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Advanced Characterization of Liposomal Vitamin C: TEM, Cryo-EM, and Caco-2 Bioavailability Studies

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Abstract

A vital nutrient, vitamin C (ascorbic acid) offers numerous physiological advantages, such as immune system support, collagen synthesis and antioxidant protection. However, its bioavailability is limited due to degradation in the gastrointestinal tract and absorption saturation. Liposomal Vitamin C formulations offer a promising approach to overcoming these limitations by encapsulating Vitamin C in lipid-based vesicles, which protect it from oxidative degradation and enhance absorption.

In this study, the structural properties of liposomal Vitamin C were assessed using Cryo-Electron Microscopy (Cryo-EM) and Transmission Electron Microscopy (TEM). The liposomes were spherical, uniform, and stable, with a well-defined bilayer structure. The bioavailability of the liposomal formulation was evaluated in vitro using a Caco-2 cell permeability model. The results demonstrated that liposomal vitamin C had a considerably higher percentage permeability and apparent permeability coefficient (P_{app}) than non-liposomal vitamin C, suggesting improved intestinal barrier absorption.

These findings suggest that liposomal encapsulation significantly improves the bioavailability of Vitamin C, making it a more efficient and tolerable option for supplementation. This formulation holds potential for improved therapeutic applications in the prevention and treatment of Vitamin C deficiency and related health conditions.

Keywords: liposomal vitamin C, Cryo-EM, TEM, Caco-2 permeability studies, ascorbic acid

1. Introduction

Vitamin C (L-ascorbic acid), is a water-soluble nutrient that is vital for a range of physiological processes in humans. As a potent antioxidant, vitamin C helps reduce reactive oxygen species (ROS) to prevent oxidative damage to cells. It also plays a pivotal role in collagen synthesis, immune system function, and the absorption of iron. Unlike most animals, humans lack the enzyme necessary for endogenous synthesis of vitamin C, and as a result, it must be obtained through dietary sources such as citrus fruits, berries, and leafy vegetables. Vitamin C deficiency is a common concern worldwide, affecting a significant portion of the population to varying degrees. This deficiency can result in a variety of health issues, from mild conditions like fatigue and weakened immunity to more severe complications, including scurvy, cardiovascular diseases, and metabolic disorders (Pehlivan, 2017; Abdullah et al., 2023). Despite the importance of adequate vitamin C intake, the body's ability to absorb and utilize it effectively can be limited by factors such as degradation in the gastrointestinal (GI) tract and the body's saturation limits for absorption. Vitamin C is prone to oxidation, especially when it comes into contact with metal ions or harsh gastrointestinal conditions, reducing its bioavailability (Michels, 2013).

To address these challenges, liposomal vitamin C formulations have emerged as a promising solution. Liposomes, which are lipid-based vesicles, provide an effective means to encapsulate vitamin C and protect it from degradation in the digestive tract. This encapsulation also facilitates enhanced absorption and bioavailability, allowing more vitamin C to reach the bloodstream and tissues. Liposomes act as a protective barrier, controlling the release of vitamin C and preventing premature degradation, making liposomal formulations more efficient than traditional vitamin C supplements (Romano., 2024).

To ensure the effectiveness of liposomal vitamin C formulations, it is essential to evaluate both their physical characteristics and their ability to improve bioavailability. Several assays and analyses can be used for this purpose, each providing important insights into different aspects of liposomal behavior. Key characteristics, such as morphology, particle size, stability, entrapment efficiency, polydispersity index, and zeta potential, are essential factors influencing the therapeutic efficacy of liposomal systems. The evaluation of liposomal vitamin C formulations requires a comprehensive understanding of their physical characteristics and their ability to improve bioavailability. To ensure optimal formulation, advanced characterization techniques, such as Cryo-EM, TEM, and Caco-2 cell assays, are crucial in assessing the structure, size distribution, stability, and functionality of liposomes (Lin et al., 2021; Wan et al., 2015).

