

## Original Article

# Studies on pH gradient loading of amphoteric drug (Doxorubicin hydrochloride) into liposome

Kishore K. Dondilkar<sup>a,\*</sup>, R.S.R. Murthy<sup>b</sup>

<sup>a</sup>Department of Pharmaceutical Research, Nobel Ilac A.S. San. Ve Tic. Duzce, Turkey.

<sup>b</sup>M.S.R. University of Applied Sciences, MSR Nagar, Bangalore, India.

\*Corresponding Author. Tel.: +91-7222968087, E-mail address: kishoredondilkar@gmail.com

### ARTICLE INFO

Received 23 Nov 2015

Revised 01 Nov 2015

Accepted 19 Nov 2015

#### Keywords:

- pH Gradient
- Ammonium Sulphate
- Doxorubicin hydrochloride
- Optimisation
- Drug Loading

### ABSTRACT

The present study was carried out with an aim to evaluate the factors influencing the active drug loading through transmembrane pH gradient using Ammonium Sulphate. (AS) The influence of various factors which influence the pH gradient loading of amphoteric drugs [Doxorubicin Hydrochloride (DH) in our case] such as incubation temperature, incubation time, transmembrane migration of ammonium ions, molarity which influence the pH gradient loading of amphoteric drug were investigated. Initially, optimization of AS (100mM) loading into HSPC liposomes by thin film hydration method were done and later influence of incubation time (0.5, 1, 2 and 3 hours) and incubation temperature (RT, 40, 50 and 60°C) on DH were investigated. Incubation time of 3 hours and incubation temperature of 60°C was found to be optimum for maximum loading of DH. AS of different molarity (20 to 300Mm) were loaded into HSPC and its effect on drug loading was investigated. Increase in molarity brought about the increase in loading of DH and liposome formulation AS (225Mm) gave maximum loading. Measurement of internal pH prior and after drug loading could correlate the influence of all the above parameters on the loading of DH through pH gradient method. Besides, physicochemical characterization of developed liposomes, in-vitro drug release studies, in-vivo pharmacokinetics studies of the prepared liposomal formulation and the related stability studies were also carried out.

## 1. INTRODUCTION

Liposomes are drug delivery vehicles that have been used to enhance therapeutic efficacy, reduce toxicity and modify the pharmacokinetics of anticancer drugs [1, 2]. Since the endothelial membrane of tumors are more “leaky” than normal membranes, liposomes with particle sizes < 200 nm easily pass through the fenestrated blood vessels and accumulate at tumor and disease sites [3]. Hence, by encapsulating anticancer agents inside the liposomes, distribution and exposure to healthy cells and tissues are minimized while the drug payload to the tumor is enhanced, thus resulting in reduced toxicity and enhanced efficacy [4]. Liposome encapsulated drugs are also known to accumulate in organs of the reticuloendothelial system [5].

Doxorubicin Hydrochloride (DH) is a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius*

var, caesius. It consists of naphthacene quinone nucleus linked through a glycosidic bond at ring atom 7 to an amino sugar daunosamine. Chemically DH is (8S-10S) -10-(3- amino 2,3,6-trideoxy- $\alpha$ -L-lyxo-hexopyranosyl) oxy-7,8,9,10-tetrahydro-6, 8,11-trihydroxy- 8-hydroxyacetyl-1-methoxy -5-,12-naphthacenedione hydrochloride. DH is an amphoteric containing acidic function in the ring phenolic group. The drug acts by binding to nucleic acid, by specific intercalation of the planar anthracycline nucleus with DNA double helix resulting in prevention of further replication.

Several method exist for improved drug loading of the drug including remote (active) loading method which load drug molecules into preformed liposomes using pH gradient and potential difference across liposomal membrane [6]. A weak base like doxorubicin has been successfully loaded into preformed liposomes via pH gradient method [7].

