PHYSICOCHEMICAL INVESTIGATION OF LOW SOLUBLE BIOCOMPOUNDS ENTRAPPED IN LIPID CARRIERS

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Abstract

Lipid carriers were prepared by thin film hydration technique using various types of lipids, cholesterol and incorporating the drug in different weight ratios. The size and physical stability for the designed lipid carriers as well as the influence of the lipidic material and the experimental conditions on the entrapment efficiency were closely followed.

All results showed that incorporation of low soluble drugs in lipid carriers is high when the appropriate formulations are used. The experimental studies were focused upon investigating the variations in the lipidic carriers characteristics (entrapment efficiency, size and stability) depending on the formulation parameters (weight ratio of phospholipids, cholesterol and drug). It was therefore shown that, by modifying the formulation, lipidic carriers with entrapment efficiencies over 90% can be obtained.

Considering their stability, the lipid carriers content in incorporated low soluble drug was stable for one month, when stored at 4-6ºC.

Rezumat

Transportorii lipidici au fost preparaţi prin tehnica hidratării filmelor lipidice subţiri folosind diferite rapoarte de masă între lipide, colesterol şi substanţă activă. A fost urmarită influenţa naturii materialului lipidic, a condiţiilor experimentale de preparare asupra eficienţei de încorporare, dimensiunii şi stabilităţii fizice a sistemelor transportoare proiectate.

Rezultatele obţinute demonstrează că folosind formulări adecvate, încorporarea în veziculele lipidice proiectate este mare. Studiile experimentale au urmărit modificările caracteristicilor veziculelor lipidice (eficienţa de încorporare, dimensiunea perticulelor şi stabilitatea acestora) funcţie de parametrii de formulare (raportul masic între fosfolipide, colesterol şi substanţa activă). S-a observat că, prin modificări corespunzătoare ale formulării se poate obţine o eficienţă de încorporare de peste 90% pentru transportorii lipidici testaţi.

Din punct de vedere al stabilităţii, transportorii lipidici proiectaţi au fost stabili pe perioada analizată (1 lună) în condiţiile menţinerii la frigider, la o temperatură de 4-6ºC.

Keywords: lipidic carriers, testosterone, entrapment efficiency, stability

Introduction

Recently, various transport systems and technologies have been studied, with the main purpose of controlling the drug release rate and to improve the effectiveness and the selectivity of the formulation. During the last decade, both the micro- and the nanospheres, as well as the polymeric micelles, the hydrogel materials and the nanocapsules proved to be efficient for improving the specific drug transportation, lowering the systemic toxicity, supplementing the treatment rate and protecting the active substances from possible biochemical degradation [1].

The vesicles, as transport systems, became of choice in drug delivery, while the lipid vesicles proved themselves to be most valuable in immunology, in the membranes biology, in various diagnosis techniques and even in genetic engineering [2-5]. The lipid transport systems (liposomes, niosomes, sfingosomes, transferosomes, etosomes and the pharmacosomes) are used in order to improve the drug therapeutic index, for both the newly introduced formulations and the existing ones, through encapsulating the drug into the vesicular structure.

Almost 75 years ago, Paul Ehrlich established the concept of the „magic bullet", envisioning a drug delivery mechanism that would deliver drugs directly to targeted cells. Liposomes, spherical vesicles consisting of one or more phospholipid bilayers, were first described in the mid 60s by Bangham and his coworkers. At first, they were used to study biological membranes; several practical applications, most notably in drug delivery, emerged in the 1970s. Liposomes have been widely studied as drug delivery systems due to their relative safety, their structural versatility concerning size, their composition and because of their bilayer fluidity, as for their ability to incorporate almost any molecule, regardless of its structure [6-8]. As drug vectors, they allow the delivery of active compounds, when the routes of administration cause problems: low therapeutic index, reduced specificity, numerous side effects (antitumorals), low stability (proteines), the target inaccessibility (DNA) [9-12]. They can be used to include a lot of lipophilic drugs (into the double lipidic layer), as well as hydrophilic substances (into the aqueous compartment) or amphiphilic ones (using both layers).