2. Materials and Methods

2.1 Preparation of Liposomal Vitamin C

The formulation of liposomal vitamin C involves a multi-phase process utilizing both organic and aqueous phases. In the aqueous phase, water is heated to 58-60°C in a reactor, and surfactant is added with continuous stirring for 20-25 minutes. The organic phase is prepared by dissolving Phospholipids and ethanol at 55-60°C until homogeneous. The organic phase is then added to the aqueous phase under vigorous stirring, maintaining the temperature at 58-60°C, and additional water is added to form a homogeneous suspension. Ascorbic acid is gradually incorporated, and mixture until fully dissolved. The temperature is then reduced to 15-20°C and held for 20-30 minutes. Excipients are added to the liposomal suspension and stirred for 20-30 minutes. The mixture is spray-dried and dried product is ground, sieved through a 60-mesh sieve, stored in airtight.

2.2 Cryo-Electron Microscopy (Cryo-EM)

A 1% (w/v) suspension of the liposomes was prepared in a mixture of water and isopropyl alcohol, followed by sonication for 30 minutes to achieve uniform dispersion. After applying a drop of the suspension to a copper grid, an excess solution was carefully removed off with filter paper. The grid was prepared by applying the sample, blotting to remove excess liquid, and plunge-freezing in liquid ethane for vitrification. The grid was then stored in liquid nitrogen until imaging. For a minimum of two hours, the grid was left to air dry at ambient temperature. The liposomal morphology was analyzed using cryo-TEM. A 200 kV Talos Arctica electron microscope with a Gatan K2 Summit Direct Electron Detector for high-resolution imaging was used for the imaging.

2.3 Transmission Electron Microscopy (TEM)

A 1% suspension of liposomal vitamin C in a water was prepared and sonicated for 30 minutes to ensure uniform dispersion. A drop of this suspension was carefully placed onto a copper grid, and excess liquid was blotted away using filter paper. To enhance the contrast, a drop of 2% (w/v) uranyl acetate aqueous solution was applied to the sample and allowed to sit for five minutes. Following this, the grid was rinsed with water to remove any residual stain and left to dry at room temperature for a minimum of two hours. The morphological characteristics of the liposomes were examined using TEM. The analysis was performed using a Tecnai 12 microscope operating at 100 kV. High-resolution TEM images of the liposomal vesicles were captured at varying magnifications to assess their size, shape, and structural integrity.

2.4 Caco-2 Permeability Study

2.4.1 Preparation of Cell Line

The $Caco_2$ permeability study was performed following the methodology outlined by (Shivaprasad H N et al., 2014). The Caco-2 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 20% heat-inactivated fetal bovine serum (FBS), along with penicillin (100 IU/mL), streptomycin (100 µg/mL), and amphotericin B (5 µg/mL). Cells were maintained in a humidified incubator at 37°C with 5% CO_2. The stock cultures were grown in 25 cm^2 culture flasks and maintained until confluent. Upon achieving confluence, cells were dissociated using Trypsin Phosphate Versene Glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS) and seeded at a density of 6×10^4 cells/cm^2 into thin layers of ThinCert wells, which had a 0.4 µm mean pore size and a growth area of 1.1 cm^2 (12-well format). Regular media changes were performed, and experiments were conducted 28 days post-seeding, when a stable monolayer was formed.

2.4.2 Cytotoxicity Study

To assess cytotoxicity, cells were harvested using trypsinization and plated at a concentration of 1.0×10^5 cells/mL in 96-well plates. After a 24-hour incubation period, test substances were added at different concentrations. Following an additional 24 hours, MTT solution was introduced to each well, and the plates were further incubated for 3 hours at 37°C.The supernatant was removed, and the formed formazan was solubilized using propanol. The

absorbance was recorded at 540 nm with a microplate reader, and the growth inhibition percentage was determined. The concentration needed to reduce cell growth by 50% (CTC_{50}) was calculated

% Growth Inhibition = 100 – [(Mean absorbance of individual test/ Mean absorbance of control) × 100]

