

Role of Cholesterol in Modifying the Physical and Stability Properties of Liposomes and In Vitro Release of Vitamin B12 [†]

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Abstract: Cholesterol has garnered significant attention in research due to its role in the structure and the fluidity/rigidity of phospholipid membranes. This property makes it an essential component in liposome formulation. Finding the right ratio of phospholipid-to-cholesterol is important for making a liposome formulation that is stable and functional. This study involved the investigation of various mass ratios between phospholipid and cholesterol. The resulting formulations were characterized in terms of mean particle size, size distribution, and ζ potential. It was observed that as the cholesterol content increased, the mean particle size also increased, with the stability of the suspensions improving up to a certain point, after which stability decreased. The optimal phospholipid-to-cholesterol ratio of 5:1 was identified and chosen for subsequent studies on the encapsulation of vitamin B12. The vitamin was encapsulated in the liposomes in the amount of 37%, and the controlled release of vitamin B12 under gastrointestinal conditions was demonstrated using the liposomes as a carrier.

Keywords: cholesterol; liposome; phospholipid; vitamin B12; controlled release



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1. Introduction

Liposomes are widely used as carriers for the encapsulation of nutraceuticals and have been extensively studied for their potential in targeted drug delivery and controlled drug release systems [1]. These lipid carriers are simplified models of biological membranes, composed of a mixture of lipids. In addition to their use in drug encapsulation, liposomes are being increasingly explored for incorporation into food for functional applications [2]. When it comes to vitamin encapsulation, various systems such as emulsions, micro/nanoemulsions, and solid lipid particles have been widely studied. However, liposomes have garnered significant attention as carriers for vitamin encapsulation due to their numerous advantages, including biocompatibility, versatility, targeted delivery, controlled release, and ease of preparation. Liposomes are typically composed of amphiphilic lipid molecules like phospholipids and sterols, with phospholipids closely resembling those found in cell membranes, making them less likely to cause adverse reactions. Despite their versatility, liposomes are prone to physicochemical instability. The lipids in their structure can degrade through oxidation or hydrolysis, and the particles themselves can form aggregates. Initially, liposomes exhibit repulsive forces between particles that provide some physical stability, but external factors such as high temperatures or pH changes

can alter the structure, affecting the bilayer's permeability and potentially leading to the release of the encapsulated compound or the formation of aggregates. Hydrolysis of ester bonds in phospholipid bilayers and the peroxidation of unsaturated acyl chains can occur, resulting in the formation of short-chain lipids and soluble derivatives. These changes can compromise the quality of liposomal products [3]. To ensure the stability of liposome formulations for both pharmaceutical and food applications, it is crucial to enhance their structural integrity. One approach is the incorporation of cholesterol, which influences membrane rigidity. Many studies have shown that cholesterol increases the packing of phospholipid molecules, reduces bilayer permeability to non-electrolytes and electrolyte solutes, improves vesicle resistance to aggregation, and alters fluidity to make the vesicles more rigid, thus allowing them to withstand high shear stress. However, the ratio of phospholipids-to-cholesterol significantly impacts liposome stability, particle size, particle size distribution, encapsulation efficiency, and controlled release [4].

Given the limited data in the literature on the influence of the phospholipid-to-cholesterol ratio on liposome formulations, this study explores the effect of various phospholipid and cholesterol concentrations on the mean particle size, zeta potential, and particle size distribution. After identifying the optimal ratio, vitamin B12 was encapsulated in the selected formulation, and its stability under gastrointestinal conditions was evaluated.

2. Materials and Methods

2.1. Materials

Soy-PC (L- α -phosphatidylcholine 95%, i.e., lecithin) was purchased from Avanti Polar Lipids (Alabaster, AL, USA), and cholesterol, cobalamin (vitamin B12), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Aldrich (Burlington, MA, USA). Chloroform was obtained from Merck (Darmstadt, Germany). In vitro gastrointestinal digestion of nanoliposomes was assessed using the pepsin (Sigma Aldrich, Burlington, MA, USA), bile salts (Biolife Italiana S.r.l, Milan, Italy), and pancreatin (MP Biomedicals, Illkirch-Graffenstaden, France). All chemicals were used as received without further purification.