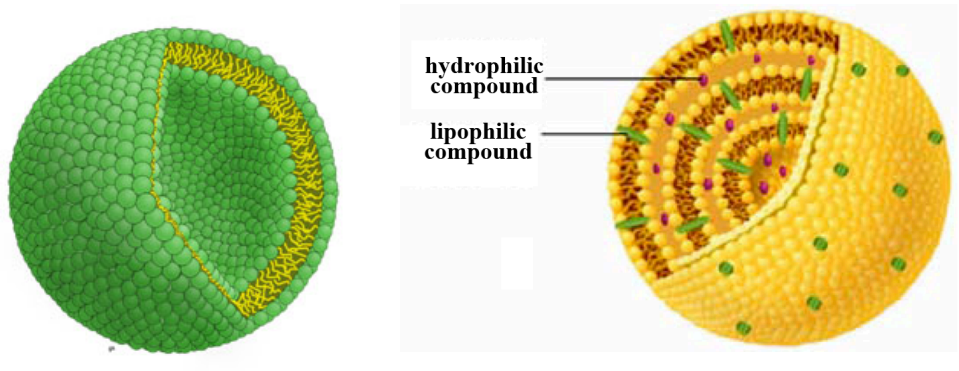

Figure 1
The structure and the incorporating technique of drug into the liposome type lipid structure [13]

Materials and Methods

The following: phosphatidylcholine from fresh egg yolk (PC), dipalmitoylphosphatidyl choline (DPPC), dimiristroylphosphatidyl choline (DMPC), dioleoylphosphatidyl choline (DOPC), dimiristroylphosphatidyl glycerol (DMPG), chloroform p.a. and methanol p.a. were obtained from Sigma. The cholesterol p.a. was purchased from Fluka. The low soluble drug (testosterone - TS) was obtained as a kind gift.

Preparation of liposomes

Multilamellar vesicles (MLV) were prepared according to the thin film hydration technique. Lipid solutions were prepared by dissolving precise amounts of phospholipids, cholesterol and drug in chloroform. 5 mL of each solution were introduced into a 100 mL round-bottomed flask. The solvent was evaporated in a Heidolph Laborota 4000 rotary evaporator**,** at 35 - 40°C, under reduced pressure (13-14 mm Hg). The organic solvent was slowly removed by this process such that a very thin film of dry lipids was formed on the inner surface of the flask. Dried lipid film was hydrated with 5 mL of distilled water. The mechanical stirring of the lipids in aqueous medium was performed with the rotary evaporator equipment at 37°C and, also, through manual stirring in the water bath, for 2 h, at the same temperature. The liposomal suspension was left to mature overnight at 4ºC, to ensure full lipid hydration [14] and in order to anneal any structural defects.

Drug:Phospholipids:Cholesterol ratios and the drug entrapment efficiency were studied [15, 16]. The effect of the various types of

phospholipids used and of the process variables (such as temperature and hydration time) were evaluated.

The MLV liposomes were sonicated in a bath-type sonicator (Sono Swiss SW 6L) for 30 min. The sonication temperature was above the critical temperature (Tc) of the lipids (a temperature under Tc determines structural defects in the bilayers of the liposomes, which conducts to the fusion of vesicles).

Purification of liposomes

The unentrappped drug was removed from the liposomal suspension by centrifugation. The vesicular suspension was centrifugated at 5ºC and at 12000 rpm for 30 minutes, using a Hettich laboratory cooling centrifuge. The supernatant was collected and the liposomes were then reconstituted by using 5 mL of distilled water.

The supernatant appeared as being slightly cloudy and contained a diluted liposomal suspension along with the unentrappped drug. This suspension was again submitted to centrifugation and, immediately after, the supernatant was collected. The operation was repeated for about three times, until obtaining a perfectly clear supernatant. All liposomes were resuspended in distilled water [14, 17].

Determining the amount of drug encapsulated in the liposomes

The quantity of drug encapsulated in the designed vesicles was measured.

In the case of purified liposomes, after the centrifugation, the supernatant was separately collected from the sediment. The supernatant was used to determine the amount of unentrappped drug in liposomes.