Numerous studies have shown that liposomal encapsulation of DH can provide a significant therapeutic benefit by decreasing the dose-limited toxicities while maintaining the antitumour potency. Although studies suggest that the therapeutic potential for DH they suffer significant problem with respect to flexibility and stability, these system exhibit variety of sizes, due to lipid ratio and lipid composition which make it difficult to select an optimal formulation [8].

Several studies have demonstrated that DH can be accumulated in LUVs, which exhibit transmembrane pH gradient. In the present study we aim to study the influence of drug loading of DH through transmembrane pH gradient method by altering some of the factors influencing the same. A simple method to estimate the AS by turbidimetry and measurement of internal pH by fluorimetry is also described.

## 2. EXPERIMENTAL

### 2.1 Materials

Doxorubicin was a kind gift from Sun Pharma (Gujarat, India), pyranine was purchased from (national chemical. Vadodara), hydrogenated soya phosphatidylcholine (HSPC) was purchased from Lipoid KG (ludwigshafen, Germany), cholesterol was obtained from Sigma (St. Louis, MO, USA), HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulphonic acid) buffer was purchased from Spectrochem, Mumbai, India, Sephadex –G 50 was a kind gift from Sun Pharma Advanced research Center, (Baroda, India). All other ingredients used were of analytical grade and used as such.

### 2.2 Methods

#### 2.2.1 Preparation of Ammonium Sulphate Liposomes

Liposomes were prepared using the conventional film method. The lipid mixture were dissolved in a 2:1 (v/v) mixture of methanol and chloroform and deposited as thin film in a round bottom flask by rotary evaporator at 60°C. The film was hydrated for 50 min by addition of 100 mM ammonium sulphate solution. The final formulation was then optimized for the entrapment of ammonium sulphate as shown in the Table 1.

**Table 1.** Effect of Total Lipid Ratio on Entrapment of  $(\text{NH}_4)_2\text{SO}_4$

Drug Lipid Ratio	% Entrapment
1: 2.5	71.0 ± 0.82
1:5	86.1 ± 1.32
1: 7.5	85.0 ± 1.22

#### 2.2.2 Estimation of Ammonium Sulphate

The prepared liposomes were sonicated in probe sonicator (Labsonic-Sartorius-Germany) to obtain liposomes of nano size. 2.5 ml of the sonicated preparation was applied to Sephadex G 50 column pre-equilibrated with 0.01M HEPES buffer. The elute which contains liposomes was collected and spectrophotometrically analyzed in Shimadzu UV Visible

spectrophotometer (UV- 1601) for ammonium sulphate content at 800nm. The amount of ammonium sulphate was estimated from calibration plot by plotting transmittance Vs known concentration of AS (1-30 µg/ml) which gave  $Y = -1.806X + 99.632$  and  $R^2$  of 0.9933

#### 2.2.3 Incubation of Liposomes

1 ml solution of DH (1 mg/ml) containing Tocopherol (0.5mM) was added to 0.5 ml of liposome dispersion, after the liposomes were gel filtered on Sephadex G 50 column. The mixture was incubated at different temperatures for different time intervals. At the end of incubation period incorporated Doxorubicin was removed by passing through Sephadex G 50 column.

#### 2.2.4 Estimation of Doxorubicin

The liposomes obtained from Sephadex G 50 column were destabilized with chloroform: methanol (1:9 v/v). The doxorubicin content was estimated spectrophotometrically in Shimadzu UV Visible spectrophotometer (UV- 1601) at 478nm. The amount of DH was estimated from calibration plot by plotting absorbance transmittance Vs known concentration of DH (1-30 µg/ml) which gave  $Y = 0.0317 - 0.0097$  and  $R^2$  of 0.9996.

