Original Article

Synthesis and Characterization of Un-encapsulated and Pterostilbene-encapsulated DOTAP: Cholesterol Liposomes

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ABSTRACT

Aim: Our study focuses on the liposome-based nanoformulation, which can encapsulate Pterostilbene for its subsequent testing in relevant, model systems for cancer. **Background:** Pterostilbene, a plant-derived, hydrophobic, dietary stilbenoid, has been studied for its ability to induce cell death and regulate caspases in the different types of cancer cells. The potential of this drug can be improved by formulating a suitable vehicle for its delivery. Biocompatible, lipid-based nanoparticles called liposomes have been studied as a potent delivery vehicle for drugs in pre-clinical as well as in clinical studies. Liposomes can improve the drug uptake and bioavailability of the drugs. **Materials and Methods:** Pterostilbene loaded liposomes were constructed using DOTAP and Cholesterol, by the Thin-Film Hydration method. Along with the loaded liposomes, blank liposomes (only DOTAP and Cholesterol, without Pterostilbene) were also constructed. The liposomes were characterized for their size, Polydispersity Index (PDI) and Zeta potential using DLS. Shape of the liposomes was analysed using TEM. Encapsulation Efficiency (EE) of the Pterostilbene loaded liposomes was determined. Also, UV-Vis spectrophotometer was used to ensure that Pterostilbene was encapsulated inside the liposomes and there was no interaction between the drug and the lipids. **Results:** Liposomes were composed of DOTAP and Cholesterol with molar ratios 2:1. The DLS showed that the size of the Pterostilbene-loaded liposomes was 435.6 ± 5 nm (n = 3), PDI was 0.5 ± 0.07 (n = 3) and Zeta potential was -16.4 ± 0.5 mV (n = 3). The drug encapsulation efficiency was found to be 97.5 ± 0.8% (n = 3). **Conclusion:** Reproducibility in the results (DLS and EE data for Pterostilbene-encapsulated liposomes) provides a sound, scientific basis for evaluating their cell death potential of Pterostilbene loaded liposomes against cancer cells in comparison with that of free Pterostilbene (parent compound). Also, the experimental flow of ours can be used as a teaching tool by educators in drug delivery and allied fields.

Key words: Pterostilbene, DOTAP, Cholesterol, Liposomes, Nanoparticles.

INTRODUCTION

Pterostilbene (IUPAC name- 4-[(E)-2-(3,5-dimethoxyphenyl)ethenyl]phenol) is a naturally occurring, hydrophobic compound and its relatively better cell membrane permeability has been attributed to be mainly due to it having two dimethoxy groups. It is mostly found in grapes, berries and fruits.[1] Pterostilbene, has numerous pharmacological properties that are similar to that of Resveratrol.[2-4] Pterostilbene has a better pharmacokinetic profile than resveratrol[5] with a logP value of 3.8. Among other pharmacological effects, Pterostilbene is also known to have anti-cancer potential.[6-8] However, its poor aqueous solubility and stability posed a real challenge that needed to be circumvented. One promising strategy has been to deliver Pterostilbene[9] using various nanoparticle-based drug vehicles including liposomes. Liposomes are spherical, micelle-like nano-carriers composed of phospholipids.[10,11] Liposomes have the potential to encapsulate a large number of hydrophobic as well as hydrophilic drugs.

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Apart from being biocompatible, liposomal-drug formulation is studied for its site-specificity and low toxicity. This nano-formulation also protects the drugs from being degraded *in vivo* and increases the efficacy and bioavailability of the drugs. Therefore, various liposomal formulations are making their way to the market.[12] DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) is a cationic surfactant, used in the formulation of liposomes. Cholesterol is a key component of cell membranes, which can be used in liposome formulation as a helper-lipid. Presence of cholesterol in liposomes is reported to provide stability to the liposomes and also can improve cellular uptake.[13] Therefore, liposomes can efficiently encapsulate drugs and deliver it to the cells efficiently.

