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RESEARCH ARTICLE

Enhancing Intracellular Uptake of Ivermectin through Liposomal Encapsulation

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Abstract

Ivermectin (IVM), an antiparasitic drug approved by the Food and Drug Administration (FDA), is widely used to treat several neglected tropical diseases, including onchocerciasis, helminthiases, and scabies. Additionally, IVM has shown potential as a potent inhibitor of certain RNA viruses, such as SARS-CoV-2. However, IVM is highly hydrophobic, essentially insoluble in water, which limits its bioavailability and therapeutic effectiveness. The use of liposomes as drug carriers offers several advantages, including enhanced solubility for lipophilic drugs, passive targeting of immune system cells, sustained release, and improved tissue penetration. To address the limitations of IVM, including its poor solubility and bioavailability, liposomal formulations were developed using a combination of soyphosphatidylcholine (SPC), dioleylphosphatidylcholine (DOPC), cholesterol (Ch), and diethylphosphate (DCP) in two distinct molar ratios (1.85:1:0.15 and 7:2:1) via the ethanol injection method. The physicochemical properties of the placebo and IVM-loaded liposomes were extensively characterized in our earlier study, including the particle size, polydispersity index, and zeta potential. The present work adds a deeper level of investigation into how to effect cellular uptake and cytotoxicity *in vitro* of both free IVM and IVM-loaded liposomes in Vero E6 cells. The half-maximal cytotoxic concentrations (CC_{50}) for free IVM and IVM-loaded liposomes were 10 µM and > 110 µM, respectively and the cellular uptake of IVM-loaded liposomes ranged from 13 to 60%, whereas free IVM showed a significantly lower uptake of only 2%. These results demonstrate that liposomal encapsulation effectively enhances IVM's cellular uptake while reducing its cytotoxicity, thus offering a promising strategy for improving the effectiveness of IVM.

Keywords cellular uptake · enhancing cellular uptake · ivermectin · liposomes · Vero E6

Introduction

Ivermectin (IVM), discovered in 1975 and commercialized in the early 1980's, is an FDA-approved antiparasitic drug widely used for treating various tropical diseases in humans and as an antiparasitic agent in animals [1, 2]. One of the major challenges in the clinical application of IVM is its extremely hydrophobic nature, which is due to its very low solubility in water. The drug's partition coefficient (logP) is a critical physicochemical property that reflects its lipophilicity and IVM has a logP value of 5.83 [3], which indicates a high affinity for lipid environments and poor solubility in aqueous solutions. This lipophilic characteristic is attributed to the drug's large, hydrophobic structure, composed of a macrocyclic lactone ring and several hydrophobic functional groups. This high logP value is associated with poor water solubility, which limits its bioavailability and therapeutic potential

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when administered via conventional routes. In pharmaceutical development, optimizing the bioavailability of such lipophilic compounds is crucial to ensuring effective treatment, especially in the case of IVM, which is used in treating serious diseases caused by parasitic infections and certain RNA viruses.

Moreover, high doses of IVM may lead to adverse effects, particularly neurotoxicity, due to its ability to cross the blood–brain barrier in certain conditions. This is especially concerning in animals, where overdose or prolonged exposure can result in severe side effects such as ataxia, tremors, and even death. In humans, although the safety profile of IVM is generally favorable when administered at therapeutic doses, side effects such as dizziness, nausea, and gastrointestinal disturbances have been reported, particularly in individuals with compromised immune systems [4]. In addition to these direct toxicological concerns, the suboptimal solubility of IVM in water limits its ability to reach therapeutic concentrations in tissues, particularly in areas where parasitic infections are most prevalent. Consequently, achieving an effective therapeutic dose without exceeding the toxicity threshold becomes a major challenge in the clinical application of IVM. Furthermore, the inability to deliver IVM in a controlled and sustained manner can result in fluctuating drug levels, which may affect both efficacy and safety. These toxicological challenges underscore the importance of developing novel drug delivery systems, which can address the poor solubility and bioavailability of IVM while minimizing systemic toxicity. The main problem of IVM, therefore, lies in the need to overcome the inherent limitations of IVM's toxicological profile, its poor solubility, and the challenges of administering effective, safe doses. To overcome these challenges, innovative drug delivery systems such as liposomes, which can encapsulate hydrophobic drugs and improve their solubility, have been explored [5–7].

