Encapsulation of a RIP II protein in liposomes

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Introduction

Liposomes, artificial spherical vesicles formed by the self-assembly of lipids, consist of closed lipid bilayers which encompass an aqueous core (Lian et al., 2001).

Liposomal preparations are generally based on phosphatidylcholine (PC), either the natural mixtures obtained from soybean or egg yolk, or chemically defined compounds. We specially focused on liposomes made from DOPC/Chol/DPPC lipid mixtures. Ternary mixtures of DOPC, cholesterol and DPPC lipids have became quite interesting for researches in recent years (Veatch 2003; Stottrup 2005). Two coexisting liquid phases over a wide range of lipid composition and temperature were found, one, so-called liquid-disordered phase, rich in the unsaturated lipid and the other, the liquid-ordered phase, rich in the saturated lipid and cholesterol. Although disoredered phase resembles the pure lipid fluid, ordered phase has intermediate properties between those of pure phospholipid fluid and gel. Behaviour of different membranes made from unsaturated, saturated phospholipids and cholesetrol have been thoroughly characterized in terms of physical properties. However, the nature and concentration of the encapsulated drug may also be of importance for molecular organisations and interactions within a bilayer and/or between membranes. In this regard, we carry out a systematic study of lipsomes encapsulationg a protein. Mistletoe lectin (ML), one of the RIPs proteins was used as a model protein.

Mistletoe lectin (ML) is a ribosome-inactivating protein (RIP) of type II isolated from Viscum album and is a major component of therapeutically relevant substances present in commercially available mistletoe extracts applied for the treatment of human cancer (Krauspenhaar 1999). All RIPs have in common to be potent inhibitors of eukaryotic protein biosynthesis.

Materials and Methods

The phospholipids were dissolved in methanol to prepare stock solutions. The adequate quantities of stock solutions and phospholipids and cholesterol needed for production of 20 mM or 100 mM liposomes were mixed together in a round bottom flask, with the addition of chloroform needed to dissolve cholesterol. The mixture of organic solvents was removed on rotary evaporator (Rotavac Senso Heildorf). The obtained dry lipid film was again redissolved in pure chloroform and then dried while rotating to form a thin, homogenous film, which was exposed to vacuum for at least 30 min to remove any remaining solvent. After an even and uniformly dry lipid film was obtained, the dry lipid layer was hydrated with PBS buffer containing ML in the desired concentration with gentle rotation for about 30 min. The exact composition is given for each experiment. Liposome suspensions were then exposed to a freeze-thaw-mixing process, freezing by immersion in liquid nitrogen, thawing at room temperature and then mixing by gentle rotation. The freeze-thaw-mixing process was repeated three times. This process is used to help break up multilamellar vesicles and to

promote the mixing of the enclosed contents with the exterior solution. All batches of vesicles were extruded through a set of two polycarbonate membrane filters (Whatman, Maidstone, UK) in series to avoid ruptures. First, 21 passes were performed with a nominal pore diameter of 200 nm, followed by 21 passes through 80 nm pores with an hand driven Liposofast extruder (Avestin Europe, Mannheim, Germany) with 0.5 ml gas tight glass syringes (Hamilton, Reno, Nevada, USA).

After preparation, liposomes were purified by gel permeation chromatography to separate encapsulated ML from free ML in the extravesicular solution. The separation of liposomes from free ML molecules was carried out on a gel permeation column (Hema Bio 1000, 300 mm x 8 mm diameter, 10 μm particle size, porosity 1,000 Å, PSS, Mainz, Germany). Quantification of lipids in the fractions was performed in two ways: a) light scattering and b) fluorescence emission measurements. Light scattering of liposomes in collected fractions was measured at 400 nm at 90° in a squared cuvette of 1 cm² cross section, while the fluorescence measurements of the lipid marker NBD-PE were performed using an excitation wavelength of 463 nm and an emission wavelength of 536 nm, both on a spectrophotometer (Perkin-Elmer 650-40). Light scattering at 400 nm depends on parameters such as lipid composition, concentration, size. Therefore, to compare different fractions, only the relative scattering intensity (compared to the intensity of the unfractionated vesicle solution of the same batch) can provide valid information. ML concentrations in the fractions were determined using an enzyme-linked immunoasorbent assay (ELISA) and a 96-well plate spectrometric reader Sunrise (Tecan, Crailsheim, Germany). In addition, fractions containing liposomes were treated with Tween 20 to destruct the lipid bilayer and subsequently quantify the released material by ELISA.