Our study involved the formulation of DOTAP-Cholesterol liposome-based delivery vehicle for Pterostilbene. Pterostilbene-encapsulated liposomes offer the possibility to entrap the drug and resolve the aforesaid solubility as well as stability-related issues, apart from possibly enabling better delivery to the target, thereby minimizing side effects. Specifically, DOTAP liposomes being cationic would be expected to target negatively charged chemical moieties on the cell membrane, necessitating site-specific drug administration for improved safety and efficacy.[14] However, any variation in the net charge from positive to negative values may be dependent on the presence of cholesterol in the DOTAP liposome nano-construct.[15] Hence, measurements of zeta potential (net charge) is an important determinant for nanoparticle behaviour in cell culture systems. Our work has reproducibly demonstrated the synthesis and characterization of Pterostilbene-encapsulated DOTAP liposomes. Our nano-constructs can be further studied to improve the ability in inducing cell death, possibly by an increase in its uptake/bioavailability.

MATERIALS AND METHODS

Materials

Pterostilbene, cholesterol and Amicon®30 kDaUltracel-PL membrane ultra-centrifugal filters were obtained from Sigma-Aldrich, India. 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) 18:1 TAP (DOTAP) was procured from Avanti Polar Lipids, Inc., USA. The remaining reagents used were of standard analytical grade.

Liposome synthesis

Pterostilbene-loaded liposomes as well as blank liposomes (unloaded) were prepared using the thin-film hydration method.[15-18] Liposomes were composed of DOTAP and cholesterol (2:1, M ratios). Stock solutions of DOTAP and cholesterol as well as Pterostilbene were prepared in a solution containing chloroform: methanol in the ratio of 2:1 (v/v). For Pterostilbene-encapsulated liposomes, the drug: lipid ratio was kept the same at 0.1 (w/w). Blank liposomes were also synthesized with the same stoichiometry as described, minus the drug. The lipid-drug solution produced by the aforesaid thin-film hydration method was bath-sonicated (20 kHz) for 15 min. Then, probe sonication was done at 25% amplitude with 5 second pulse and 1 sec. pause for another 2 min. The samples were then passed through a 0.2-µm filter to ensure sterility and were stored at 4°C.

Liposome characterization-size, PDI, zeta potential

The samples were subjected to 15 min. of bath sonication, followed by dilution with ultra-pure, sterile (Milli-Q) water. Samples were further centrifuged at 5000xg in Ultracel-PL membrane ultra-centrifugal filter (Amicon® 30 kDa) for 5 min. The top phase comprised of liposomes was collected and was again dispersed in ultra-pure (Milli-Q), sterile water and was characterized for its size (Z-average), PDI and zeta potential using Malvern's Zetasizer Nano ZS (ZEN 3600).[16,19]

Liposome morphology- TEM

Morphology of the liposomes was assessed using TEM. The samples were subjected to 15 min. of bath sonication, followed by dilution with ultra-pure, sterile (Milli-Q) water. Samples were centrifuged at 5000xg in Ultracel-PL membrane ultra-centrifugal filter (Amicon® 30 kDa) for 5 min. The top phase comprised of liposomes was collected and was again dispersed in sterile, ultra-pure (Milli-Q) water. An FEI-Tecnai G2 20S-TWIN high-resolution TEM was used to analyse the samples.[16,18]

Drug-lipid interactions in liposomes

In order to study whether Pterostilbene interacts with DOTAP and cholesterol during and after liposome formation, we performed qualitative tests using Shimadzu UV-1280 UV-Vis spectrophotometer. Individual UV-Vis scans of DOTAP, cholesterol and Pterostilbene, dissolved in the same solvent that was used for the liposome synthesis (chloroform:methanol (2:1 v/v)), were recorded from 800 nm to 190 nm. The liposome samples were subjected to 15 min. bath sonication, followed by centrifugal filtration, so as to separate the free and encapsulated Pterostilbene. Individual UV-Vis scans of the top and the bottom phase of DOTAP-cholesterol blank as well as Pterostilbene-loaded DOTAP-cholesterol liposomes were recorded from 300 nm to 190 nm. Finally, the top phase of

Pterostilbene-loaded DOTAP-cholesterol liposomes was lysed by diluting it 20-times with methanol followed by probe sonication at 25% amplitude and 5 sec pulse for 30 min.[20] UV-Vis scan of this lysate was also recorded from 800 nm to 190 nm.