Liposomes are microscopic structures consist of an aqueous core surrounded by a lipid bilayer promising nanocarrier systems due to their biocompatibility, low toxicity, high drug-loading capacity, and ability to enhance drug bioavailability [8, 9]. Because of these properties, liposomal drug delivery systems have played a crucial role in the formulation of both hydrophobic and hydrophilic drugs, significantly improving therapeutic outcomes [10–12]. Liposomes, as carriers, provide a controlled release system, enhance the stability of hydrophobic drugs, and improve the therapeutic index by increasing drug accumulation at the target site while minimizing systemic toxicity. Additionally, liposomes can improve the hydrophilic charge transfer of molecules, facilitating better tissue penetration [13, 14]. This approach capitalizes on the high logP of IVM to encapsulate the drug in a lipid bilayer, improving its solubility and delivery to the site of action. Therefore, the high logP of IVM plays a pivotal role in guiding the development of nanoencapsulation strategies aimed at optimizing the pharmacological effects of the drug [5–7].

Various methods are available for preparing liposomes, each with distinct techniques and mechanisms that affect the final product [15, 16]. The choice of preparation method can influence characteristics such as particle size, charge, and surface hydration. Proper characterization of these properties is essential to optimize liposome performance and minimize their rapid clearance from the bloodstream [17, 18]. Among the different preparation techniques, the ethanol injection method is preferred due to its simplicity, safety, and reproducibility [19–21]. This method produces unilamellar liposomes with smaller particle sizes and better monodispersity, without the need for additional steps such as sonication or extrusion, which are commonly required for liposomes prepared by the thin-film hydration method. The ethanol injection technique involves injecting a phospholipid solution in ethanol into a stirred aqueous solution, allowing for the spontaneous formation of liposomes [21, 22]. This method affects the physical, chemical, and biological properties of the resulting liposomes, which are directly influenced by the formulation parameters. These characteristics include particle size, lipid composition, and surface charge, all of which play a crucial role in the performance and application of liposomal drug delivery systems [23]. And also, the lipid composition used in liposome formulations significantly affects these properties, including cellular uptake [24, 25].

In our previous study, we demonstrated that the encapsulation of IVM into liposomes enhanced the antiviral efficacy against SARS-CoV- 2 in Vero E6 cells compared to free IVM [26]. In this study, we aimed to extend the characterization and application of IVM-loaded liposomes, following our previous research [26], where we introduced the initial liposomal formulations and provided fundamental insights into their physicochemical properties. While the previous study focused on the preliminary formulation and characterization of IVM-loaded liposomes, the current work builds upon these findings to explore the drug's intracellular uptake, cytotoxicity, and its potential for enhanced antiviral activity. To further investigate whether this improvement in antiviral activity was associated with increased cellular uptake of the drug, Vero E6 cells were chosen for the uptake assay due to their relevance in studying the interaction between antiviral agents and host cells. As a well-established model for evaluating the susceptibility of kidney epithelial cells to viral infection, Vero E6 cells provide a reliable system for assessing the intracellular delivery and uptake of drug-loaded nanoparticles, particularly for hydrophobic drugs like IVM [27].

In this study, the influence of different lipid types and ratios on the cellular uptake of liposomes was investigated. The cellular uptake of free IVM was compared with that of IVM-loaded liposomes to elucidate the effects of various lipid compositions on drug delivery efficiency and cellular internalization. Through a systematic examination of lipid formulations, an understanding of the relationships between lipid characteristics and the performance of liposomal drug delivery systems was sought. The findings of this study are anticipated to yield valuable insights into optimizing liposome formulations for enhanced therapeutic efficacy, potentially leading to improved treatment outcomes in clinical applications. Furthermore, the knowledge gained from this research may contribute to the development of more effective drug delivery strategies, thereby addressing the challenges associated with conventional drug administration methods.

Materials and Methods

Materials

Ivermectin (IVM) was obtained from Sigma-Aldrich (9041–08-1, St. Louis, MO, USA.), Cholesterol (Ch) was obtained from Nacalai Tesque (Kyoto, Japan), 1,2-dioleoyl-sn-glycero- 3-phosphocholine (DOPC), soy-phosphocholine (SPC) and dicetyl phosphate (DCP) were obtained from Waken Hd. Co.Ltd. (Japan).

Reagents

Eagle's medium (MEM) was obtained from Nacalai Tesque (Kyoto, Japan), Penicillin–Streptomycin (PS) (10,000 U/mL), GlutaMAX [TM] Supplement and Fetal Bovine Serum (FBS) were obtained from Invitrogen (Life Technologies Japan Ltd., Tokyo, Japan), Acetonitrile for LC/MS was obtained from Wako Chemicals (USA).

Cell Culture

Vero E6 (JCRB0111) African Green Monkey kidney normal cells were genereously gifted from JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank) and maintained in Modified Eagles's medium (MEM) supplemented with 1% penicillin/streptomycin, 1% GlutaMAX and 5% fetal bovine serum (FBS) in a humidified 37°C and 5% CO_2 incubator. The cells were subcultered 1:9 ratio at every 3–4 days [28].

