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Original Research Article

Enhancement of Ribonuclease A encapsulation in egg lecithin–cholesterol liposomes

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Abstract

Purpose: *To characterize Ribonuclease A (RNase A)-loaded liposomes formulated with egg lecithin and cholesterol, and determine the effect of total lipid concentration on particle size and encapsulation efficiency.*
Methods: *Two liposomal formulations of RNase A with total lipid concentrations of 10 mg/mL and 15 mg/mL were prepared. Physicochemical properties, including particle size and encapsulation efficiency (EE), were evaluated for each formulation.*
Results: *Increasing the lipid concentration slightly increased the mean hydrodynamic diameter of the liposomes from 200 to 211.9 nm. The polydispersity index also rose from 18.2 % to 21.5 %. The 10 mg/mL lipid formulation exhibited an encapsulation efficiency of 11.5 %, while the 15 mg/mL formulation showed a higher encapsulation efficiency of 25 %. Increasing the total lipid concentration significantly increased both particle size and EE.*
Conclusion: *Increasing the total lipid concentration improves encapsulation efficiency and particle size of RNase A-loaded liposomes, providing a basis for optimizing liposomal carriers for therapeutic applications.*

Keywords: *Cholesterol, Egg lecithin, Encapsulation Efficiency, Lipid concentration, Liposomes, Particle size, Ribonuclease A*

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INTRODUCTION

Therapeutic proteins and enzymes have attracted increasing attention as promising agents for the treatment of a wide range of diseases. Despite their significant therapeutic potential, their clinical application remains challenging because of poor membrane permeability, rapid enzymatic degradation, and limited circulation time in the bloodstream [1,2]. To overcome these limitations, various nanocarrier systems have been investigated, among which liposomes are considered one of the most effective and versatile delivery platforms [3,4]. Liposomes are spherical vesicles consisting of one or more phospholipid bilayers surrounding an aqueous core. Owing to their biocompatibility and biodegradability, liposomes can protect encapsulated biomolecules from degradation, improve their stability, and facilitate controlled release. These properties make liposomes attractive carriers for the delivery of proteins and enzymes [5-7].

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Ribonuclease A (RNase A) is a well-characterized enzyme capable of degrading RNA and has been investigated for potential therapeutic applications. However, the administration of free RNase A is associated with several limitations. Due to its hydrophilic nature and relatively large molecular size, cellular uptake is inefficient, resulting in limited access to intracellular targets [8]. Furthermore, the enzyme is susceptible to degradation and clearance under physiological conditions, which reduces its therapeutic effectiveness [1,2]. Encapsulation of RNase A into liposomal carriers may help overcome these drawbacks by improving enzyme stability, prolonging circulation time, and enhancing intracellular delivery [9].

It is well known that the composition of the lipid bilayer plays a crucial role in determining the performance of liposomal formulations. Egg lecithin (phosphatidylcholine) is widely used as a primary phospholipid because of its excellent biocompatibility. Cholesterol is frequently incorporated into liposomal membranes to improve structural stability, reduce membrane permeability, and increase bilayer rigidity, which can enhance the retention of encapsulated hydrophilic molecules [10,11]. The cholesterol content of the bilayer therefore strongly influences both the physical properties of the vesicles and their loading capacity [12]. In this study, the effect of the concentration of egg lecithin–cholesterol on the vesicle size and on the encapsulation efficiency of liposomes containing RNase A was investigated, with the aim of developing a stable and effective liposomal delivery system for RNase A.

EXPERIMENTAL

Materials

Liposomes were prepared using a rotary evaporator (Laborota 4000, Heidolph, Germany), a vortex mixer (Vortex-Genie 2, Scientific Industries, USA), and a mini-extruder (Avanti Polar Lipids, USA). Separation of liposome-associated and free RNase A was performed by size-exclusion chromatography using a Sephadex G-50 Fine column. UV absorbance measurements were carried out with a UV-1800 spectrophotometer (Shimadzu, Japan) at 280 nm. The hydrodynamic diameter and polydispersity index of the liposomes were measured by dynamic light scattering using an Anton Paar Litesizer 500 particle analyzer (Anton Paar GmbH, Graz, Austria). Ribonuclease A (RNase A, 20 mg/mL) was purchased from Invitrogen (Thermo Fisher Scientific, USA). Egg lecithin, Sephadex G-50 Fine (Sigma-Aldrich), Phosphate-buffered saline (PBS, pH 7.4). All chemicals and reagents were of analytical grade and used without further purification.