2.4.3 Permeability Study

The test substances for permeability studies were dissolved in Ringer's solution to achieve a stock concentration of 10 mg/mL, followed by dilution to prepare lower concentrations for testing. For the assay, the Caco-2 monolayer was washed with Ringer's solution on both the apical and basolateral sides, then incubated with transport buffer (Ringer's solution) at 37°C for 30 minutes. Pre-warmed test solutions in transport buffer were added to the apical side of the ThinCert wells, and the basolateral side was treated with transport buffer. The plate was incubated at 37°C in a 5% CO_2 environment. At specified time intervals, 100 µL samples were withdrawn from the basolateral side, and an equal volume of fresh transport buffer was added to maintain the volume. The samples were then stored at -20°C for subsequent analysis.

Vitamin C content in the collected samples was quantified using HPLC as per in-house protocols to assess the permeability and absorption of the test substances. Chromatographic separation was performed on a C18 column (250 × 4.6 mm, 5 µm), using water as the diluent. The mobile phase consisted of water adjusted to pH 3.0 with ortho-phosphoric acid and methanol (670 mL water: 330 mL methanol). A flow rate of 1.0 mL/min was maintained, and the analytes were detected at a wavelength of 280 nm, with a column temperature of 30°C and a run time of 20 minutes.

Determination of Permeability Coefficient

The apparent permeability coefficient (P_{app}, cm/s) was determined using the equation:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times Co}$$

where dQ/dt indicates the rate of change in the cumulative concentration of the compound in the receiving chamber over time, commonly referred to as the steady-state flux (mol/sec).

Co represents the initial concentration of the compound applied to the apical side (mol/mL)

A is the membrane surface area with pores, which is 1.13 cm^2.

Statistical analysis

The experimental data for each sample were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad InStat Version 5.0.

3. Results

3.1 Cryo-Electron Microscopy (Cryo-EM)

Cryo-EM was performed for the formulated liposomal Vitamin C, and the results shown in Figure 01 revealed that the morphology of the liposomes was spherical, with the liposomes remaining intact.

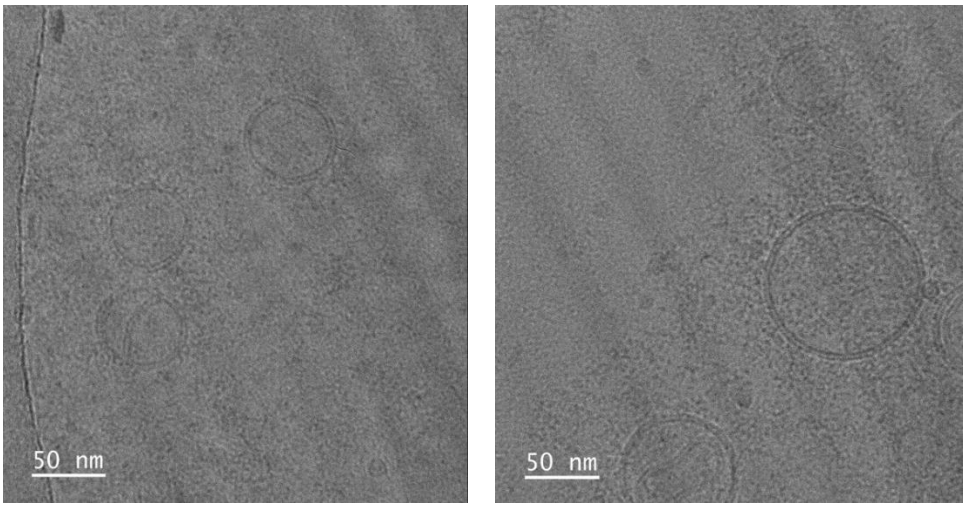

Figure 1. Cryo-EM images of Liposomal Vitamin C

3.2 Transmission Electron Microscopy (TEM)

The TEM analysis of Liposomal Vitamin C was conducted at magnifications of 60KX, 135KX, and 300KX. At 60KX, the general particle morphology, including size and spherical shape, was observed. At 135KX, enhanced structural details of the liposomal formation were visible, providing a clearer view of the liposomal bilayer structure. At 300KX, high-resolution imaging allowed for the visualization of fine structural details, including the uniformity and integrity of the bilayer, further confirming the structural stability of the liposomes.