#### 2.2.5 Measurement of Internal pH

The pH of internal compartment of lipid vesicle was measured with the membrane impermeant pH sensitive fluorescent molecule, pyranine. The liposomes were prepared in 100 mM ammonium sulphate pH 5.5. The ammonium concentration in the external medium was adjusted with serial dilution with the solution used to prepare the liposome. The internal pH was calculated from the entrapped pyranine fluorescence estimated in a spectrofluorometer (Shimadzu RF-540, Japan) at excitation wavelength of 460 nm and emission wavelength of 550 nm. The liposomes were prepared containing 1µM pyranine and 100 mM of ammonium sulphate and untrapped pyranine was removed by gel extrusion chromatography on Sephadex G–50 columns pre-equilibrated with solution of ammonium salts used to prepare the liposomes (i.e., 100 mM ammonium Sulphate pH 5.5). The fluorescent emission intensity at 550 nm was measured at excitation wavelength of 460 nm.

#### 2.2.6 Formation of the Transmembrane difference in Ammonium Concentration

The concentration of ammonium sulphate salt in the external compartment was controlled by dilution of liposome suspension in 100 mM  $(\text{NH}_4)_2\text{SO}_4$ . The salt concentration was 100mM to maintain the same osmolality on both side of lipid bilayer. The osmolality of the solutions used in this study were measure with Osmometer. (Advanced Instrument (c) 2005 A .I. Inc. Model 3250, Version 1.1 serial # 05050593).

#### 2.2.7 In-vitro Drug release Study from Liposomes

*In-vitro* drug release was performed using dialysis membrane, in which drug release was observed in phosphate buffer saline,

pH 7.4 (PBS). The temperature of the external media was kept at  $37 \pm 2^\circ\text{C}$  for the simulation of the body conditions. The volume of the external medium was 25 ml. The plain drug solution and liposomal dispersion was taken directly in diffusion cell and placed in beaker containing release medium.

### 2.2.8 In-vivo Studies

In-vivo studies was performed by administering control (plain drug solution of doxorubicin hydrochloride) and the liposomal formulation formed by thin film hydration technique using pH gradient method for loading of the drug. The dose was given intravenously to the albino rats (1.5 mg/kg/day) and compared the pharmacokinetics parameters like  $C_{\max}$ ,  $T_{\max}$ , volume of distribution, area under curve and clearance etc. Blood samples were collected at different time intervals for the period of upto 24 hrs and estimation of DH in blood sample was done by fluorimetric method using spectrofluorophotometer.

### 2.2.9 Stability Studies of Liposomes

The prepared liposome dispersion was subjected to stability studies, in triplicate, at conditions according to ICH guidelines i.e.  $2-8^\circ\text{C}$  with ambient humidity and  $30 \pm 2^\circ\text{C}/60 \pm 5\%$  RH. The prepared were taken in 5 ml amber colored glass vials (USP Type I glass). The vials were then sealed using 20 mm grey bromobutyl rubber stopper and 20 mm aluminium seals. For the condition of  $2-8^\circ\text{C}$  with ambient humidity, the vials were placed in refrigeration. For the stability studies of the vials at  $30 \pm 2^\circ\text{C}/60 \pm 5\%$  RH, the vials were taken in desiccator  $30 \pm 2^\circ\text{C}/60 \pm 5\%$  RH desiccators, containing 60 w/v sodium nitrite which was then placed in an oven maintained at  $30^\circ\text{C}$ . At the intervals of 10, 20 and 30 days liposomal dispersion samples were withdrawn from vials placed at both the conditions and subjected to the analysis of the size distribution and drug retention efficiency.

## 3. RESULTS AND DISCUSSION

### 3.1 Preparation and Optimization of AS

The AS was loaded into the HSPC liposomes by thin film hydration as previously reported. Initially we identified the process as well as formulation factors influencing the loading of AS into HSPC liposomes, we selected LIPID: CHOL and hydration volume as two important parameter that significantly influence the loading, Trials were conducted by taking three different ratios of HSPC: CHOL and three different volumes of hydration. It was observed that as the cholesterol content decreases the entrapment was found to be  $70.0 \pm 0.23$  and with the increase in the hydration media the entrapment was found to be  $86.1 \pm 1.22$ . The LIPID: CHOL molar ratio of (3.5:1.5) and hydration media of 2.5 ml gave the maximum loading of AS i.e.  $86.1\% \pm 1.22$  (Table 2).