Preparation of liposomes

Liposomes were prepared by the thin-film hydration method followed by membrane extrusion. Two formulations were prepared: a lower-concentration formulation containing 40 mg egg lecithin and 10 mg cholesterol (50 mg total lipid; ratio 40:10), and a higher-concentration formulation containing 60 mg egg lecithin and 15 mg cholesterol (75 mg total lipid; ratio 40:10). The lipids were dissolved in chloroform, and the solvent was removed under reduced pressure at 40 °C using a rotary evaporator to form a uniform lipid film on the wall of a round-bottom flask. The lipid film was further dried under vacuum for 1 h to remove residual solvent traces. Hydration was carried out with 5 mL of 10 mM PBS (pH 7.4) containing RNase A (2 mg/mL), resulting in lipid concentrations of 10 mg/mL and 15 mg/mL, respectively. The resulting suspension was vortexed for 1 h and subjected to five freeze–thaw cycles using liquid nitrogen and a water bath, maintained at 37 °C. The obtained multilamellar vesicles were subsequently extruded eleven times through a 200 nm polycarbonate membrane using a mini-extruder to produce unilamellar vesicles.

Determination of encapsulation efficiency

Encapsulation efficiency (EE) was determined by size-exclusion chromatography on a Sephadex G-50 Fine column (1.5 × 40 cm) pre-equilibrated with 10 mM PBS (pH 7.4). A 0.5 mL aliquot of the liposomal suspension was applied to the column and eluted with the same buffer at a flow rate of 0.5 mL/min; fractions of 1 mL were collected sequentially. The void-volume (liposome) peak and the retarded free-RNase peak were identified using reference runs: blue dextran defined the void volume, empty (protein-free) liposomes confirmed the position of the liposome peak (fractions 5–8), and free RNase A confirmed the position of the free-protein peak (fractions 9–11). Because the absorbance of the liposome peak is dominated by light scattering from the lipid vesicles, encapsulation efficiency was determined from the scattering-free free-RNase peak rather than from the liposome peak. For each formulation, a parallel mixture control was prepared in which RNase A was simply combined with pre-formed liposomes, without encapsulation; in this control all of the protein elutes in the free-RNase peak, which was therefore taken to represent non-encapsulated enzyme. The free-RNase peak of each loaded

sample and of its mixture control was quantified by summing the A280 values of fractions 9–11, and EE was calculated using Eq 1.

$$EE\ (\%) = \{1 - (A_{free,\ loaded} / A_{free,\ control})\} \times 100 \quad \ldots\ldots\ldots (1)$$

where A_{free} is the summed absorbance at 280 nm of the free-RNase fractions. This calculation uses only the free-protein peak and is therefore not affected by the light scattering of the liposomes.

RESULTS

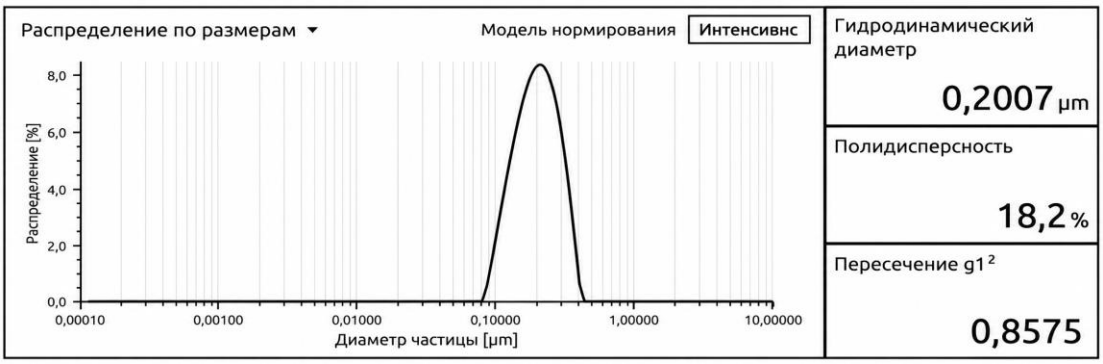
Particle size and polydispersity of liposomes

The larger vesicle size and slightly broader size distribution observed at the higher lipid concentration may be attributed to the greater lipid availability, which can promote the formation of larger vesicles. In addition, the higher cholesterol content of this formulation may have contributed to these changes, as cholesterol is known to increase bilayer thickness and rigidity [10,11]. Dynamic light scattering (DLS) showed that both formulations produced vesicles in the expected sub-micron range with a single, monomodal size distribution (100 % of the intensity in a single peak). The higher-concentration formulation produced larger vesicles, with the mean hydrodynamic diameter increasing from 200 nm to 211.9 nm and the polydispersity index increasing from 18.2 % to 21.5 %. The intensity-weighted size distributions of the two formulations are shown in Figure 1.