2.2. Methods

2.2.1. Preparation of Liposomes

Liposomes were prepared using the thin-film hydration method, as previously described by Pavlović et al. [5]. Phospholipid-to-cholesterol mass ratio of 20:1, 9:1, 7:1, 5:1, and 4:1 have been used. A mixture of lipid and cholesterol was dissolved in chloroform and dried to form a thin lipid film under vacuum using a rotary evaporator (Rotavapor® R-210, BÜCHI Labortechnik AG, Flävil, Switzerland) at 50 °C and 323 mbar. After chloroform evaporation, the flasks with thin films were placed on a vacuum system for 1 h at 40 °C to remove residual chloroform, and then the thin film was hydrated with HEPES buffer pH 7.4. After hydration, five heating (60 °C) and cooling (room temperature) cycles were performed for 2 min each, with intensive vortex during the cooling cycles, using 3 mm diameter glass beads. Liposome size reduction and standardization were performed using high-intensity ultrasound waves generated by an ultrasound probe (frequency: 20 kHz, amplitude: 30%) (Sonopuls MS72, Ultrasonic Homogenizers, HD 2200 Bandelin, Germany) in three cycles. Each cycle included 20 s of sonication followed by 1 min of cooling. During sonication, the liposome suspension was maintained at a constant temperature of 5–6 °C to avoid thermal effects.

The phospholipid mass was then optimized following the selection of the most suitable cholesterol mass. The preparation procedure was conducted in the same manner, except that 0.075, 0.150, 0.300, and 0.450 g of phospholipids were dissolved in 5 mL of chloroform.

When the most suitable phospholipid-to-cholesterol ratio was selected, vitamin B12 was encapsulated in the liposomes. The thin lipid film was hydrated with a solution of vitamin B12 (3 mg/mL) in HEPES buffer (pH 7.4). After the hydration phase, the remaining procedure was carried out as described above.

2.2.2. Liposome Physicochemical Characterization

The size distribution (mean diameter and polydispersity index) and the zeta (ζ) potential of the liposomes were measured by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments Ltd., Malvern, UK), which provided the mass distribution of particle size as well as electrophoretic mobility. Using pure deionized water, liposome samples were first diluted 50-fold. Measurements were made at 25 °C with a fixed angle of 90°. The average of at least 8 consecutive measurements was used for particle size analysis, with each measurement being the average of 14 individual readings.

2.2.3. Encapsulation Efficiency

The mass of encapsulated cobalamin was determined indirectly by measuring the concentration of non-encapsulated cobalamin in the fabricated nanoliposome suspension, which was centrifuged at 40,000 rpm for 30 min at 4 °C (Optima™ L-100 XP Ultracentrifuge, Beckman Coulter, Brea, CA, USA). Cobalamin encapsulation efficiency (*EE*) was calculated as the ratio of the mass of cobalamin introduced at the beginning of the particle preparation process to the unencapsulated cobalamin mass measured in the supernatant. Encapsulation efficiency (*EE*) was calculated using the following equation (Equation (1)):

$$EE(\%) = \frac{\text{Total amount of B12} - \text{amount of B12}_{sn}}{\text{Total amount of B12}} \cdot 100 \quad (1)$$

where *B12_{sn}* represents the cobalamin mass in the supernatant.

