presence within the carrier of the antitumoral agent. After an incubation of 48 h, free gemcitabine did not elicit any cytotoxic effect at the investigated concentrations on Caco-2 cells, which presented a vitality of >95 %. A significant ( $p < 0.001$ ) improvement of the drug anticancer activity with respect to the free drug was achieved by using the various gemcitabine-loaded liposome formulations. All the three



**Fig. (4).** Dose-dependent antitumoral activity expressed as Caco-2 cell vitality (MTT test) after 48 h treatment with various gemcitabine-loaded liposome formulations or the free drug. All unloaded liposome formulations did not influence the Caco-2 cell vitality at the investigated concentrations. Each value is the average of nine different experiments ± standard deviation.

liposome formulations containing gemcitabine showed a dose-dependent anticancer activity versus Caco-2 cells.

The various formulations presented a different cytotoxic efficacy as a function of the lipid composition (Fig. 4). Liposome B showed the lowest efficacy by producing a significant cytotoxic effect only at a concentration of 10 µM, which was ten times higher than that of liposomes A and C. Therefore, liposome B did not seem to have suitable features for a potential therapeutic use as a gemcitabine delivery system. A significant ( $p < 0.001$ ) difference was also observed between liposomes A and C at a concentration of 1 µM, i.e. Caco-2 vitality of 93.2% and 81.2% after 48 h incubation, respectively (Fig. 4). This difference was even greater at a 10

**Table 6. Key Parameters of the Caco-2 Intracellular Gemcitabine Uptake from the Three Liposome Formulations Prepared with the pH-gradient Method and Extruded Through 200 nm Pore Size Polycarbonate Membranes<sup>a</sup>**

Key parameters	Free Drug	Formulations		
		Liposome A	Liposome B	Liposome C
Total time (h) <sup>b</sup>	4	>48	>48	>48
$C_{max}$ <sup>c</sup> (ng/ml)	11.3±1.3	112.1±8.3	48.7±3.4	267.5±16.3
$T_{max}$ <sup>d</sup> (h)	0.5	2	1	1
AUC <sub>0-48</sub> (ng·ml <sup>-1</sup> ·h)	23.7±4.9	876.6±122.9	220.4±58.0	944.8±127.1

<sup>a</sup>Data represent the mean value of five different experiments ± standard deviation.

<sup>b</sup>Total time when the drug was still detectable.

<sup>c</sup>Maximum drug concentration.

<sup>d</sup>Time when maximum concentration of the drug was detected.

μM drug concentration. In particular, liposome C provided a noticeable increase of the anticancer activity of gemcitabine (10 μM) vs. Caco-2 cells, thus showing an improvement of efficacy of 2, 1.9 and 1.7 fold with respect to the free drug ( $p < 0.001$ ), liposome B ( $p < 0.001$ ) and liposome A ( $p < 0.01$ ). The improvement of gemcitabine antitumoral efficiency versus Caco-2 cells provided by pegylated liposomes (liposome C), as well as their long circulating properties [43,18], suggested that this liposome formulation could be used as a possible successful carrier for *in vivo* gemcitabine delivery and treatment of solid cancer diseases [44,7].

The findings regarding cytotoxic effects of free or liposomally entrapped gemcitabine could be justified by and correlated with the intracellular gemcitabine uptake and retention. This effect can be modulated by the various liposome formulations [45] and particularly by their vesicular features, which can allow a greater and more rapid cellular entrance of the delivered anticancer agent.

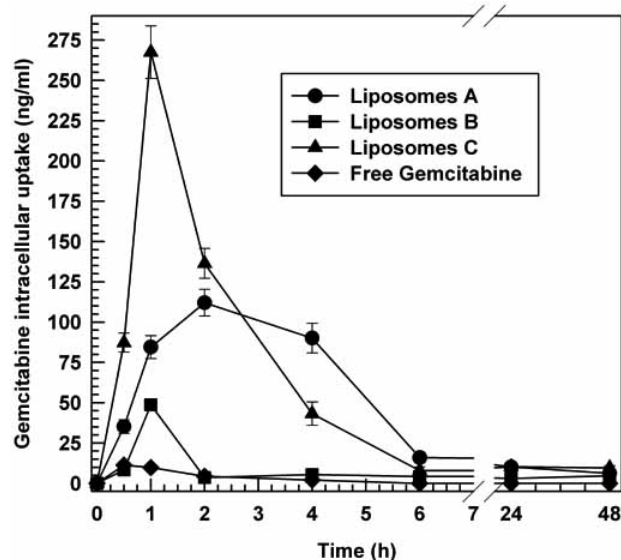
### 3.4. Intracellular Uptake of Gemcitabine

The intracellular uptake of free or liposomally entrapped gemcitabine as a function of time was investigated. As shown in Fig. 5, free gemcitabine, due to its physicochemical property and to the biological features of Caco-2 cells (presence of specific nucleotide transporters and efflux pumps) [46,47], did not easily pass through Caco-2 cell membranes and hence low levels were detected in the intracellular compartments. These data were in agreement with findings of MTT test experiments (Fig. 4), which showed a poor antitumoral efficacy of the free drug versus Caco-2 cells after 48 h of treatment.