Table I Formulation code and composition of liposomes ($n = 3$)

Formulation Code*	PC (mM)	Ch (mM)	DCP (mM)	IVM (μM)
SPC1.85-Ch1-IVM3	1.85	1	0.15	3
SPC7-Ch2-IVM3	7	2	1	3
DOPC1.85-Ch1-IVM3	1.85	1	0.15	3
DOPC7-Ch2-IVM3	7	2	1	3
SPC1.85-Ch1	1.85	1	0.15	-
SPC7-Ch2	7	2	1	-
DOPC1.85-Ch1	1.85	1	0.15	-
DOPC7-Ch2	7	2	1	-

*The numbers provided in the formulation codes refer to the molar ratios of DOPC, SPC, and Ch contained in the liposomes

Preparation and Characterization of IVM-Loaded Liposomes

IVM-loaded liposomes were prepared using the ethanol injection method as described in previous study [26]. Various ratios of SPC and DOPC were used in the formulations, along with different molar ratios of cholesterol to the charged lipid DCP. Specifically, the molar ratios employed in the study were 1:0.15 and 2:1, representing the Ch to charged lipid ratio (Table I).

The particle size (nm), polydispersity index (PDI), and zeta potential (mV) of the liposomes were determined using dynamic light scattering (DLS) with a Malvern ZetaSizer Nano ZS (Malvern Instruments Ltd., UK). To ensure accurate measurements and minimize inter-sample variability, all liposomal formulations—including placebo and IVM-loaded variants—were dispersed in ultrapure water at a dilution ratio of 1:10 (v/v), optimizing the concentration for DLS analysis while preventing multiple scattering effects. Measurements were conducted at 25°C under standard conditions, and each formulation was analyzed in triplicate to assess reproducibility and minimize experimental error.

The encapsulation efficiency (EE%) of IVM was quantified using an ultrafiltration method to separate unencapsulated IVM from the liposome-encapsulated fraction. Specifically, a 500 µL aliquot of each formulation was subjected to centrifugation at 4000 rpm for 30 min using an ultrafiltration device with a molecular weight cutoff (MWCO) membrane appropriate for retaining liposomes while allowing the free drug to pass through. The resulting filtrate, containing unencapsulated IVM, was collected and analyzed using high-performance liquid chromatography (HPLC) with a validated method to determine the free drug concentration. The encapsulation efficiency (EE%) was then calculated using the following equation (Eq. 1):

$$EE\ (\%) = \frac{Total\ amount\ of\ IVM\ in\ liposomes - IVM\ Amount\ of\ drug\ in\ the\ filtrate}{Total\ amount\ of\ IVM\ in\ liposomes} \times 100 \quad (1)$$

The *in vitro* release profile of IVM from the liposomal formulations was evaluated using the dialysis bag diffusion method. Briefly, 1 mL of the IVM-loaded liposome suspension was placed in a pre-soaked dialysis bag (12–14 kDa molecular weight cutoff) and immersed in 50 mL of phosphate-buffered saline (PBS) pH 7.4 with 0.2% sodium dodecyl sulfate (SDS) to maintain sink conditions. The release study was conducted within a shaking water bath set to 37°C and 50 rpm. At predetermined time intervals, 1 mL of the release medium was withdrawn and replaced with an equal volume of fresh medium to maintain a constant volume. The collected samples were analyzed for IVM content using LC. The cumulative percentage of IVM released was plotted as a function of time to determine the release profile ($n = 3$).

The morphology of the nanoparticles was imaged using transmission electron microscopy (TEM) (Tecnai G2 Spirit BioTwin, FEI Co.). The TEM sample preparation involved depositing a thin film of carbon on the TEM grid, placing the liposome suspension on the carbon film, and then blotting away the excess liquid to ensure even sample distribution [29].

For each formulation, a total of three independent batches were prepared to ensure reproducibility and consistency of the results. Detailed results of liposome preparation and *in vitro* characterization parameters are provided in our previous study [26].

IVM Assay with LC–MS/MS

The LC–MS/MS analysis was performed using an LCMS-8040 system (Shimadzu, Kyoto, Japan). The system was operated in multiple-reaction monitoring (MRM) mode to ensure high sensitivity and specificity for IVM detection. Electrospray ionization (ESI) was employed as the ionization source, operating in positive ion mode to enhance the ionization efficiency of IVM. The chromatographic separation was achieved using columns maintained at a temperature of 40°C to ensure consistent and reproducible retention times.

Data acquisition and instrument control were managed using LabSolutions LC–MS software (Shimadzu), which facilitates both instrument operation and data processing. The software provided comprehensive tools for method development, data analysis, and quantification.

Detailed conditions for liquid chromatography (LC) and mass spectrometry are provided in Tables SI and SII [30]. These tables include specific parameters such as the column type, mobile phase composition, flow rate, ion source settings, collision energies, and other critical parameters necessary for optimizing assay performance and ensuring accurate and reliable quantification of IVM.