2.2.4. Vitamin B12 Release Under Simulated Gastrointestinal Conditions

In vitro gastrointestinal (GI) digestion catalyzed by pepsin and pancreatin was performed in a batch system, following the method described by Liu et al. [6] with minor modifications. Gastric juice was prepared by dissolving 0.5 g of NaCl in 1.25 mL of 6 M HCl solution, adding 200 mL of distilled water, and adjusting the pH to 1.4–1.5 and the volume to 250 mL. The stock solution was incubated for 30 min at 37 °C in the incubator shaker (Incubator shaker KS 4000 i control, IKA-Werke GmbH & Co. KG, Staufen, Germany). Gastric juice was prepared by dissolving pepsin (3.2 mg/mL) in the preheated stock solution. Pancreatic juice was prepared by dissolving 6.8 g of K₂HPO₄ in 190 mL of 0.1 M NaOH solution, adjusting the pH to 7.4 and the volume to 1000 mL. After that, the bile salts at a concentration of 0.2 mg/mL were added. This solution was incubated at 37 °C for 30 min with constant stirring. The pancreatin solution was prepared immediately before use by dissolving pancreatin (3.2 mg/mL) in the preheated stock solution.

For digestion, 1 mL of liposome was mixed with the corresponding digestive juice (20 mL). Pepsin-catalyzed digestion was carried out at 37 °C while stirring at 120 rpm for 1.5 h, followed by pancreatin-catalyzed digestion at 37 °C while stirring at 150 rpm for 4 h. After each sampling (0.2 mL), the same amount of heated gastric and pancreatic juice solution was returned to the vessel. The proportion of released vitamin B12 was determined spectrophotometrically by measuring the change in absorbance at 350 nm in each sample, calculated as the ratio of the mass of vitamin released to the mass of the ingested vitamin B12.

3. Results and Discussion

3.1. Effect of Cholesterol on the Physical Characteristics of Liposome

Table 1 shows the results describing the effect of cholesterol on the physical characteristics of liposome.

Table 1. Influence of different mass ratios of phospholipid-to-cholesterol (PC:Chol) on the particle properties of the liposomal formulation.

Phospholipid, g	Cholesterol, mg	Mass Ratio PC:Chol	Particle Size, nm	PdI	ζ Potential, mV
0.150	7.5	20:1	311.3	0.521	−15.4
	16.7	9:1	323.6	0.515	−18.7
	21.4	7:1	347.2	0.533	−18.8
	30.0	5:1	380.0	0.424	−21.7
	37.5	4:1	422.7	0.498	−14.4
0.075	30.0	2.5:1	323.4	0.518	−9.6
0.300		10:1	425.8	0.514	−14.2
0.450		15:1	522.4	0.585	−16.3

The results shown in Table 1 indicate that as the cholesterol mass increases, the mean diameter of the obtained particles also increases. Specifically, increasing the cholesterol mass from 7.5 mg to 37.5 mg resulted in an increase in mean particle size from 313 nm to 422 nm. The introduction of cholesterol into the lipid bilayer leads to the formation of both cholesterol-depleted and cholesterol-rich domains within the membrane, which then coalesce into larger vesicles. Cholesterol incorporation increases Van der Waals forces between lipid particles and alters short-range repulsive interactions. As a result, the inclusion of cholesterol likely contributes to changes in particle size [7]. Additionally, cholesterol molecules improve the permeability barrier properties of the lipid bilayer. They orient themselves in the bilayer with their hydroxyl groups directed toward the polar head groups of the phospholipid molecules. By reducing the mobility of the first few CH₂ groups of the phospholipid hydrocarbon chains, cholesterol makes the lipid bilayer less deformable in this region, reducing the permeability of the bilayer to small, water-soluble molecules.

The particle size distribution, i.e., polydispersity index (PdI) value, indicates that the most uniform distribution was achieved with 30 mg of cholesterol, while a mass of 37.5 mg resulted in a bimodal distribution. As seen in Figure 1, a uniform distribution is present only in the sample with 30 mg of cholesterol. All other samples exhibited a bimodal distribution with two peaks, which is reflected in the PdI values. Based on the results in Table 1, it can be seen that the most optimal mass ratio of PC:Chol is 5:1 when the volume of the hydration medium is 5 mL.