Encapsulation efficiency of RNase A in liposomes

The encapsulation of RNase A was assessed by size-exclusion chromatography. For each formulation, the chromatogram of the RNase A-loaded liposomes was compared with that of a non-encapsulated mixture control, in which the protein elutes entirely in the free-RNase A peak (fractions 9–11). A protein-free (empty) liposome run was also recorded as a reference: it shows the void-volume peak (fractions 5–8), which arises purely from light scattering of the lipid vesicles, together with the absence of any free-RNase peak at fractions 9–11. A reduction of the free-RNase peak in the loaded sample relative to the mixture control indicates the fraction of enzyme that was encapsulated. For the lower-concentration phospholipid formulation (10 mg/mL), the encapsulation efficiency was 11.5 %. The chromatograms for this formulation are shown in Figure 2.

(a)



(b)

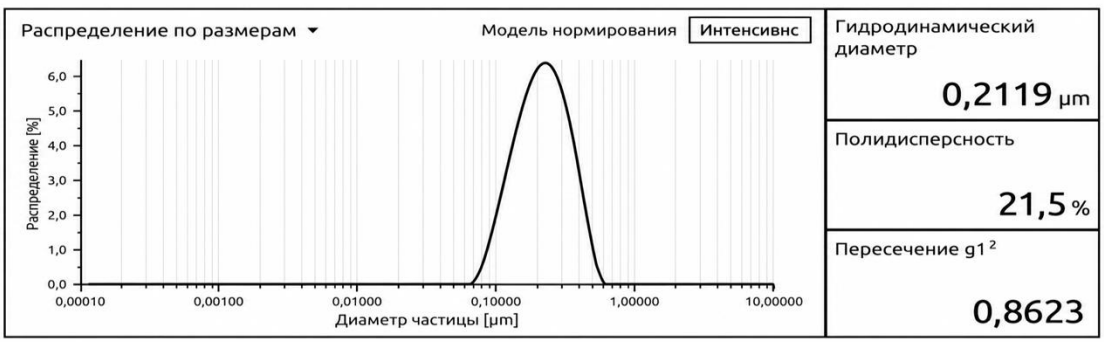


Figure 1: Dynamic light scattering intensity-weighted size distributions of the egg lecithin–cholesterol liposomes: (a) low-concentration formulation (10 mg/mL) and (b) high-concentration formulation (15 mg/mL)