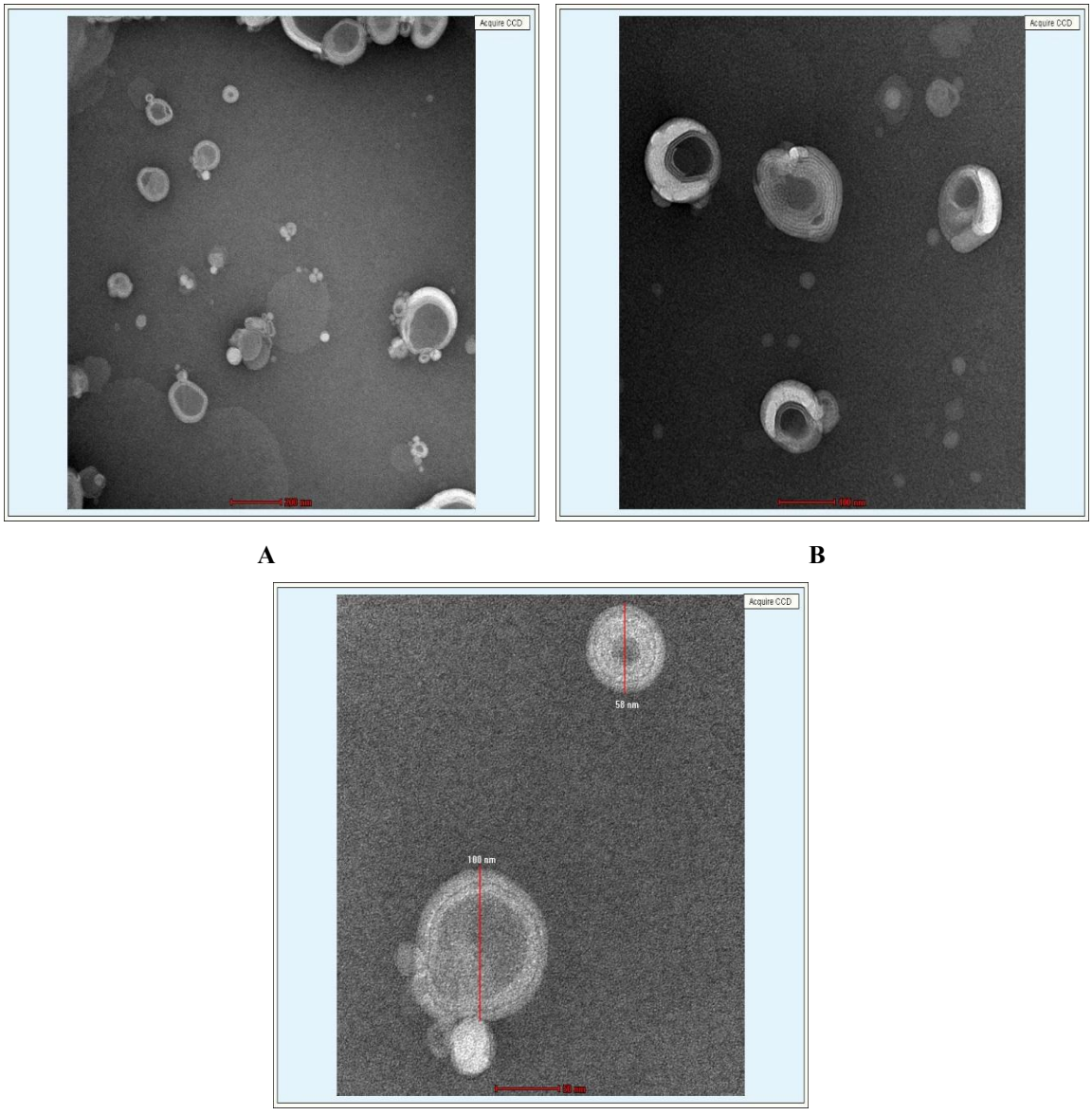

Figure 2. A. Liposomal Vitamin C at 60KX magnification, B. Liposomal Vitamin C at 135KX magnification, Liposomal Vitamin C at 300 KX magnification.

