# LIPOSOMES AS DELIVERY SYSTEMS FOR PROTEINS

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## ABSTRACT

Proteins were incorporated into different types of liposomes: small unilamellar vesicles (SUV), multilamellar vesicles (MLV), lyophilized multilamellar vesicles (LMLV) and dehydration/rehydration vesicles (DRV). According to the maximal encapsulation efficiency and protein to lipid ratio, LMLV and DRV were selected as the most appropriate systems for protein encapsulation. Protein molecular weight values from 40.000 to 480.000 daltons, do not affect protein encapsulation. Entrapped protein to lipid ratio of liposomes increases with protein concentration at the incorporation step until saturation is reached. Proteins with enzymatic activity were incorporated into liposomes. The activity of the encapsulated form is 50 to 85% that of the free form according to the encapsulation method used. The stability of enzymes encapsulated into liposomes tested in human plasma decreased with incubation time as judged by their activity. However after 84h of incubation 70% of the activity is still present in the encapsulated form.

Key words: Liposomes, protein, delivery system.

## INTRODUCTION

In recent years natural physiologically active compounds, namely peptides and proteins have attracted increasing interest as novel therapeutic agents (1,2). This fact is quite understandable if we consider the number of protein related diseases which, theoretically, can be cured either by replacement of the missing or deficient protein or by protein action. As examples of diseases that can be treated by administration of enzymes, we can refer: metabolic diseases (by administration of the missing enzyme to remove accumulated undesirable substrates); thrombosis and cardiac diseases (by the action of urokinase or streptokinase to solubilize thrombus); cancer (by the enzymatic degradation of nutrientes that are essential for tumor growth) gout, renal deficiency and arthrites (by the degradation of toxic compounds accumulated either in the blood or in joints) (3,4). In spite of the potencialities of enzymatic therapy and the advantages of higher specificity and efficiency of enzymes compared to most of the drugs there are severe limitations to the clinical use of free enzymes. These are due to the low availability and high cost of pure enzymes, poor absortion across epithelia, fast clearance from the organism, quick innactivation under physiological conditions, antigenicity, and impossibility of obtaining high local concentrations without increasing total dose. To overcome these problems, liposomes have been preferentially used as carriers for several kinds of therapeutic agents, including proteins and enzymes, (1,2,5). Comparison of studies concerning protein encapsulation into liposomes are difficult as wide variations are found due to different proteins, methodology and lipid composition of liposomes used.

The purpose of the present paper is to investigate in a systematic way some factors which may affect hydrophilic protein encapsulation into liposomes, retention of enzymatic activity and stability in human plasma.

## MATERIAL AND METHODS

Lipids, albumin and urease were obtained from Sigma.

Liposomes were prepared with egg phosphatidylcholine and cholesterol (2:1, molar ratio) in a protein containing aqueous phase. MLV and SUV were prepared as previously described (6). Briefly a thin layer of dried lipid (30μmoles) was hydrated with apropriate buffers containing 1-30mg of protein. To obtain SUV, the MLV suspension was sonicated for periods of 3 min with intervals of 1 min, the total sonication time being 9 min. LMLV were prepared as described elsewhere (7); a dried film of 10μmoles of lipid was freezed and lyophilized and then aqueous phase containing protein was added. To obtain DRV (8), SUV made of plain lipid (16 μmoles) was added to water, being the mixture freezed and lyophilized followed by another hydration phase with a 10 fold reduced volume. In all preparations, intra and extraliposomal phases were separated by centrifugation at

30.000gfor 30' at 10ºC repeated 3-5 times after suspension of pellet with 0,5M NaCl.

Protein measurements were carried out according to the method of Lowry et.al., with some modifications (9). The total phospholipid content was determined by modified Fisk and SubbaRow technique (10). Enzymatic activity was estimated by following the ammonia production, which was measured by the Berthelot method modified by Chaney and Marbach (11). The media contained 250 mM ureia and 20µg/ml of urease or 20 mM of asparagine and 10µg/ml of asparaginase. Liposomes permeability was estimated by turbidimetry measurements, as described elsewhere (6). Liposomes were added to an isosmotic solution containing the substance whose permeation rates were to be determined. The osmotic swelling of liposomes was followed at 420 nm. The change of-A/min gives the initial rate of swelling and is proportional to the permeability of liposomes to the substance.