The incorporation of cholesterol into the system also leads to an increase in the negative zeta potential up to a cholesterol mass of 30 mg. However, at 37.5 mg, the zeta potential begins to decrease, suggesting that aggregate formation and a decrease in particle stability may have occurred. The increase in stability, particularly the negative zeta potential, with the introduction of cholesterol, is justified. Cholesterol incorporated into the bilayer reduces the surface binding affinity. At high activation energy, the phospholipid bilayer is in a gel phase because the presence of cholesterol reduces intermolecular Van der Waals forces [8]. This lowers activation energy and increases bilayer fluidity. However, once the cholesterol content exceeds a certain threshold, its effect on increasing the viscosity of the lipid bilayer becomes more dominant. This explains the observed decrease in phospholipid bilayer fluidity. The probable cause for this is the supersaturation of phospholipid molecules, leading to decreased stability and aggregation formation.

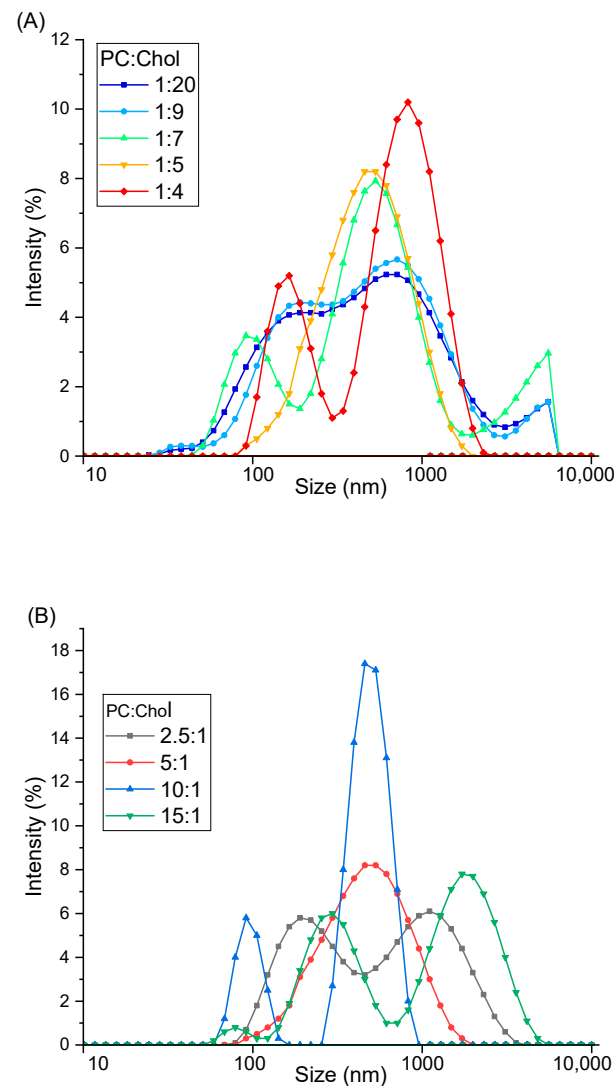


Figure 1. Particle size distribution of different mass ratios of phospholipid-to-cholesterol: fixed mass of cholesterol (A), and fixed mass of phospholipid (B).

Regarding the variation in phospholipid mass, increasing the mass from 0.075 g to 0.450 g results in an increase in average particle size from 323 nm to 522 nm. This can be attributed to the higher concentration of phospholipids, which occupy more space in the aqueous medium, formulating larger particles. The sample with 0.075 g of phospholipids yielded unstable particles, as shown by the zeta potential value. This is likely due to the low phospholipid mass relative to the hydration medium, which caused the formation of weakly bound vesicles that were prone to disintegration. The optimal phospholipid mass is 0.15 g, considering both the particle size distribution and zeta potential (380 nm, -21.7 mV). Higher masses of phospholipids (0.30 and 0.45 g) produced larger particles with bimodal distributions and lower stability, suggesting a higher propensity for aggregation.