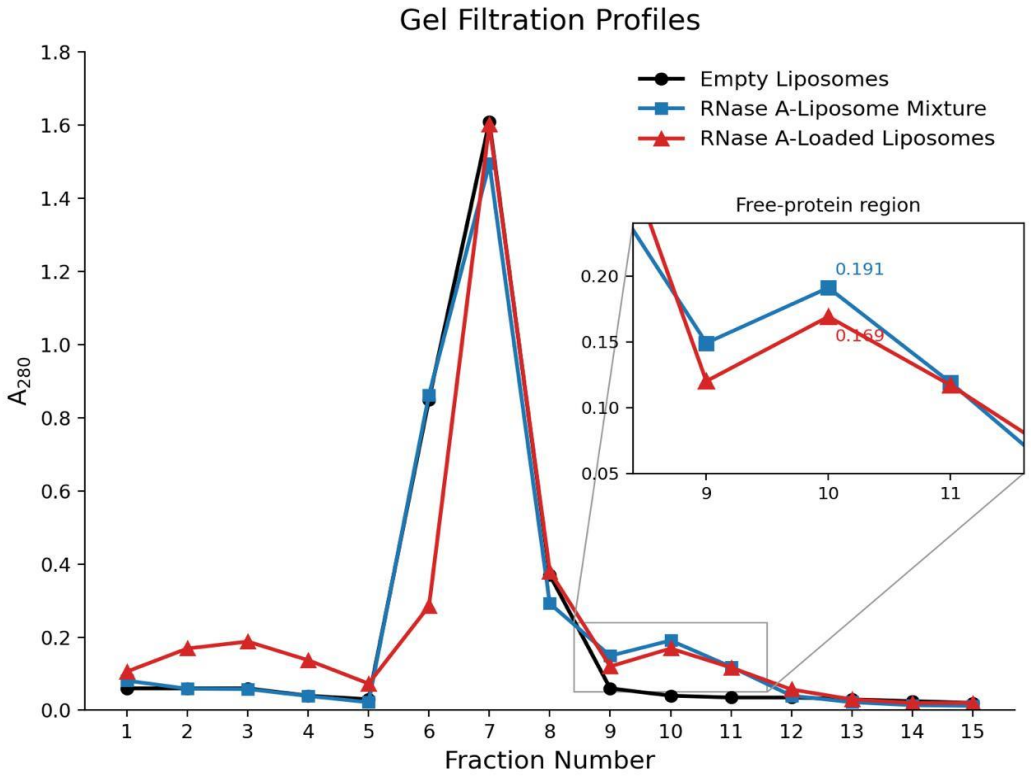


Figure 2: Size-exclusion chromatography profiles of the lower-concentration formulation (10 mg/mL): empty (protein-free) liposomes, the non-encapsulated mixture control, and RNase A-loaded liposomes

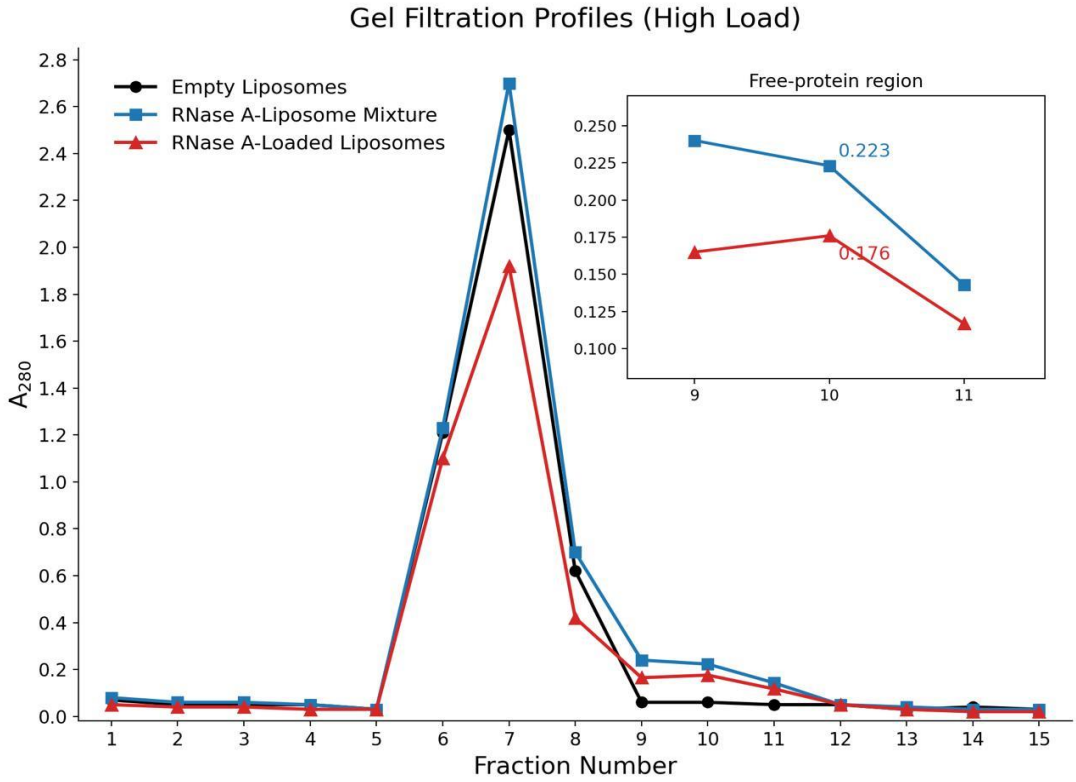


Figure 3: Size-exclusion chromatography profiles of the higher-concentration formulation (15 mg/mL): empty (protein-free) liposomes, the non-encapsulated mixture control, and RNase A-loaded liposomes (mean ± SD, n = 3)

Table 1: Composition, lipid concentration, particle size and encapsulation efficiency (EE) of the egg lecithin–cholesterol liposomes

Formulation	Egg lecithin (mg/mL)	Cholesterol (mg/mL)	Total lipid (mg/mL)	EE (%)	Diameter (nm)	PDI (%)
1 (low)	8	2	10	11.5	200	18.2
2 (high)	12	3	15	25	211.9	21.5