The test of stability was performed by incubation of enzymes containing liposomes in human plasma at 37ºC. After appropriate time periods, liposomes and plasma were separated by centrifugation at 30.000g for 30' and enzyme and lipid content analysed in pellet and supernatant.

## RESULTS

### 1 - Effect of incorporation methods

Table 1 shows the characteristics of different types of liposomes and the incorporation of bovine serum albumine into these systems. All multilamellar vesicles have similar apparent internal space (1.9 to 3µl/µmole of lipid), similar population distribution (55% of the liposome diameters range from 0.2 to 0.6µ). The protein encapsulations is strongly dependent on the characteristics of the vesicles and the incorporation method. For a protein concentraition of 1mg/ml at incorporation step, the encapsulation efficiency increases from 1% for SUV to about 10% for LMLV and MLV and to 25% for DRV. The protein concentration inside liposomes increases in the same direction, 7,44,48 and 230µg/ml for SUV, LMLV, MLV and DRV respectively. These vesicles also show the highest protein to lipid ratio (27 µg/ /µmole) and so they seem to be the most appropriate system for proteins encapsulation.

### 2 - Effect of protein molecular weight

Table II shows the effect of the protein molecular weight on the encapsulation of proteins. Proteins of molecular weight ranging from 40.000 to 480.000 daltons were incorporated into MLV prepared with egg lecithin and cholesterol (2:1). For an initial protein concentration of 1mg/ml and a constant protein/lipid ratio the amount of protein encapsulated into liposomes is not affected by the M.W. of protein. In these conditions the protein encapsulation varies from 2.5 to 3.0µg/mg. Also the encapsulation efficiency is not significantly affected by the M.W. of proteins, ranging from 5.8 to 10.8%.

TABLE I

PROTEIN ENCAPSULATION INTO LIPOSOMES

| TYPE OF LIPOSOMES | APARENT INTERNAL SPACE (µl/µmole) | DIAMETER µm (%) | PROTEIN (µg/ml) | PROTEIN/LIPID (µg/µmole) | ENCAPSULATION EFFICIENCY (%) |
|---|---|---|---|---|---|
| SUV | ---- | 0.2 | 7 | 24 | 1 |
| MLV | 1.9 | 0.2-0.6 (55) | 48 | 2 | 9 |
| LMLV | 3 | 0.2-0.6 (52) | 44 | 8 | 8 |
| DRV | 2.8 | 0.2-0.4 (54) | 230 | 27 | 25 |

Apparent internal space represents the volume captured inside liposomes refered to µmol of lipid. Encapsulation efficiency represents the percentage of protein entrapped refered to the total initial protein in the incorporation media.

TABLE II

EFFECT OF MOLECULAR WEIGHT ON PROTEIN ENCAPSULATION

| PROTEINS | MOLECULAR WEIGHT (daltons) | ENCAPSULATED PROTEIN/ /LIPID (µg/mg) | ENCAPSULATION EFFICIENCY |
|---|---|---|---|
| PEROXIDASE | 40000 | 3.0 | 9.0 |
| ALBUMIN | 65000 | 2.7 | 5.8 |
| URICASE | 120000 | 2.8 | 6.7 |
| GLUCOSE OXIDASE | 200000 | 2.5 | 10.8 |
| UREASE | 480000 | 2.5 | 7.1 |

## 3 - Effect of protein to lipid ratio

Figure 1 shows the bahaviour of protein encapsulation and encapsulation efficiency into MLV as a function of the initial protein concentration until saturation is reached, after 25mg/ml. The encapsulation efficiency dropped from 7 to 1% at 1mg/ml and 25mg/ml. Similar behaviour was observed in other systems, DRV and MLMV (data not shown).