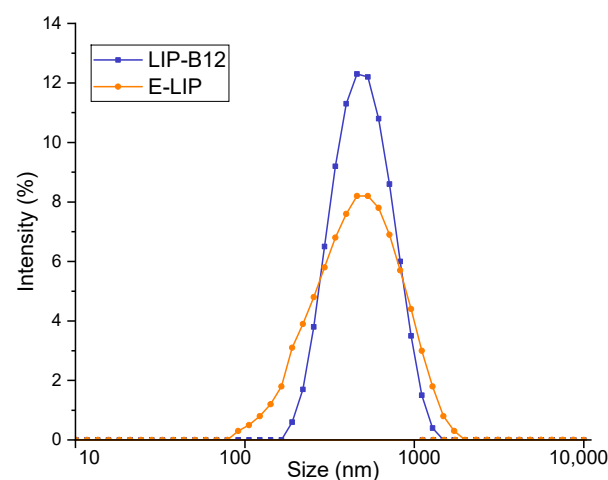
3.2. Encapsulation Efficiency of Vitamin B12

After optimizing the masses of cholesterol and phospholipids based on parameters such as mean particle size, particle size distribution, PDI, and zeta potential, a sample with 30 mg of cholesterol and 0.15 g of phospholipid was selected. This formulation was then used to encapsulate vitamin B12, and Table 2 shows the key properties of the resultant formulations.

Table 2. Characteristics of empty liposomes (E-LIP) and liposomes with vitamin B12 (LIP-B).

Liposomal Formulations	Particle Size, nm	PdI	ζ Potential, mV
E-LIP	380.0	0.424	−21.7
LIP-B12	382.4	0.352	−10.5

Vitamin B12 was encapsulated in liposomes using the thin-film method, with an encapsulation efficiency of 37%. Although the thin-film method is one of the simplest encapsulation techniques, its main disadvantage is low encapsulation efficiency, particularly for water-soluble substances. The incorporation of vitamin B12 into liposomes did not result in a significant increase in particle size. However, when examining the PdI value, it can be observed that the particles became more uniformly distributed (Figure 2 and Table 2). The zeta potential of the empty liposomes (E-LIP) decreased significantly (from −21.7 mV to −10.5 mV) upon the introduction of B12 into the system, indicating that vitamin molecules are likely located on the surface of the liposomes.

**Figure 2.** Particle size distribution of empty liposome (E-LIP) and liposome with vitamin B12.

Another study confirmed that the encapsulation of vitamin B12 does not significantly change the particle size and that the negative zeta potential is reduced upon encapsulation, with an encapsulation efficiency of 14% [9]. Many studies on the encapsulation of water-soluble vitamins report an encapsulation efficiency of approximately 30% when using the thin-film method. For example, the entrapment efficiency of pyridoxine hydrochloride in unilamellar liposomes was about 30% [10], and similar values were found for the encapsulation of niacin and thiamine (~30%) [11]. As observed, the encapsulation efficiency in this study aligns with these findings, as it concerns water-soluble vitamin B12. However, compared to previous studies, the particle sizes obtained in this research were larger. For instance, a similar particle size (~400 nm) was obtained when encapsulating vitamin C using the thin-film method, though the encapsulation efficiency was slightly lower at 23% [12].

3.3. Controlled Release of Vitamin B12 in Simulated Gastrointestinal Condition

The kinetics of vitamin B12 release were examined using a digestion model in a batch system with pepsin and pancreatin. As shown in Figure 3, approximately 30% of the encapsulated vitamin B12 was released under gastric conditions. In the first 30 min, only a small amount of vitamin was released, as the liposomes demonstrated good stability in the acidic environment of the stomach. Over the next 4 h, digestion under intestinal conditions was monitored, showing a gradual release that reached approximately 90% by the end of the digestion process.