The liposomes from the sediment were resuspended in distilled water until further processing. Afterwards, they were soaked in 10 mL of ethanol and sonicated for 10 min. The vesicles were then broken to release the drug, thereby we were able to spectrophotometrically estimate (λ=241 nm) the absorbance for the computed drug content.

The entrapment efficiency was then determined by using the following equation:

$$\%EE = \frac{W - S}{W} x100$$

where: EE – the entrapment efficiency, W – the total amount of drug from supernatant and sediment (measured after disruption of liposomes with ethanol) and S – the quantity of drug from supernatant [18].

Storage stability of the liposomal formulations

The stability studies were carried out throughout a one month period, as it is well known the property of liposomal systems of keeping the incorporated substances, in certain external conditions, for determined periods of time. The experimental tests were focused on the weekly evolution of the size and entrapment efficiency of all experimental liposomes. The dispersions of liposomal formulations were sealed in 10 mL glass vials and stored either at refrigeration temperature (2-8ºC), at room temperature (22±2°C) and at 40°C. At certain time intervals, the liposomes were evaluated considering their drug content and the size distribution. The residual amount of drug in the vesicles was spectrophotometrically determined, after separation from the unentrappped drug [19, 20]. The evaluation of liposomes average size was performed with a particle size analysizer Zetasizer^{TM}.

Results and Discussion

Preparation of liposomes

The influence of the formulation variables upon the liposome properties

Using the described technique, several liposomal formulations were prepared, in which the ratio between the component weight was varied (phospholipids: cholesterol: drug) and their influence on the entrapment efficiency were analyzed. In each case we were able to obtain a milky dispersion of large multillamelar liposomes, that was sonicated for 30 minutes.

As liposomes properties (entrapment degree, stability, active substance release rate) are highly influenced by the nature of lipids used, a series of phospholipids with distinct properties such as: phosphatidylcholine (PC - a natural phospholipid that appears in most of the liposome formulas), the synthetic saturated phosphatidylcholines dipalmitoylphosphatidyl choline (DPPC) and dimiristylphosphatidyl choline (DMFC), as well as dioleoilphosphatidyl choline (DOPC - a synthetic unsaturated phospholipid), dimiristroilphosphatidyl glicerol (DMPG- a synthetic saturated phospholipid negativelly charged), and last, but not least, cholesterol (C) have been chosen. We focused on the nature of phospholipids as they are one main factor in the entrapment efficiency (EE) (Table I). Liposomes characterization was performed immediately after preparation. The influence of certain parameters, such as the hydrating temperature and the hydrating time, on the physical and chemical properties of the designed liposomes (Table II) was studied.

Table I

The influence of the lipidic component nature and of the qualitative ratio in between components on the entrapment efficiency (EE)

Sample	Weight Ratio							*EE (%)*
	PC	*DPPC*	*DMPC*	*DMPG*	*DOPC*	*T*	*C*	
LTS1	10	-	-	-	-	1	1	**59.20**
LTS2	20	-	-	-	-	1	1	**79.10**
LTS3	10	-	-	-	-	1	0.5	**73.70**
LTS4	10	10	-	-	-	1	0.5	**89.30**
LTS5	10	10	-	-	-	1	1	**85.20**
LTS6	-	-	7	3	-	1	0.5	**55.10**
LTS7	10	-	7	3	-	1	0.5	**83.30**
LTS8	10	-	7	-	-	1	0.5	**81.80**
LTS9	10	-	-	-	10	1	0.5	**75.30**
LTS10	10	-	-	-	10	1	1	**67.20**

By analyzing the data shown in Table I we are able to state that:

- an increase in the amount of phospholipids leads to a significant increase in the amount of entrappped substance
- an increase in the cholesterol amount leads to a decrease in the quantity of entrappped substance
- the presence in the liposome formulations of various phosphatidylcholines of synthesis, either saturated or unsaturated (DMPC, DMPG) next to cholesterol, significantly diminishes the value of the entrapment efficiency
- PC presence, along with the previously mentioned components (DMPC, DMGP and C) significantly improves the entrapment efficiency (from 55 to 83%)
- the mixture PC:DMPC:DMPG does not improve the entrapment efficiency, in comparison to the case in which only PC:DMPC is used
- then using unsaturated lipids, the entrapment efficiency is rather small (less than 75% in all experimental versions).