Cytotoxicity Determination Using WST-8 Assay

Cytotoxicity was assessed using the WST- 8 assay with Vero E6 cell line [31]. For the assay, Vero E6 cells were seeded at a density of 5×10^3 cells per well in 96-well plates [32]. The cells were cultured either in the absence of treatment (control group) or in the presence of IVM at varying concentrations, ranging from 0.625 to 20.0 µM, in 100 µL of 0.5% dimethyl sulfoxide (DMSO)-containing Vero E6 cell culture medium. The use of DMSO as a solvent is common in drug screening studies, as it helps dissolve hydrophobic compounds like IVM, without causing significant toxicity at low concentrations [33]. After a 48-h incubation at 37°C in a 5% CO_2 atmosphere, the culture medium was removed from each well. Then, 100 µL of the fresh culture medium and 10 µL of the cell counting reagent SF (Nacalai Tesque, Japan) were added to each well and incubated for 1 h [34]. After 1-h incubation the cell viability detected colorimetrically using microplate spectrophotometer (Thermo Scientific, Varioskan LUX, USA) at a wavelength of 450 nm [35].

CC50 Calculation

This value was calculated using nonlinear regression analysis of the dose–response data, which was plotted as a sigmoidal curve in GraphPad Prism 9 software. The CC_{50} value represents the concentration at which the cell viability is reduced to 50% of the control, providing a measure of the compound's potency in terms of cytotoxicity [36].

Uptake of Free IVM and IVM-Loaded Liposomes

Vero E6 cells were seeded in 12-well plates at a density of 1×10^5 cells/mL per well and allowed to reach 100% confluence within 24 h [37, 38]. Prior to treatment, the medium was removed, and to synchronize the cell cycle, 800 µL of fresh medium without FBS was added to each well containing cells and incubated for 2 h [39]. This step ensured that the cells were in a consistent phase of the cell cycle, providing more reliable uptake measurements.

After synchronization, 200 µL of the medium containing either IVM solution or IVM-loaded liposomes was added to achieve a final concentration of 0.75 µM IVM equivalent. The incubation was carried out for 6 h at 37°C [38]. Following incubation, the medium was removed, and the cells were washed twice with 1 mL of ice-cold phosphate-buffered saline (PBS) to remove any unbound IVM or liposomes.

Subsequently, 200 µL of trypsin–EDTA solution was added to each well to detach the cells, which were then scraped using a cell scraper (Iwaki AGC Techno, Japan) to facilitate cell collection [40]. The cell suspensions were transferred to 1.5 mL centrifuge tubes, and the cells were pelleted by centrifugation at 1500 rpm for 2 min. The supernatant was discarded, and 0.5 mL of acetonitrile was added to the cell pellet [41]. The suspension was vortexed thoroughly to ensure complete cell lysis and the extraction of IVM from the cells.

The cell suspension was then centrifuged again at 1500 rpm for 2 min, and the supernatant was collected for analysis. The supernatant was filtered through a 0.22 µm polyvinylidene fluoride (PVDF) filter into LC vials to remove any particulates before injection. The concentration of IVM in the supernatant was determined using LC–MS/MS. The results were calculated using the following equation (Eq. 2) [42], which allows for the quantification of IVM uptake and comparison between free drug and liposome-encapsulated drug.

$$Uptake\ (\%) = \frac{Total\ amount\ of\ IVM\ in\ the\ cells}{Total\ amount\ of\ IVM\ in\ the\ liposomes} \times 100 \quad (2)$$

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). The Design-Expert V13 software was used to evaluate the effects of lipid type, lipid ratio, and production method on particle size, PDI value, zeta potential, and encapsulation efficiency. The lipid type, ratios, and production methods were set as independent variables, while particle size, zeta potential, PDI value, and encapsulation efficiency were selected as response variables. To analyze the significance of these factors, a one-way analysis of variance (ANOVA) was performed, and the results were considered statistically significant at $p < 0.05$ [43].

To compare the mean uptake of IVM in cells treated with free IVM *versus* cells treated with IVM-loaded liposomes, the Dunnett's multiple comparison test was applied as a post-hoc analysis. This test was employed to determine whether the differences in cellular uptake between the two conditions were statistically significant ($p < 0.05$) [44]. Including both normality testing and appropriate post-hoc analysis ensures a robust statistical evaluation of the data.

Results

Characterization of IVM-Loaded Liposomes

The particle size distribution was also relatively monodisperse, as depicted in Fig. 1, CTEM images revealed that the liposomes exhibit a spherical shape with a relatively rough surface. The liposomes had a size range from approximately 100 to 500 nm when the typical particle size of placebo liposomes had a size range from 100 to 200 nm, which was in agreement with the DLS measurements. The zeta potential of liposomes was negative, approximately − 50 mV, as shown in Table II and the zeta potential of placebo formulations is typically in the range of − 20 to − 40 mV. The PDI value of placebo formulations ranged from 0.1 to 0.3 when the IVM-loaded liposomes showed a 0.2–0.6 PDI value.