3.3 Caco-2 Permeability Study

Liposomal Vitamin C permeability was evaluated using a Caco-2 cell monolayer model. The optimal concentration of the test compound was selected based on cytotoxicity analysis using the MTT assay on Caco-2 cells. The test samples were added to the apical side of the Caco-2 monolayer and incubated for 2 hours to assess Vitamin C permeability. The outcomes of both the cytotoxicity and permeability studies are presented below

3.3.1 Cytotoxicity Study

The cytotoxicity of Vitamin C and Liposomal Vitamin C was assessed using the MTT assay, and the CTC_{50} values were determined to be approximately 22.7 µg/mL and 32.8 µg/mL, respectively. These values were calculated by performing a two-fold serial dilution of the test substances, ranging from 100 µg/mL to 6.25 µg/mL. Based on the CTC_{50} values, a test concentration of 25 µg/mL was selected for the permeability study to ensure the dose remained below the cytotoxic threshold while allowing effective evaluation of permeability.

3.3.2 Permeability Study

In this study, the permeability of liposomal vitamin C 70% and non-liposomal vitamin C 70% across Caco-2 cell monolayers was evaluated by collecting samples from the basolateral side at different time intervals. The percentage permeability and P_{app} values were calculated for both formulations. Liposomal vitamin C 70% showed a significantly better permeability compared to non-liposomal vitamin C 70% (Table 1). The P_{app} value of liposomal vitamin C was 5.98×10^{-2} cm/sec, which was 1.42 times higher than the P_{app} value of non-liposomal vitamin C, which was 4.21×10^{-2} cm/sec. The permeability of liposomal Vitamin C was measured at 41.40%, which was 1.42 times higher than the permeability of non-liposomal Vitamin C (29.16%). This indicates that liposomal encapsulation significantly enhanced the ability of Vitamin C to pass through the membrane.

Table 1. Permeability potential of test substances

Sl no	Sample	Percentage Vitamin C	Apparent Permeability (P_{app}) in cm/sec.	Percentage Permeability
1	Liposomal Vitamin C	70%	5.98×10^{-2}	41.40
2	Non-Liposomal Vitamin C	70%	4.21×10^{-2}	29.16

4. Discussion

Liposomal Vitamin C significantly enhances bioavailability by improving absorption and protecting the active ingredient from degradation, ensuring more efficient delivery to target tissues. The liposomal encapsulation also provides sustained release, allowing for longer-lasting effects and reducing the gastrointestinal irritation often associated with high doses of free ascorbic acid. This formulation offers a more effective and tolerable option for Vitamin C supplementation (Lukawski et al., 2020).

The findings of this study demonstrate the superior bioavailability and permeability of liposomal Vitamin C compared to non-liposomal formulations, supporting the potential of liposomal encapsulation in enhancing the therapeutic efficacy of Vitamin C. The liposomal formulation showed a P_{app} value of 5.98×10^{-2} cm/sec and permeability of 41.40%, which were 1.42 times higher than the P_{app} value (4.21×10^{-2} cm/sec) and permeability (29.16%) of non-liposomal Vitamin C. These results align with previous studies, emphasizing the role of liposomal encapsulation in improving drug delivery systems.

(Żmuda et al., 2024) reported that liposomal Vitamin C in powder form showed a 30% greater bioavailability compared to non-encapsulated Vitamin C, exhibiting higher peak plasma concentrations and prolonged systemic retention. This prolonged retention minimizes rapid elimination and ensures stable plasma levels, which is critical for therapeutic applications. Our findings are consistent with these observations, as the protective lipid bilayer of liposomes prevents degradation in the gastrointestinal tract, enabling efficient transport across intestinal membranes.