The influence of the lipidic composition on the entrapment efficiency

In order to obtain liposomes with the highest possible degree of entrapment, several batches were prepared, as the sole modification was in the ratio between the lipids and the low solubility substance. In the experimental formulations we also added α-tocopherol (α-TOC) (2%) as stabilizer. The EE experimental values, as the entrapped quantity of TS, are shown in Table II.

Table II

The influence of the lipid component weight ratio on drug entrapment efficiency

Sample	Weight Ratio					EE (%)
	PC	DPPC	C	T	α-TOC	
LTS11	10	10	1	1	0.5	**95.93**
LTS12	10	10	1	2	0.5	**100**
LTS13	10	10	1	4	0.5	**91.83**
LTS14	10	10	2	1	0.5	**85.67**
LTS15	10	10	2	2	0.5	**89.27**
LTS16	10	10	2	4	0.5	**96.73**
LTS17	10	10	6	3	0.5	**80.13**

It is shown that, for the same ratio PC:DPPC:C, a modification of the EE appears, according to the used amount of TS. The EE increases at a mass ratio C:TS most comparable to 1:1, as it later diminishes to another ratio in between the formulation components. Generally, the increase in the cholesterol quantity practically leads to a decrease of EE.

The influence of temperature and of hydrating time upon the designed liposomes properties

The influence of time and hydrating temperature on the entrapment degree was also analyzed. The hydration was performed at temperatures between 40 and 50°C (Table III, formulations marked with "T") while for the other formulations the hydration was performed at room temperature.

Table III

Influence of temperature and hydrating time on the entrapment efficiency

Sample	Weight Ratio					t (h)	EE (%)
	PC	DPPC	C	T	α- TOC		
LTS12	10	10	1	2	0.5	12	**100**
LTS12(T)	10	10	1	2	0.5	12	**96.73**
LTS12(t)	10	10	1	2	0.5	6	**98.93**
LTS11	10	10	1	1	0.5	12	**95.93**
LTS11(T)	10	10	1	1	0.5	12	**84.32**
LTS11(t)	10	10	1	1	0.5	6	**89.88**
LTS16	10	10	2	4	0.5	12	**96.73**
LTS16(T)	10	10	2	4	0.5	12	**88.35**
LTS16(t)	10	10	2	4	0.5	6	**91.81**

Analyzing the data shown in Table III, it's obvious that, for most formulations, an increase in the hydrating temperature leads to a small decrease in the entrapment degree. Meanwhile, it is also shown that the hydrating time does not significantly influence the results.

Purification of liposomes. Studies of the most efficient way in which the liposomes can be separed from the unentrappped substance

In order to establish if the technique of separating the liposomes from the unentrappped material, using centrifugation, is in fact, reproducible and, therefore, applicable in the studies concerning the determination of the entrapment efficiency, a series of parallel tests were performed on two sets of liposomes with the same composition, prepared in the same experimental conditions. The results of these determinations are shown in Table IV.

Table IV

Determination of the entrapment degree, after liposomes were separated from the unentrappped substance, through centrifugation

Sample	No. of Determ.	EE (%)	Mean EE (%)
LTS12	1	98.32	**100.04**
	2	99.68	
	3	102.12	
LTS16	1	98.07	**96.83**
	2	96.78	
	3	95.65	

The data shown in table IV led us to conclude that, in the case of liposoluble substances, alongside some large entrapment coefficients, the centrifugation is recommended as the separating technique. More, based on the gathered results after the tests took place, we can easily state that there is not mandatory to wash the liposomes after the separation process as the differences that appeared in the samples with 0 - 3 washes perfectly fit in between the experimental error limits for the following dosing technique.

Storage stability of the liposomal formulations

The results of the stability study, based on the weekly determination of the entrapment efficiency are shown in Figure 2. The graph shows the percentage of TS that remained in the liposomes, out of the tested suspensions, kept at various temperatures, at 1, 2, 3 and 4 weeks after their preparation.

We see that the liposomes are quite stable in refrigerated storage conditions. The entrapment efficiency has a value close to the initial one even after one month from preparation, in the case of the suspension kept in the refrigerator.