In Vitro Release Test

The release of the drug from the liposomes was examined in a phosphate-buffered solution at pH 7.4. Release profiles of IVM loaded liposomes were given in Fig. 2 [26].

Cytotoxicity Determination of Free IVM and IVM-Loaded Liposomes

After a 48-h incubation period, free IVM exhibited a CC_{50} value of 10.32 µM, indicating that at this concentration, half of the cells were affected by the drug. In contrast, the IVM-loaded liposomal formulations showed significantly higher CC_{50} values, exceeding 110.2 µM (Table III).

Uptake of Free IVM and IVM-Loaded Liposomes

We compared the %uptake by Vero E6 cells of IVM and IVM-loaded liposomes. 2% of the free IVM was internalized by the cells, whereas for the IVM-liposomes, it was as high as 66%.

Discussion

Characterization of IVM-Loaded Liposomes

In this study, liposomes were successfully prepared using the ethanol injection method, with a narrow size distribution [6, 18]. Liposomes with a phosphatidylcholine (PC) molar ratio of 1.85 exhibited a smaller average particle size

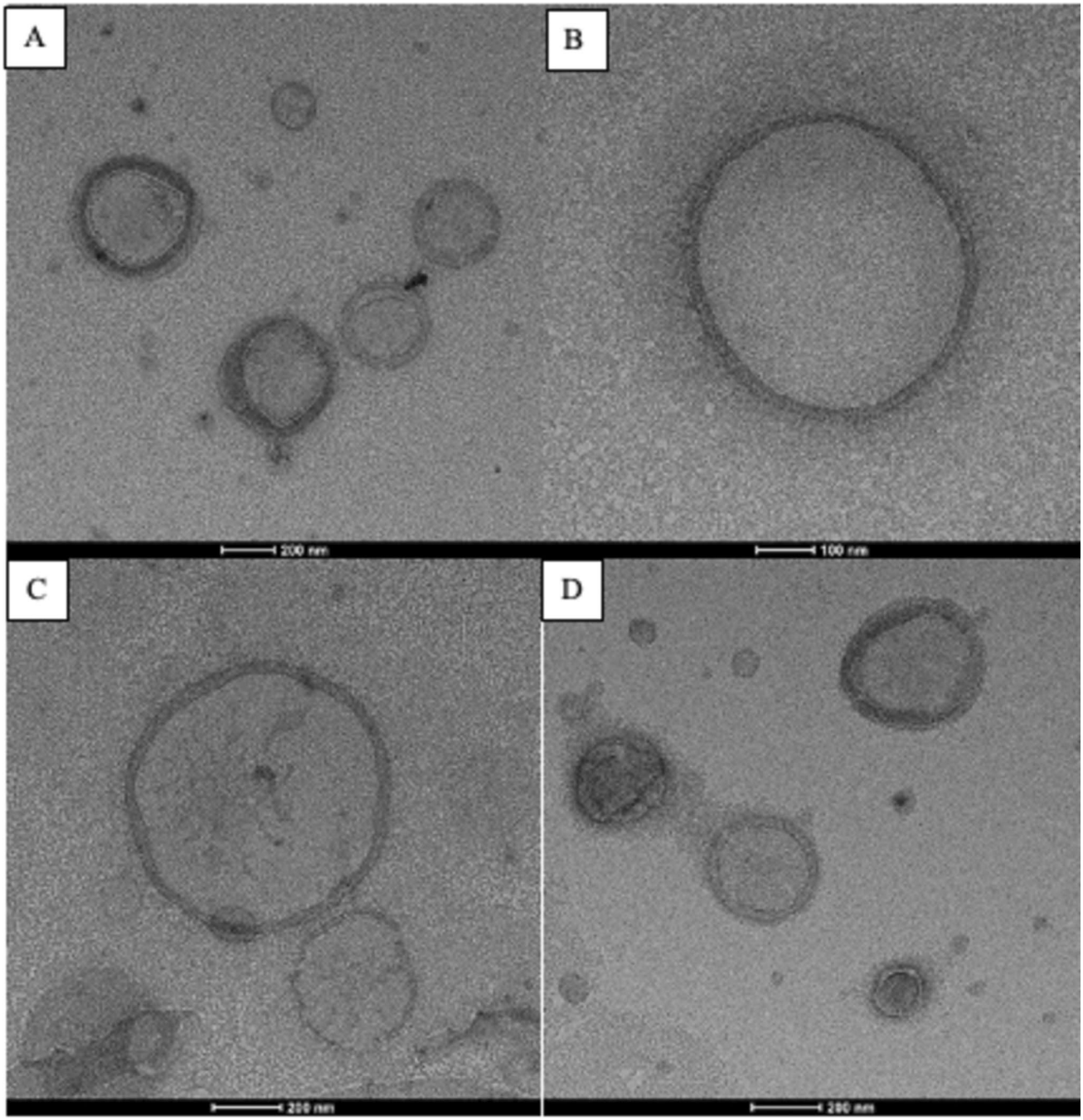

Fig. 1 TEM images of the IVM-loaded liposomes; #SPC1.85-Ch1-IVM3 (**A**), #SPC7-Ch2-IVM3 (**B**), #DOPC1.85-Ch1-IVM3 (**C**), #DOPC7-Ch2-IVM3 (D) [26]

compared to those with a higher molar ratio of 7 (Table II). This can be attributed to the excess PC, which increases the fluidity of the lipid bilayer. SPC, with its unsaturated fatty acid chains, further enhances this fluidity, leading to looser lipid packing and, consequently, larger liposome