Vesicle size plays an important role on the wide variety of therapeutic liposomes. As the small liposomes are able to accumulate in the tumor cell and exhibit extended circulation life time. DH has been reported to accumulate readily into LUVs exhibiting pH gradient. For obtaining nano sized liposomes the MLVs formed from the preparation were sonicated to get particle size ranging from 104 – 208 nm.

**Table 2.** Effect of cholesterol concentration and hydration volume

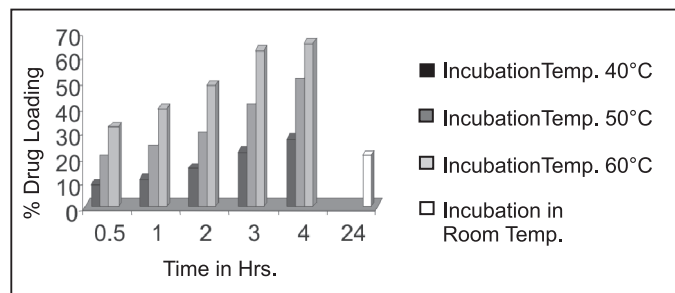
HSPC: CHOL	Hydration Vol. (ml)	% Entrapment	Avg. Vesicle Size (nm)
4:1	1.5	$70.0 \pm 0.38$	$284 \pm 9.6$
3.5:1.5	2.5	$76.0 \pm 0.33$	$263 \pm 5.7$
3:2	3.5	$72.0 \pm 0.29$	$269 \pm 7.8$
4:1	1.5	$78.2 \pm 0.35$	$264 \pm 4.4$
3.5:1.5	2.5	$75.0 \pm 0.22$	$212 \pm 2.5$
3:2	3.5	$79.0 \pm 0.27$	$205 \pm 7.4$
4:1	1.5	$71.0 \pm 0.82$	$270 \pm 8.8$
<b>3.5:1.5</b>	<b>2.5</b>	<b><math>86.1 \pm 1.22</math></b>	<b><math>208 \pm 2.2</math></b>
3:2	3.5	$70.0 \pm 0.23$	$290 \pm 3.3$

**Note:** Ammonium Sulphate concentration used in all batches = 0.1M, \* $n = 3$

### 3.2 Influence of Incubation Time, Incubation Temperature, and Molarity of AS on Drug Loading

The first objective of this study was to evaluate the various factors for the encapsulation of doxorubicin that relied on the presence of pH gradient across the liposome bilayer. pH gradient was established directly. To directly establish the pH gradient the liposomes were prepared in the presence of 100 mM ammonium Sulphate salt pH 5.5. The exterior salt solution was then replaced with 0.01 M HEPES buffer pH 7.5 by using Sephadex G-50 column chromatography. The ammonium sulphate loading procedure was first described by Barenholz to encapsulate doxorubicin Following the encapsulation of ammonium sulphate the external solution is exchanged for an iso-osmotic solution to establish the ammonium sulphate gradient. Due to high permeability of  $\text{NH}_3$  ( $1.3 \times 10^4$  cms/s), it readily crosses the liposome bilayer, leaving behind one proton for every molecule of  $\text{NH}_3$  lost. This creates a pH gradient, the magnitude of which is determined by  $\text{NH}_{4\text{IN}}/\text{NH}_{4\text{OUT}}$  gradient. The existing pH gradient (interior acidic) across these liposomes therefore can drive the uptake of proportion of drug however once the drug uptake occurs, the pH gradient collapse. For the encapsulation of doxorubicin into HSPC/CHOL liposomes incubation time, temperature and molarity of the salt solution was determined. Drug loading was determined in the condition were drug and lipid

was mixed in the ratio (1:5) (Mm/Mm). The doxorubicin loading data summarized in the Fig.1 indicates that at 40°C there is no appreciable drug uptake over the entire time course regardless of ion gradient used. Doxorubicin encapsulation was found to be temperature dependent with fast rate of loading being observed at 60°C. High level of entrapment at high temperature are also observed by DSPC: CHOL system The DSPE: CHOL has a high uptake between 15 and 60 minutes.