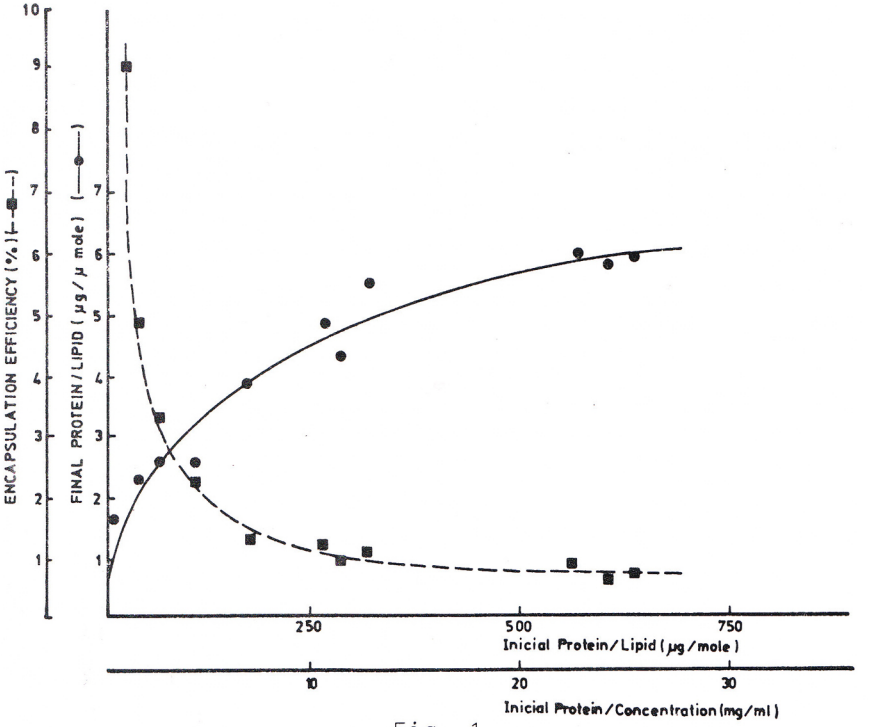


Fig. 1

Effect of initial protein concentration on Final protein to lipid ratio (●) and on Encapsulation Efficiency (■).

## 4 - Encapsulation of biologically active enzymes

Biologically active enzymes, urease and asparaginase, were encapsulated into SUV and MLV.

Table III compares the percentage of enzymes encapsulation and the specific activities of encapsulated and free forms. The measured specific activities of the encapsulated enzymes are lower than that of the free forms for both systmes. But still 50% of the total specific activity was found in the enzymes encapsulated into SUV.

For MLV encapsulated enzymes 65 and 85% of the specific activity was found for asparaginase and urease respectively. The decrease observed for SUV might be due to sonication during the vesicles preparation.

TABLE III

INCORPORATION OF ENZYMES INTO LIPOSOMES

| LIPOSOME | ASPARAGINASE | | UREASE | |
|---|---|---|---|---|
| | ACTIVITY (U/mg) | RECOVERY (%) | ACTIVITY (U/mg) | RECOVERY (%) |
| SUV | 118 | 51 | 95 | 49 |
| MLV | 150 | 65 | 160 | 85 |
| FREE ENZYME | 230 | 100 | 190 | 100 |

The enzymes activity is referred to mg of protein either encapsulated into liposomes or in free form. Recovery represents the percentage of the activity found in encapsulated form comparad to free form.

## 5 - Permeability of liposomes

In order to investigate the possibility of degrading external substrates with the encapsulated enzyme, the permeability of MLV to urea was determined.

Figure 2 shows the decrease in turbidity of urea solutions with time after liposome injection. The permeability is estimated by the initial rate of swelling and given by $\frac{\Delta A}{min}$.

## 6 - Stability of liposomes in human plasma

Figure 3 shows the stability of MLV in human plasma at 37ºC. The percentage of enzyme present in the encapsulated form decreases with time. The maximal decrease is observed after a few hours of incubation in human plasma. The enzymatic activity of liposomes decreased 5 and 10% after 2 and 6 hours of incubation respectively. This decreasing rate is smaller for longer time periods. After an incubation time of 24h, the activity of the encapsulated enzyme is 80% of the initial value and after 84h is still 70%. This decrease is followed by the amount of Lipid in Liposome form which decreases to 70% after 24h and 60% after 84h. By analysing the extraliposomal fluid (upper part of the plot), we can see that, concomitantly with the decrease of lipid and enzyme in liposomes an increase of the

free forms is observed.