sizes. The difference in particle size between liposomes with varying PC molar ratios can be better understood by considering the role of lipid composition and molecular interactions during liposome formation. The results clearly demonstrate that the lipid-to-chol ratio significantly influences the particle size of the liposomes. The lipid-to-chol ratio directly influences the packing and organization of lipid molecules, and consequently, the size of the liposomes [45]. Cholesterol plays a stabilizing role by reducing membrane fluidity and enhancing the mechanical strength of the bilayer, thus contributing

Table II *In vitro* characterization results of IVM-loaded liposomes (mean ± S.E.M., $n = 3$)

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)	Drug loading capacity (%w/w)
SPC1.85-Ch1-IVM3	164.00 ± 40.90	0.46 ± 0.13	− 48.40 ± 2.31	98.10 ± 1.21	4.80 ± 0.22
SPC7-Ch2-IVM3	271.30 ± 03.80	0.49 ± 0.08	− 49.90 ± 2.31	86.98 ± 0.43	6.40 ± 0.19
DOPC1.85-Ch1-IVM3	190.20 ± 01.20	0.27 ± 0.01	− 40.00 ± 0.50	95.92 ± 0.55	3.10 ± 1.00
DOPC7-Ch2-IVM3	498.00 ± 67.30	0.58 ± 0.03	− 45.20 ± 2.00	98.51 ± 0.40	4.10 ± 0.80

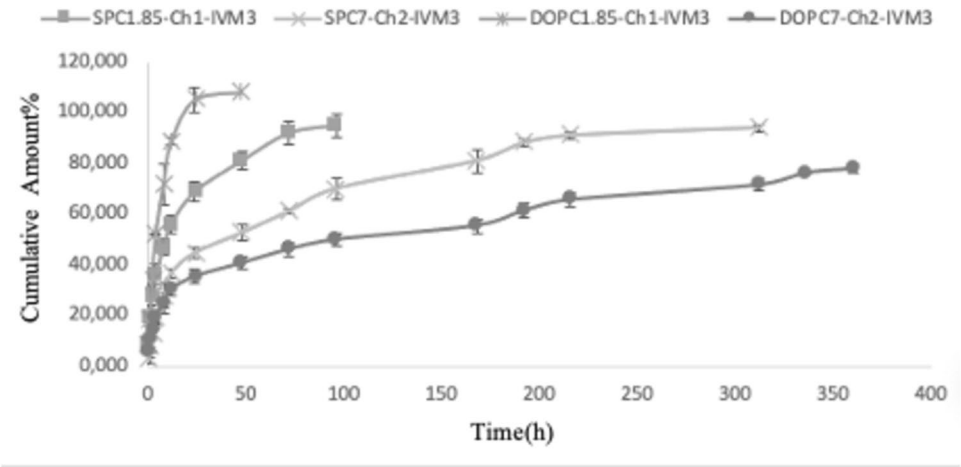

Fig. 2 Release profiles of IVM loaded liposomes (mean ± S.E.M., $n = 3$) [26]

Table III CC_{50} expressed in μM for IVM and IVM-loaded liposomal formulations ($n = 4$)

Sample	CC_{50} (μM)
IVM	10.32
SPC1.85-Ch1-IVM3	440.5
SPC7-Ch2-IVM3	2.6930E + 12
DOPC1.85-Ch1-IVM3	~ 110.2
DOPC7-Ch2-IVM3	~ 171.0

to the formation of smaller liposomes when present in appropriate amounts [46].