The structural advantages of liposomal Vitamin C were corroborated by Cryo-electron microscopy (Cryo-EM) and transmission electron microscopy (TEM) analyses, which confirmed the spherical morphology, uniform size distribution, and well-defined bilayer structure of the liposomes. These characteristics are essential for maintaining the stability of the encapsulated Vitamin C and controlling its release. (Baxa et al., 2018) highlighted the significance of bilayer integrity in preventing leakage and ensuring the consistent delivery of hydrophilic substances such as Vitamin C. Our TEM analysis revealed the absence of aggregation and structural damage, further demonstrating the stability of the liposomal formulation during preparation and storage.

In addition to improving absorption, liposomal encapsulation protects Vitamin C from oxidative degradation. Liposomal Vitamin C retained more of its active compound under oxidative conditions, compared to free Vitamin C (Favarin et al., 2022). This enhanced stability ensures a higher proportion of the administered dose reaches systemic circulation, thereby maximizing its therapeutic efficacy.

The *in vitro* permeability results obtained from the Caco-2 cell monolayer model further validate the benefits of liposomal formulations. Caco-2 cells, widely recognized as an effective model for predicting intestinal

permeability, demonstrated enhanced absorption of liposomal Vitamin C compared to non-liposomal formulations (Keemink et al., 2018)

From a clinical perspective, the enhanced bioavailability of liposomal Vitamin C holds significant promise for therapeutic and preventative applications, particularly in managing oxidative stress, supporting immune function, and as an adjunct in cancer therapies. The sustained-release nature of liposomal formulations reduces the gastrointestinal irritation often associated with high doses of free ascorbic acid, making it a safer and more tolerable option for long-term supplementation. However, additional studies comparing various liposomal formulations and long-term clinical trials are necessary to further substantiate these findings and optimize delivery systems.

In conclusion, the results of this study, supported by existing literature, highlight the superior bioavailability, stability, and efficacy of liposomal Vitamin C over non-liposomal formulations. The protective encapsulation offered by liposomes not only enhances absorption but also ensures prolonged systemic availability, making it a promising approach for improving the delivery and therapeutic potential of Vitamin C. Future research should focus on comparative evaluations of different liposomal systems, encapsulation efficiency, and in vivo studies to explore the full spectrum of benefits offered by this innovative formulation.

5. Conclusion

In this study, the characterization of liposomal Vitamin C and its enhanced bioavailability was assessed. The structural integrity and uniformity of the liposomes were confirmed through advanced imaging techniques such as Cryo-EM and TEM. These imaging results revealed that the liposomes were predominantly spherical and stable, with a well-defined bilayer structure that facilitates the encapsulation of Vitamin C, offering protection from degradation in the gastrointestinal tract.

The Caco-2 permeability study revealed that liposomal Vitamin C substantially enhances intestinal absorption compared to non-liposomal Vitamin C. The apparent permeability coefficient (P_{app}) and percentage permeability of liposomal Vitamin C were notably higher, indicating enhanced absorption and more efficient delivery of Vitamin C to the bloodstream and tissues. The results indicate that liposomal Vitamin C delivery may offer a more efficient and better tolerated supplementation approach, especially for individuals needing higher doses or experiencing gastrointestinal issues linked to free Vitamin C.

In conclusion, the findings suggest that liposomal Vitamin C offers a promising approach to enhance the bioavailability of this crucial nutrient, potentially leading to improved therapeutic results in preventing and managing conditions related to Vitamin C deficiency.

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Authors contributions

Dr. H. N. Shivaprasad, Madhu Krishnamani, and Gaurav Soni were involved in conceptualizing the study and providing critical revisions to the manuscript. Dr. Arnab Chatterjee took charge of collecting the data. Tharun M. and Cherupalli Sree Ramya composed the initial draft of the manuscript, which was subsequently refined by T. Sravani. All contributors reviewed and approved the final manuscript. Equal contributions were made by the drafting authors, and appropriate agreements were established to reflect this.

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Competing interests

The authors declare that they have no competing interests.

Informed consent

Not applicable.

Ethics approval

Not applicable.

Provenance and peer review

Not commissioned; externally double-blind peer reviewed.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Data sharing statement

No additional data are available.

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