The particle size was measured by Photon correlation spectroscopy (PCS) using Zetasizer 3 (Malvern Instruments, Herrenberg, Germany) particle analyser. Vesicles suspensions were analysed by Cryo-TEM (CEM 902, Zeiss).

Results and Discussion

The ML content of intact liposome preparations was measured to determine the total amount of ML entrapped. Results of encapsulation efficiencies for different liposomal compositions are compiled in Table 1. Most of data present the average of three to six batches with 15-100 μg/ml initial ML concentration, since we observed that the initial ML concentration added to dry lipid film, prior to liposome formulations does not affect on final entrapping efficiencies. In general, the lipid structure such as chain length, chain unsaturation, headgroup type and cholesterol component, as well as various environmental conditions, such as temperature and pH affect the trapping capability of liposomes. The main conclusion drawn from our measurements is that DPPC contributes the most to capture capabilities of bilayer membranes. Increasing DPPC content, while decreasing unsaturated DOPC portion and keeping constant cholesterol ratio (30 mol%), we succeed to achieve a better ML retention inside the aqueous core of liposomes, from 0.23±0.16 % encapsulation efficiency for 60:30:10 mol% DOPC/Chol/DPPC to 0.80±0.19 and 1.54±0.12 % for 40:30:30 and 20:30:50 mol% DOPC/Chol/DPPC, respectively (Table 1).

We observed that repetitively freezing/thawing cycles significantly contributed to the enhancement of the drug uptake. For all checked samples, after applying three freeze-thaw cycles, EE higher than 1% were obtained. Freeze/thawing procedure causes internal solute concentrations that are much higher than those present initially in the vesicle forming solution, as well as more homogenously distributed. Bilayer flexibility, capacity of reorganization during the freeze and thawing cycles and affinity with the ML contribute to the differences observed with the three liposomal formulations. Mixtures of palmitic and oleoyl acyl chains in different portions provide different order and rigidity

to the bilayer. During vesicle formation, solutes which are bound to phospholipids destined to become part of the inner monolayer will become trapped when the vesicle closes. Those features may alter their response to perturbation and to ML loading as it may happen during the freeze and thawing cycles. Thus, solute-phospholipid binding interactions are another factor to consider when interpreting entrapment ratios.

Lipid composition	Molar fractions [mol%]	Conc. [mM]	F/T	Mean diameter [nm] ± s.d. (n)	PI ± s.d. (n)	EE [%] ± s.d. (n)
DOPC/Chol/DPPC	60:30:10	20	- +	134.0±9.5 (4) n.a.	0.11±0.042 (4) n.a.	0.23±0.16 (4) 1.01±0.15 (3)
DOPC/Chol/DPPC	40:30:30	20	- +	165.0±4.0 (5) n.a.	0.18±0.038 (5) n.a.	0.80±0.19 (5) 1.61±0.16 (3)
DOPC/Chol/DPPC	20:30:50	20	- +	187.4±12.4 (4) n.a.	0.21±0.075 (4) n.a.	1.54±0.12 (4) 2.2±0.14 (3)
DOPC/Chol/DPPC	40:30:30	100	+	146.2±3.5 (2)	0.12±0.2 (2)	3.11±0.45 (2)

n.a. – not analysed
F/T: freeze/thaw cylces
- numbers in brackets represent number of samples used for averaging of data

Table 1. Effective diameters with polydispersity indexes, entrapment (EE) of liposomes encapsulating mistletoe lectin made from different lipids or lipid mixtures.

Poor binding affinity of MLs to phospholipid molecules as well as large dimensions of ML monomer and dimer molecules makes them difficult to integrate in aqueous compartments of lipid vesicles. Therefore, encapsulation efficiencies were below 4% for all investigated lipid mixtures.