The parameters of the placebo formulation, including particle size, PDI, and zeta potential, were assessed to establish a baseline for the liposomal formulations without the drug. In accordance with the literature, the typical particle size of placebo liposomes ranges from 100 to 200 nm, with a good uniformity. The zeta potential of placebo formulations is typically in the range of − 20 to − 40 mV, which is indicative of stable formulations due to electrostatic repulsion [47]. The addition of IVM influenced these parameters, leading to an increase in particle size and a change in zeta potential due to the interaction of IVM with the lipid components.

When comparing placebo and IVM-loaded formulations, it was observed that the introduction of IVM significantly increased the particle size. This increase can be attributed to the hydrophobic nature of the drug, which may cause aggregation or modification of the lipid bilayer structure. Literature reports also support this observation, noting that similar effects are seen with other hydrophobic drugs in liposomal formulations [45]. Furthermore, encapsulating IVM in liposomes can decrease the zeta potential if the drug interacts with the lipid headgroups, as has been observed with other hydrophobic drugs [45].

This decrease in zeta potential could negatively impact the stability of the formulation, which is a crucial factor for ensuring sustained drug release and minimizing premature drug leakage. These changes may pose challenges, especially for formulations intended for controlled release. However, we believe that further characterization tests are needed to gain a deeper understanding of these effects. Similar findings in the literature indicate that hydrophobic drugs often cause a loss in liposomal stability and adversely affect release profiles [46]. Therefore, these findings highlight the potential challenges associated with the use of IVM in liposomal delivery systems and their impact on formulation strategies.

In essence, the size differences arise from the balance between membrane rigidity, governed by cholesterol, and membrane fluidity, influenced by the amount and type of PC. As PC concentration increases, the bilayer becomes more

flexible, which promotes the formation of larger liposomal vesicles. Therefore, the interplay between lipid composition, cholesterol content, and molecular packing largely dictates the final particle size [26].

Cytotoxicity Determination of Free IVM and IVM-Loaded Liposomes

The results demonstrate that encapsulating IVM within liposomes markedly reduced its cytotoxicity compared to the free drug. The reason for this is believed to be because free IVM interacts directly with the cell membrane, leading to higher immediate intracellular concentrations due to its rapid uptake by cells. This rapid uptake can induce cytotoxic effects, primarily through the activation of the AMPK/mTOR signaling pathway, which plays a central role in regulating autophagy [48]. IVM's activation of this pathway disrupts cellular homeostasis, leading to increased autophagy, cell stress, and ultimately, cell death [49]. In the case of Vero E6 cells, this direct interaction and rapid accumulation of free IVM cause significant cell damage, as reflected by the lower CC_{50} value of 10.32 µM.

The Vero E6 cell line was selected for cytotoxicity and uptake studies due to its extensive use in virological research and its relevance to the primary pharmacological target of IVM. These cells provide a robust and reliable model for evaluating the interaction between drug-loaded nanoparticles and host cells due to their well-characterized membrane properties and stable growth. Moreover, Vero E6 cells have been used in numerous studies to evaluate the cytotoxicity of antiviral agents, including those with hydrophobic properties, making them particularly suitable for assessing the intracellular delivery and cytotoxic potential of IVM-loaded liposomes [31].

In contrast to free IVM, liposomal encapsulation alters the way IVM interacts with the cells. Liposomes provide a protective carrier, limiting the direct exposure of the cells to IVM by controlling the release of the drug over time. Instead of a sharp rise in intracellular drug concentration, the gradual release from liposomes leads to a more sustained, lower concentration of IVM within the cells. This gradual release minimizes the acute cytotoxic effects of IVM, as evidenced by the significantly higher CC_{50} values (> 110.2 µM) observed with liposomal formulations. The controlled release reduces the likelihood of overwhelming the cellular mechanisms that manage stress and autophagy, thereby allowing the cells to maintain higher viability for a longer period.

In accordance with previous studies, the cytotoxicity decreased (CC_{50SPC} > CC_{50DOPC}) as the phase-transition temperature (Tm) of the main lipid component increased (DOPC < SPC), with approximate Tm values of − 17°C and 5°C, respectively [50–53]. This trend can be attributed to the increased rigidity of liposomal membranes composed of SPC compared to DOPC. Lipids with higher phase-transition temperatures result in more rigid bilayers, which may provide enhanced stability and controlled drug release, thereby contributing to reduced cytotoxic effects.