The entrapment of gemcitabine within liposomes determined an improvement of the Caco-2 intracellular accumulation (Fig. 5). This effect was correlated to the ability of liposomes to penetrate through the cell membrane and thus to obtain a significant concentration in cytoplasm compartment. As shown in Fig. 5, the levels of gemcitabine intracellular uptake were in very good agreement with the anticancer activity of the various formulations. Namely, liposome C provided the highest gemcitabine intracellular levels followed by liposome A and liposome B (Fig. 5 and Table 6). The differences between various formulations were

not only in terms of intracellular total drug uptake but also in terms of intracellular uptake profile as a function of time.

As reported in Table 6, liposome C showed a maximum intracellular gemcitabine accumulation after 1 h ( $T_{max}$  value) followed by a gradual reduction of the intracellular drug accumulation up to 6 h. Whereas, liposome A showed (Fig. 5) a sustained uptake profile. In the case of liposome B, which showed the lowest intracellular gemcitabine levels, a  $T_{max}$  value of 1 h was observed. These different behaviours of the



**Fig. (5).** Caco-2 intracellular uptake of free and liposomally entrapped gemcitabine as a function of time. Experiments were carried out at 37 °C at a final drug concentration of 10 μM. Each point represents the mean value of five different experiments ± standard deviation.

three liposome formulations with Caco-2 cells could be due to the different bilayer composition, which could modulate the interaction between liposomes and biological membranes. Also the liposome drug delivery properties (gemcitabine loading and drug leakage) can influence and modulate the intracellular drug uptake. In particular, the presence of a cone-shaped lipid, such as oleic acid, which induces a nega-

tive curvature in phospholipid bilayer [48], may rapidly modulate the rearrangement of lipid moieties of bilayer phospholipids, thus promoting a fusion with cellular membranes [33], which may achieve a sustained intracellular drug uptake.

#### 4. CONCLUSION AND FUTURE PERSPECTIVES

The possibility of using liposomes as a drug delivery system for anticancer therapeutic applications was correlated to their physicochemical and drug delivery properties. Data herein reported show that mean size, polydispersity index, zeta potential, loading capacity, drug release, antitumoral activity and drug intracellular uptake were influenced by liposome lipid composition and preparation methods. However, MTT experiments showed that gemcitabine-loaded liposomes elicited a dose-dependent improvement of the gemcitabine cytotoxic effect versus Caco-2 cells with respect to the free drug. This achievement in therapeutic activity promoted by liposomal delivery systems was correlated to the ability to increase the intracellular drug uptake.

Due to the high modularity (shown in this paper) of the liposomal carrier both in terms of lipid composition and colloidal carrier features and to the encouraging *in vitro* results on antitumoral activity of gemcitabine-loaded liposomes, various applications of potential therapeutic relevance in different fields can be envisaged for liposomes. In particular, the deformable properties and the improved percutaneous features of oleic acid containing liposomes [31] together with the ability of oleic acid to modify the skin barrier permeability [15] prompt the use of liposome A (particularly multilamellar ones) as a topical formulation [49] to answer the increasing demand for topical treatments of hyperproliferative skin diseases, such as psoriasis or melanoma [50,17]. Whereas, the improved colloidal features, the suitable drug delivery properties, the cell interaction behaviors and the possibility of long circulation properties, due to the presence of PEG moieties conjugated to liposome surface [43,17], make liposome C suitable as a drug delivery system for systemic administration and treatment of colon carcinoma and other solid tumors.

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#### ABBREVIATIONS

Caco-2	=	Human colon carcinoma cell line
Chol	=	Cholesterol
DMEM	=	Dulbecco minimal essential medium
DPPC	=	Dipalmitoylphosphatidylcholine
DPPS	=	Dipalmitoylphosphatidylserine
DRVs	=	Dehydrated rehydrated vesicles

DSPE-MPEG 2000	=	N-(carbonyl-methoxypolyethylene glycol-2000)-distearoylphosphoethanolamine
EC	=	Encapsulation capacity
EY	=	Encapsulation yield
FAT	=	Freeze and thaw
FBS	=	Fetal bovine serum
HPLC	=	High performance liquid chromatography
MLVs	=	Multilamellar vesicles
MTT	=	[4,5-Dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide
OA	=	Oleic acid
PBS	=	Phosphate buffer saline solution
PEG	=	Polyethylene glycol
SDS	=	Sodium dodecyl sulphate
TLE	=	Thin layer evaporation
SUVET	=	Small unilamellar vesicles by extrusion technique.

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