The quantity of TS of the experimental liposomes decreases as the temperature increases. This is due to the modifications in the lipidic bilayers fluidity at higher temperatures (which will lead to great losses of the entrappped drug) or due to some deffects in their membrane, as a result to the effect of temperature on the gel over liquid transition of lipid bilayers together with a chemical degradation of phospholipids.

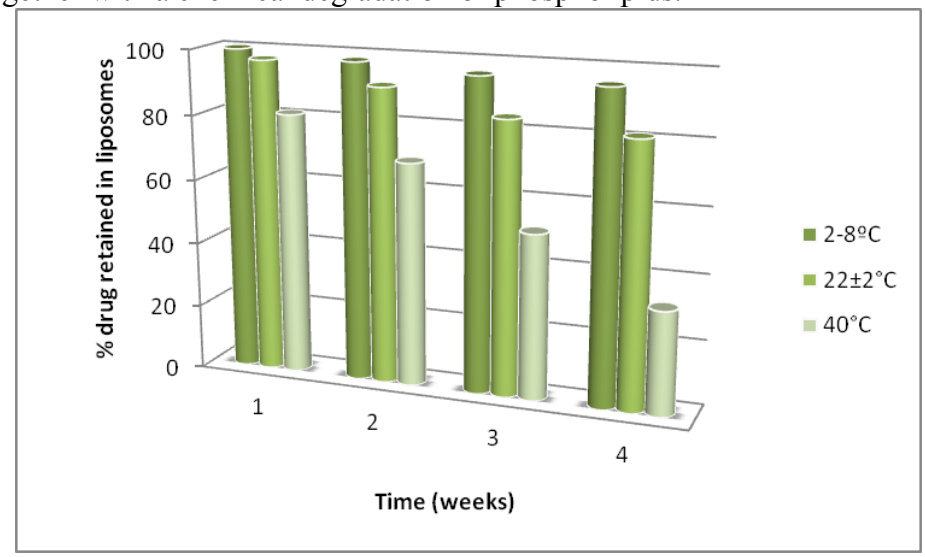

Figure 2

Stability studies - % drug retained in liposomes under different storage conditions

The range of particle size of the designed liposomes was between 190 and 300 nm.

Table V

Stability studies – Modifications in designed liposomes particle sizes under different storage conditions

Sample	t (^{0}C)	Particle Size (nm)		
		1 day after preparation	1 week after preparation	4 weeks after preparation
LTS12	4	194±96.59	202±102.32	208±106.09
LTS16	22±2	247±92.75	263±96.55	293±112.05
LTS17	40	278±105.16	312±117.36	398±125.82

*All values are shown as mean ± SD (n=3).

The analysis of the liposome sizes, performed once every week for every liposomal suspension, kept at the three submitted temperatures, allows us to see that even the particle size suffers a modification similar to the content of entrappped drug. The most significant decrease in liposomes

dimension is recorded at 40°C, while for the suspensions kept in the refrigerator the modifications in size are not significant (Table V).

Conclusions

The paper closely follows the designing of vesicular lipid structures, liposomes, that show a convenient incorporation efficiency, a good polydispersity and a proper stability, for the new therapeutic systems to come. Here, the liposomes were prepared using a thin film hydration technique. By following the way in which the nature of the lipidic material, its composition and the ratio in between its various components directly influences the entrapment efficiency, we can recommend the LTS12 and the LTS16 formulations for testosterone encapsulation. Also, we can state that, in the case of liposoluble substances, with large entrapment coefficients, there is no need to wash the liposomes after separation. The differences that appeared between the analyzed samples lay within the experimental errors of the chosen dosing method.

The stability studies performed have shown that the maximum percentage of drugs was encapsulated at refrigerated temperature of 2 to 8°C. Therefore, the drug entrapment efficiency is due to the phospholipids' ability to create vesicles as they have two non polar bulky lipid chains, as they present a polar head group. Because of the characteristics mentioned above, they are able to form closed bilayer systems. Based on the stability studies carried out, we recommend keeping the liposomal system refrigerated before the final formulation takes place, in order to develop the therapeutic system.

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