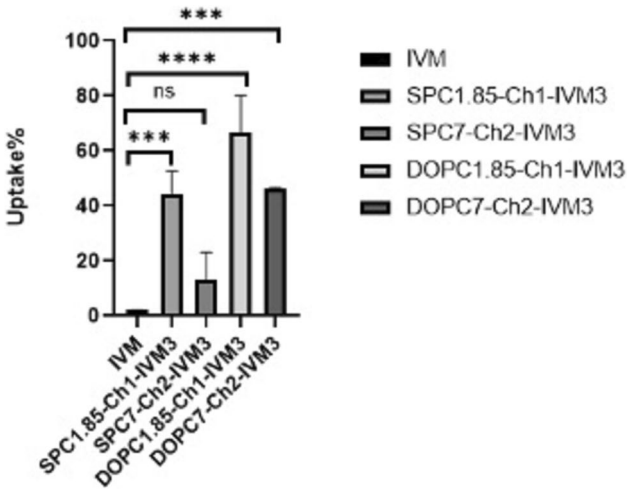

Fig. 3 Uptake% results of liposome formulations and IVM

The combination of liposomal encapsulation and the selection of a relevant cell model such as Vero E6 cells enhances the reliability and translational value of the study. The results underscore the potential of liposomal formulations to mitigate the cytotoxic effects of hydrophobic drugs like IVM by altering drug delivery kinetics and reducing immediate intracellular drug concentrations.

Uptake of Free IVM and IVM-Loaded Liposomes

The results of the cellular uptake study demonstrated that IVM was more efficiently taken up by Vero E6 cells when encapsulated in liposomes compared to the free drug (Fig. 3). This enhanced uptake indicates that the encapsulation of IVM in liposomes significantly increases its cellular internalization [54]. Understanding the mechanisms of this cellular uptake is essential for optimizing the design of liposomes as drug carriers, as it directly impacts their efficacy.

There are several mechanisms by which liposomes act within and outside the body which are as follows; liposome attaches to cellular membrane and appears to fuse with them, releasing their content into the cell; some times they are taken up by the cell and their phospholipids are incorporated into the cell membrane by which the drug trapped inside is released and in the case of phagocyte cell, the liposomes are taken up, the phospholipid walls are acted upon by organelles called lysosomes and the active pharmaceutical ingredients are released [55–58].

Among the tested formulations, #DOPC1.85-Ch1-IVM3 showed the highest cellular uptake, suggesting that the lipid composition and membrane fluidity play a crucial role in modulating the efficiency of drug delivery. When comparing the IVM-loaded liposomal formulations, the #DOPC1.85-Ch1-IVM3 formulation exhibited the highest intracellular uptake, whereas the #SPC1.85-Ch1-IVM3 formulation showed the lowest uptake. Andar *et al*., found that the liposome uptake is size dependent and increases with a decrease in diameter. They showed that the smaller 40.6 nm liposomes seem to depend on a dynamin dominant pathway. However the slightly larger diameter liposomes (97.8 nm to 162.1 nm), when compared to the smaller sizes, did not show any significant dependency on any particular uptake pathways that were tested, but when the larger liposomes were compared among themselves they showed slight dependency on the clathrin-mediated pathway [59]. It seems unlikely that this differential uptake of liposomes is due to a mismatch in the surface density, size distribution, encapsulation efficiency, and stability of the liposomes because the particle size and zeta potential of these two formulations did not differ significantly. Therefore, the differences in cellular internalization between various liposomal formulations are most likely due to differences in cellular uptake pathways of lipid composition and membrane fluidity [60–62]. Moreover, identifying similarities and distinctions among liposomes is a challenging task owing to the disparities in experimental design across various studies. These factors include lipid formulations, the absence or presence of serum in the experiment's culture, the use of pharmacological inhibitor(s), exposure times, and the type of recipient cell tested [54].

This study demonstrated that encapsulating IVM within liposomes significantly enhanced its cellular uptake and internalization in Vero E6 cells compared to the free drug, while simultaneously reducing its cytotoxicity. Therefore, while the liposomal formulations enhanced cellular uptake, the encapsulation acts as a modulator of cytotoxicity, providing a delayed and controlled release of IVM. This helps prevent the rapid activation of the cytotoxic autophagy pathway and protects cells from the immediate toxic effects seen with free IVM [63].

This is most likely due to a slower and incomplete uptake of IVM from the liposomal formulation, which might be a result of lower extracellular availability of IVM when dosed as IVM-liposome as showed before another hydrofobic drug Doxorubicin and Docetaxel liposomal formulation [39]. In this context, the higher uptake of liposomal IVM does not correlate with increased cytotoxicity because the release kinetics are different, allowing for better cellular tolerance [64].

Conclusion

This research provides a foundation for further investigation into optimizing liposomal drug delivery systems. Future studies could explore the benefits of surface modification techniques, such as PEGylation, or the use of ligand-targeted and immune liposomes to improve specificity and enhance antiviral efficacy. These advanced liposomal formulations could offer targeted delivery to specific cell types or tissues, further minimizing off-target effects and maximizing therapeutic outcomes. Additionally, exploring the mechanisms of cellular uptake in greater detail, including identifying the specific endocytic pathways involved, could provide insights into the rational design of even more efficient liposome-based drug delivery systems.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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