**Fig. 1.** Effect of Incubation Time and Temperature on Drug Loading. For the encapsulation of doxorubicin into HSPC/CHOL liposomes incubation time, temperature was determined, at 40°C there is no appreciable drug uptake over the entire time course regardless of ion gradient used. Doxorubicin encapsulation was found to be temperature dependent with fast rate of loading being observed at 60°C. High level of entrapment of DH was observed at 3 hrs though this did not increase further.

**Table 3.** Effect of incubation time and temperature on drug loading for 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

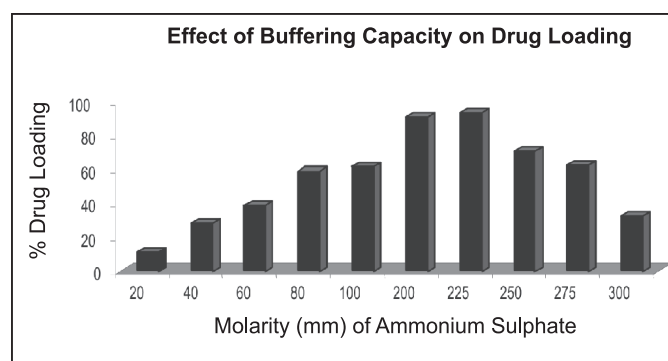
Incubation Temp.	Time in Hrs.	% Loading
40°C	0.5	9.00
	1.0	11.15
	2.0	15.41
	3.0	22.00
50°C	0.5	20.39
	1.0	24.65
	2.0	29.62
	3.0	40.95
60°C	0.5	31.75
	1.0	38.86
	2.0	48.41
	3.0	62.34
Room Temp.	24	20.39

In the present study the high level of entrapment of DH was observed at 3 hrs though this did not increase further. It is commonly assumed that lipophillic amines permeate membrane in the neutral (deprotonated) form. The uptake in response to pH relies on permeation of neutral species and subsequent reprotonation in the acidic internal medium which depletes the internal proton pull. The resulting dependence for doxorubicin on the internal buffer capacity was studied by monitoring drug

accumulation while varying the concentration of ammonium sulphate inside the liposomes. For initial drug to lipid mM ratio of 1:5 increasing the AS concentration from 20 to 225 mM produces an increase in the doxorubicin trapping efficiency from 11% to 93%. However increasing the trapped ammonium sulphate concentration to 300mM did not alter the entrapment efficiency over those compared to 225 mM. This may be due to the decreased buffering capacity and from activity coefficient effect or alternatively leakage due to the osmotic forces experienced at this high ammonium sulphate concentration (Fig. 2).

**Table 4.** Effect of buffering capacity on drug uptake

Temperature	Molarity (mM) of (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	% Entrapment
60°C	20	11.0
	40	28.0
	60	39.0
	80	59.0
	100	62.0
	200	91.2
	225	93.5
	250	70.5
	275	63.0
	300	32.46