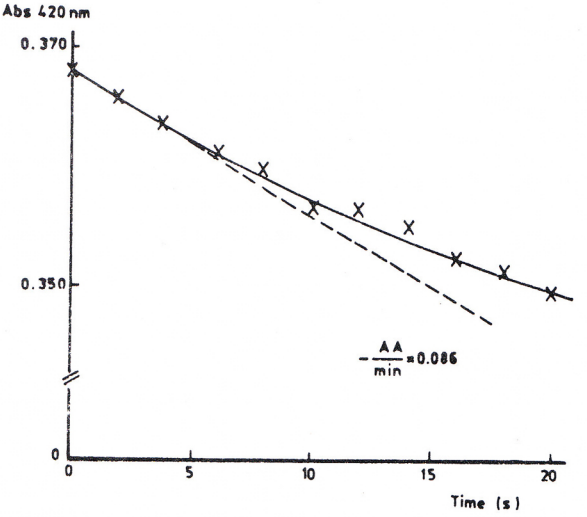


Fig. 2

Permeability of MLV to urea.

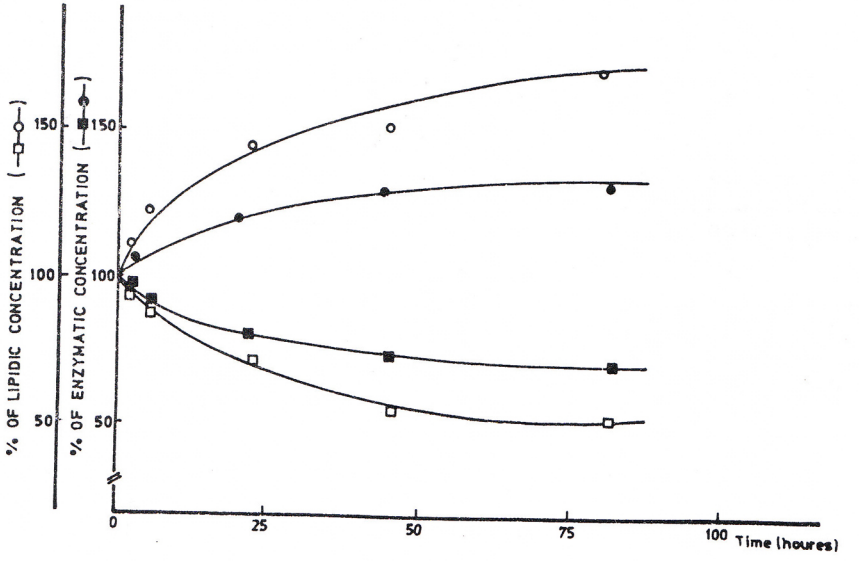


Fig. 3

Stability of liposomes in human plasma. Liposomal lipidic (□) and enzymatic (■) concentration; free lipidic (o) and enzymatic (●) concentration.

## DISCUSSION

The application of liposomes to therapy, requires the knowledge of the factors that affect their physical and chemical properties either on storage, *in vitro* and *in vivo* (12). The present study demonstrates that, within a similar classe of vesicles (multilamellar), the methods of encapsulation, namely the hydration phase, is critical in order to obtain a high protein concentration inside liposomes with high recoveries. Also protein concentration at the hydration phase influences the protein encapsulated either in MLV, LMLV and DRV. For a low concentration at the hydration phase, M.W. does not influences protein encapsulation. Lyophilized systems (LMLV and DRV) present the advantage of higher protein concentration and recoveries when compared to the classical MLV (13,14). Besides, as the encapsulation method includes lyophylization steps, it is possible to store the systems for long periods before use.

The decrease on encapsulated enzymatic activity observed in the presence of human plasma, accounted by the increase of enzyme and lipid free forms, indicates that, a disruption of liposomes must occur with consequent release of entrapped material. The destabilization of vesicles, generally attributed to the removal of phospholipids by plasma components(15) is strongly dependent on the properties of the lipid matrix of liposomes.

## CONCLUSIONS

Our results demonstrate that several factors affect protein encapsulation into liposomes. Maximization of encapsulation and stability can be achieved by a correct choice of several parameters and lipid composition (on progress).

We hope that, the free availability of pure enzymes by recent advances on Biotechnology, improved methodology, for proper evaluation of liposomes associated enzymes behaviour, and the development of appropriate liposome systems for enzymes encapsulation will open a new generation of enzymes as Novel Therapeutic Agents.

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