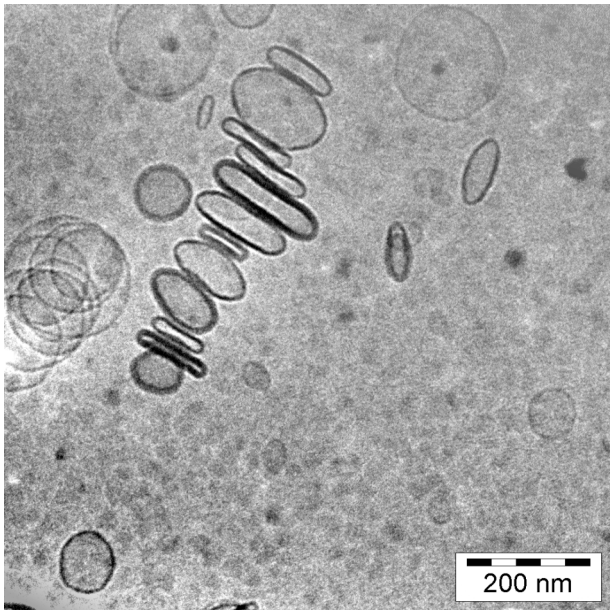

Figure 1. Cryo-TEM images of DOPC/Chol/DPPC liposomes loaded with mistletoe lectins.

PCS revealed liposomes with a polydispersity index PI ≤ 0.21 for all investigated lipids and lipids mixtures used. Compared to other reports of liposomes produced by extrusion through 80 nm membranes (Frisken et al., 2000), the obtained liposomes are considerably larger. The increase in liposome size could be due to the creation of a more swollen membrane structure in the presence of

ML molecules. The increase in DPPC led to significant increase in diameter, from 134±9.5 for 60:30:10 mol% DOPC/Chol/DPPC to 165±4 and 187.4±12.4 nm, for 40:30:30 and 20:30:50 mol%, respectively. The PI also increases with the molar content of DPPC, from 0.11 for 10 mol% to 0.18 and 0.21 for 30 and 50 mol%, respectively (Table1).

Cryo-TEM analyses were performed to gain information on the form and structure of liposomes, as well as on morphological changes and aggregation phenomena taking place upon interaction with ML. Cryo transmission electon microscopy shows that with an increase of the saturated phospholipid DPPC the number of deformed discoidal SUV augments and that these discoidal SUV tend to aggregate in piles, see figure. Aggregation is proportional to the number of viscumin molecules per vesicle for the same lipid mixture. For 30 mol% DPPC only about one viscumin molecule is needed to induce adherence of two vesicles. Aggregation is reversible as is disappears upon dilution and can also occur without proteins, e.g. for membranes with DSPC/cholesterol = 6:4. It is therefore assumed that aggregation is produced by hydrophobic interaction of the bilayer membrane.

Conclusions

The extrusion of raw liposomes followed by freezing/thawing cycles is a suitable preparation method for the generation of liposomal formulations of ML. Encapsulation efficiency depends on the lipid composition; we could show a strong influence of the ratio of saturated to unsaturated phospholipids ratios at an equal cholesterol content of 30 mol%. Thus, an increase in the DPPC/DOPC ratio in vesicle suspensions leads to a better ML uptake, owing to enlargement of liposomes, which are, in addition, less homogenous. This is a less favorable feature of liposomes from an applicational point of view. Another effect of the increase in DPPC content is more pronounced phase separations (domain formation) of the membrane and consequently, visualizable more obvious deformation of initially spherical structures to elongated shapes and formation of agglomerates. In conclusion, the results indicate complex membrane interactions of ternary phospholipid/cholesterol bilayers, where the role of ML molecules in membrane behaviour still needs to be completely resolved.

Bibliography

T. Lian et al. (2001) *Trends and developments in liposome drug delivery systems.* Journal of Pharmaceutical Science 90 667-680

R. Krauspenhaar et al. (1999) *Crystal Structure of Misteltoe Lectin I from Viscum album*. Biochememical and Biophysical Research Communications 257 418-424

S.L. Veatch et al. (2003) *Separation of Liquid Phases in Giant Vesicles of Ternary Mixtures of Phospholipids and Cholesterol*. Biophysical Journal 85 3074-3083.

B.L. Stottrup at al. (2005) Miscibility of Ternary Mixtures of Phospholipids and Cholesterol in Monolayers, and Application to Bilayer Systems. Biophysical Journal 88 269-276

B.J. Frisken et al. (2000) *Studies of vesicle extrusion*. Langmuir 16 928-933.