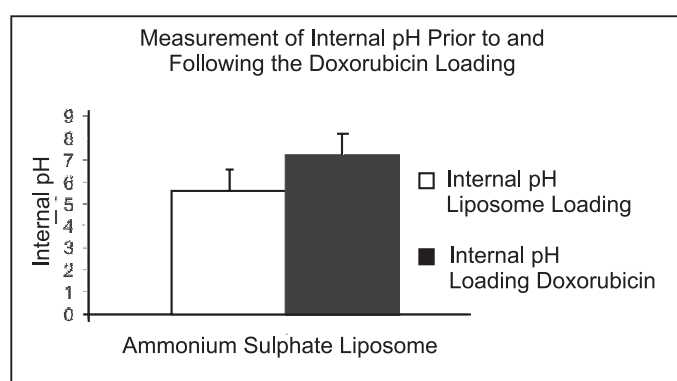
**Fig. 2.** Effect of buffering capacity on drug loading: For initial drug to lipid mM ratio of 1:5 increasing the AS concentration from 20 to 225 mM produces an increase in the doxorubicin trapping efficiency from 11% to 93%. However increasing the trapped ammonium sulphate concentration to 300 mM did not alter the entrapment efficiency over those compared to 225 mM.

### 3.3 Measurement of Internal pH

In order to determine if difference in the loading efficiency were due to magnitude of transmembrane pH, gradient before and after drug loading membrane impermeant pH sensitive probe pyranine was used to measure the transmembrane pH gradient. Formulation was evaluated prior to and following drug uptake and results are summarized in Fig. 3. The formulation with encapsulated ammonium sulphate exhibited internal pH of about 5.6. This demonstrated that sufficient transmembrane pH gradient (inside acidic) was present prior to drug encapsulation.

### 3.4 Increase in the Liposome inner pH in Response to Transmembrane difference in Ammonium Sulphate Concentration

The measurement using the membrane impermeant pH sensitive probe pyranine clearly show that the internal pH of liposome was linearly dependent on the logarithm of ammonium concentration ratios  $C_{in}/C_{out}$ . In this study initially the internal and external concentration of AS was equal to that used for the preparation of liposomes (100 mM, pH 5.5) which was later diluted with the AS. The internal pH was calculated from the fluorescence intensity ratio of trapped pyranine. This experiment thus gave the method for measuring the pH of the aqueous space trapped inside the lipid vesicle. The pH increase depends only on the ratio of internal to external concentration of ammonium ions and shows no dependence in the nature of counter ion (in our case sulphate).



**Fig. 3.** Measurement of internal pH prior to and following the Doxorubicin loading. The formulation with encapsulated ammonium sulphate exhibited internal pH of about 5.6. This demonstrated that sufficient transmembrane pH gradient (inside acidic) was present prior to drug encapsulation

The linear relationship inner pH and log of the concentration ratio is in good agreement with Eq. 1 derived from acid base equilibrium and difference in the permeability coefficient.

$$pH_{in} = pH_{out} + \log (C_{in}/C_{out})$$

### 3.5 *In-vitro* drug release study from liposomes

The plain drug took 8 hours for about 75.5% of drug release whereas only 49.98% drug released from the liposomal formulation after 48 hrs.

### 3.6 *In-vivo* Studies

The result shows the liposomal formulation of doxorubicin hydrochloride has  $C_{max}$  11138 ng/ml,  $T_{max}$  0.5,  $AUC_{0-24}$  98.20  $\mu\text{g}\cdot\text{hr}/\text{ml}$ ,  $AUC_{0-\infty}$  146.97 ( $\mu\text{g}\cdot\text{hr}/\text{ml}$ ), clearance 2.5 ml/hr/gram, volume of distribution 81.06 ml/hr/gram,  $K_{el}$   $\text{h}^{-1}$  0.031, and  $T_{1/2}$  (hrs) 22.01. Whereas free drug has  $T_{1/2}$  (hrs) 7.27,  $K_{el}$   $\text{h}^{-1}$  0.095,  $V_d$  78.09 ml/hr/gram, clearance 7.4 ml/hr/gram,  $AUC_{0-24}$  29.71 ( $\mu\text{g}\cdot\text{hr}/\text{ml}$ ),  $AUC_{0-\infty}$  50.38 ( $\mu\text{g}\cdot\text{hr}/\text{ml}$ ),  $C_{max}$  12010 ng/ml,  $T_{max}$

0.5 hrs. From the *in-vivo* liposomal formulation shows more area under curve and stay more time in the body as compared to plain drug solution.