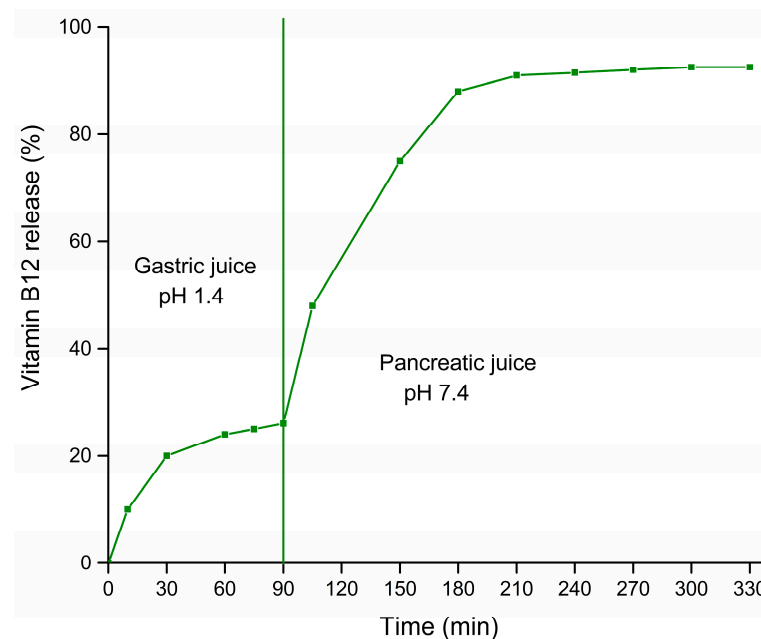


Figure 3. Controlled release of vitamin B12 from liposome.

This high release percentage in intestinal conditions is likely due to the hydrolysis of phospholipids by pancreatin, which contains lipolytic enzymes. These enzymes induce lipolysis, leading to the permeabilization of the lipid bilayer and the dissolution of the vesicles. This process increases membrane permeability, facilitating the release of the encapsulated active substance [13].

Considering that this experiment was designed to simulate the conditions prevailing in the gastrointestinal tract, including temperature, pH, and enzyme activity during digestion, it can be concluded that a controlled release of vitamin B12 was successfully achieved. This is significant because it allows vitamin B12 to be effectively incorporated into food formulations, ensuring an optimal concentration of this essential vitamin in the human body. Similar observations were made by Sugiyama et al. [14], whose study showed that the release rate of vitamin B12 under gastric conditions was 40%. They also monitored the release under intestinal conditions for 120 min, achieving a release rate of about 30%, which is slightly lower than the percentage obtained in this study. In addition, one study recorded a 10% release after 2 h in gastric juice, where a protein–lipid system was used as the matrix. This slower release in gastric juice is due to the presence of proteins, whose hydrophobic groups form a compact network that is quite stable. When the release under intestinal conditions was observed, a 50% release was recorded after one hour, indicating that this system is more sensitive to the action of pancreatin [15].

While B12 deficiency was once thought to be extremely rare, it is now known to be relatively common in the elderly and senile population and is a global issue, affecting approximately 10–15% of the elderly. Additionally, people with gastrointestinal problems often experience difficulty absorbing vitamin B12, which can lead to a deficiency of this vitamin. Therefore, encapsulating vitamin B12 in liposomes presents a highly effective strategy for compensating for this deficiency.

4. Conclusions

This study examined various mass ratios of phospholipid-to-cholesterol in the formation of liposomes. After characterization, including mean particle size, Pdl, particle size distribution, and ζ -potential, it was determined that a 5:1 ratio of phospholipid to cholesterol yielded the best results. Vitamin B12 was encapsulated in this optimized formulation,

and it was found that its encapsulation did not cause a significant change in particle size but led to a decrease in the negative ζ -potential value. The liposome formulations containing the encapsulated vitamin were also tested for stability under gastrointestinal conditions, where it was demonstrated that liposomes effectively achieved controlled release of the encapsulated vitamin. These findings are of particular significance, as such formulations can be incorporated as functional additives into food products, offering a potential solution for addressing vitamin B12 deficiencies.

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