Encapsulation efficiency was obtained from the free-RNase peak (sum of fractions 9–11) of each loaded sample relative to its mixture control. For example, for one replicate of the lower-concentration formulation (10 mg/mL) the free-peak (liposome free) sum was 0.120 + 0.169 + 0.117 = 0.406; while its mixture control gave 0.149 + 0.191 + 0.119 = 0.459. Substituting these values into the equation gives:

$$EE\ (\%) = \{1-(A_{free,\ loaded}/A_{free,\ control})\}100 \quad \ldots\ldots\ (2)$$

$$= \{1-(0.406/0.459)\}\ 100 = 11.5\ \%$$

Increasing the total lipid concentration 10 mg/mL to 15 mg/mL raised the encapsulation efficiency to 25 %. The chromatograms for this formulation are shown in Figure 3.

Encapsulation efficiency was obtained from the free-RNase peak (sum of fractions 9–11) of each loaded sample relative to its mixture control. For example, for one replicate of the higher-concentration formulation, the free-peak sum was 0.165 + 0.176 + 0.117 = 0.458, while its mixture control gave 0.609. Substituting these values into the equation gives:

$$EE\ (\%) = \{1-(A_{free,\ loaded}/A_{free,\ control})\}\ 100 \quad \ldots\ldots\ (3)$$

$$= \{1-(0.458/0.609)\}\ 100 = 25\ \%$$

Averaging the three replicates gave EE of 25 % for the higher-concentration formulation (15 mg/mL), while the lower-concentration formulation (10 mg/mL) evaluated in the same way gave EE of 11.5 %.

Characterization of RNase A-loaded liposomes

The particle size and encapsulation efficiency of egg lecithin–cholesterol liposomes were influenced by the total lipid concentration. Two formulations were evaluated: a lower-concentration formulation containing 10 mg/mL total lipid and a higher-concentration formulation containing 15 mg/mL total lipid. The main physicochemical characteristics of both formulations, including particle size and encapsulation efficiency, are presented in Table 1.

DISCUSSION

Increasing total lipid concentration from 10 to 15 mg/mL approximately doubled the estimated RNase A encapsulation efficiency, with only a small increase in hydrodynamic diameter. Since the hydration volume and protein concentration were unchanged, the initial lipid-to-protein mass ratio increased from 5:1 to 7.5:1. Greater lipid availability may have favored aqueous entrapment or protein association during vesicle formation. Colletier *et al.* similarly observed concentration-dependent encapsulation of acetylcholinesterase, although differences in protein and lipid composition limit direct comparison [13]. The lecithin-to-cholesterol ratio remained 80:20 in both formulations; therefore, the higher EE cannot be attributed to an increased cholesterol fraction. Both formulations had mean diameters close to the 200 nm extrusion pore size. The higher lipid concentration also produced a slightly broader size distribution. Although each preparation showed a single DLS peak, this does not establish unilamellarity. Multilamellar structures can persist after extrusion, as demonstrated by Nele *et al* [14].

Comparisons with other loading studies require similar caution. Ullmann *et al* achieved high encapsulation efficiencies using a nanoemulsion transfer method [15], but these values are not directly comparable with those obtained here by film hydration. The indirect EE measurement also depends on comparable protein recovery from the loaded samples and controls. Reduced free-protein recovery could overestimate encapsulation, while surface-associated protein cannot be distinguished from internally entrapped RNase A.

The higher-loading formulation provides a starting point for further evaluation. Protein recovery, retained enzyme activity and storage stability should be established before assessing biological performance. For intracellular applications, membrane interactions and cytosolic delivery present additional requirements [16]. The present results support further formulation work but do not yet demonstrate improved delivery of active RNase A.

CONCLUSION

The characterization of RNase A-loaded liposomes revealed that total lipid concentration plays a pivotal role in determining particle size and encapsulation efficiency. The higher lipid concentration formulation results indicate that lipid content must be carefully controlled to optimize liposomal performance. The enhanced EE at higher lipid concentrations suggests improved loading capacity and potential stability of the liposomal carriers. These insights contribute to the rational design of effective liposomal delivery systems for RNase A and other therapeutic enzymes, emphasizing the importance of formulation parameters in achieving desired encapsulation and stability outcomes.

DECLARATIONS

Acknowledgement/Funding

None.

Ethical approval

Not applicable.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

Aygul Yarilkaganova and Oybek Ashirov. participated in all experiments and contributed to manuscript preparation; O. All. Otabek Allaev participated in all experiments; H.P. participated in preparation of reagents; T.S. participated in measuring size of liposomes and S.A. provided overall supervision and guidance for the research.

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