### 3.7 Stability Studies of Liposomes

The evaluation of the particle size and the drug retention of the liposomal dispersion showed that the liposomal formulation stored at 2-8°C was more stable than at 30±2°C with less leakage of the drug from the liposomal formulation. Storage at 2-8°C also maintained the suitable particle size for the period of one month. There was gradual leakage of the drug from the liposomal formulation and also increase in mean particle size when stored at 30±2°C showing their instability for 1 month. Thus from the above data it can be concluded that prepared liposomal formulation must be freeze dried as an alternative for long time stability.

## 4. CONCLUSION

Doxorubicin being an effective anticancer agent used in treatment of many malignancies its incorporation in the liposomal formulation has always been desired. Many attempts in its incorporation in liposome by conventional method had been failed due to its hydrophilicity. Loading of water soluble drugs like Doxorubicin Hydrochloride had been a challenging till the introduction of pH gradient loading of amphiphatic drugs by Mayer et al., 1986, 1993. DH had been extensively worked by many researchers using this technique on different lipidic substances. In the present study we tried to explore the influence of various factors such as incubation time, incubation temperature, migration of ammonium ions, and molarity on HSPC: CHO liposome. Its characterizations, release profiles, optimization of the final formulation and its stability parameters, had significant influence on loading. Temperature of 60°C and time of 3hrs and molarity of 225 mM of ammonium sulphate were found to be optimum to incorporate 1mg/ml DH (Table 5). This has been well correlated with the internal pH measurement using the fluorimetric method. The above study showed that the DH loading by pH gradient method was possible in HSPC: CHO system by controlling/optimizing various optimizing parameters. The above study showed that the DH loading by pH gradient method was possible in HSPC: CHO system by controlling/optimizing various optimizing parameters.

**Table 5.** Effect of molarity on percent loading of water soluble drugs Doxorubicin Hydrochloride

Drug Conc.	Molarity (mM)	% Loading
1 mg/ml	225	93.5
	250	70.5
	275	63.0

## REFERENCES

---

---

- [1] Crawley, J.C.; Gregoriadis, G. Tissue distribution of liposomes exhibiting long half-lives in the circulation after intravenous injection. *Biochim. Biophys. Acta.* 1985, 839, 1-8.
- [2] Senior, J.; Gregoriadis, G. Is half-life of circulating liposomes determined by changes in their permeability? *FEBS Lett.* 1982, 145, 109-114.
- [3] Gabizon, A.; Papahadjopoulos, D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci.* 1988, 85, 6949- 6953.
- [4] Santos, N.D.; Mayer, L.D.; Abraham, S.A.; Gallagher, R.C.; Cox, K.A.; Tardi, P.G.; Bally, M.B. Improved retention of idarubicin after intravenous injection obtained for cholesterol- free liposomes. *Biochim. Biophys. Acta.* 2002, 1561, 188– 201.
- [5] Munn, M.W.; Parce, J.W. Antibody-dependent phagocytosis of haptenated liposomes by human neutrophils dependent on the physical state of the liposomal membrane. *Biochim. Biophys. Acta.* 1982, 692, 101-108.
- [6] Lasic, D.D.; Martin, F.J.; Gabizon, A.; Huang, S.K.; Papahadjopoulos, D. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. *Biochim. Biophys. Acta.* 1991, 1070, 187-192.
- [7] Lasic, D.D.; Ceh, B.; Stuart, M.C.A.; Guo, L.; Frederik, P.M.; Barenholz, Y. Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery. *Biochim Biophys Acta.* 1995, 1239, 145-156.
- [8] Straubinger, R.M.; Hong, K.; Friend, D.S.; Papahadjopoulos, J.K. Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles. *Cell.* 1983, 32(4), 1069-79.