Liposome Encapsulation efficiency

The liposome samples (DOTAP-cholesterol blank and Pterostilbene-loaded liposomes) were subjected to 15 min. of bath sonication. Free Pterostilbene was separated from the Pterostilbene-loaded liposomes by centrifuging it at 5000xg for 5 min. at 4°C using Ultracel-PL membrane ultra-centrifugal filter (Amicon® 30 kDa). Free Pterostilbene was obtained at the bottom and the Pterostilbene-loaded liposomes were obtained at the top. In order to lyse the liposomes and measure the amount of encapsulated drug, the top phase was diluted 20-times with methanol, followed by 30 min. of probe sonication at 25% amplitude and 5 sec. pulse. The bottom layer was also diluted with methanol and was mixed thoroughly. Standard graph of Pterostilbene in methanol was plotted (318 nm) and the top and bottom phases were read using Shimadzu UV-1280 UV-Vis spectrophotometer at 318 nm.[16,20]

$$\text{Encapsulation Efficiency (\%)} = (\text{Pterostilbene}_{(\text{Top})} / \text{Pterostilbene}_{(\text{Top})} + \text{Pterostilbene}_{(\text{Bottom})}) \times 100$$

$\text{Pterostilbene}_{(\text{Top})}$ is the amount of Pterostilbene in the top phase, $\text{Pterostilbene}_{(\text{Bottom})}$ is the amount of Pterostilbene in the bottom phase.

RESULTS

Liposome characterization-size, PDI, zeta potential

The reproducibility of our measurements was demonstrated based on their size, PDI and zeta potential. The size of DOTAP-Cholesterol blank liposome was 415.1±41nm (n=3), PDI was 0.477±0.02 (n=3) and the zeta potential was - 21.6±1.4mV (n=3). The size of DOTAP-Cholesterol Pterostilbene-loaded liposome was little more than the blank liposome and was 435.6±50nm (n=3). The PDI was 0.506±0.07 (n=3), whereas the zeta potential was - 16.4±0.5mV (n=3).

Liposome morphology- TEM

The morphology of the liposomes was observed using TEM. Both, DOTAP-Cholesterol blank liposomes (Figure 1 (A)) as well as DOTAP-Cholesterol Pterostilbene-loaded liposomes (Figure 1 (B)) were circular in shape. Pterostilbene-loaded liposomes were slightly larger in size.

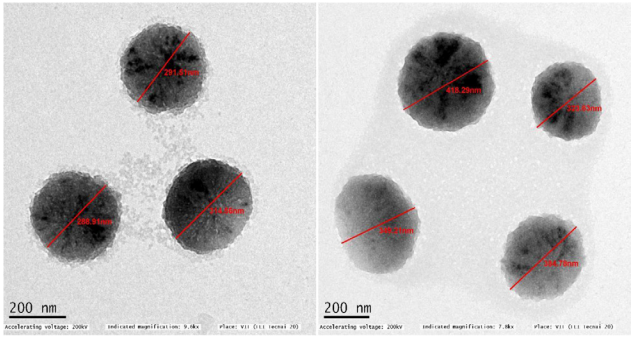

Figure 1(A): DOTAP-Cholesterol blank liposomes. Figure 1(B): Pterostilbene loaded DOTAP-Cholesterol liposomes.

TEM images reviled the circular morphology of liposomes. DOTAP-Cholesterol blank liposomes were smaller in size than Pterostilbene-loaded DOTAP-Cholesterol liposomes.

Drug-lipid interactions in liposomes

In order to study if there is any interaction between the liposome components, namely DOTAP, Cholesterol and Pterostilbene, UV-Vis spectrum scans were carried out for individual components as well as during and after liposome synthesis. UV-Vis λ_{max} for DOTAP was 238.5nm (Figure 2 (A)), for that of cholesterol was 234nm (Figure 2 (B)), whereas, when DOTAP and cholesterol were combined in 2:1 (M ratios), the λ_{max} was found to be 239nm (Figure 2 (C)). The UV-Vis λ_{max} for Pterostilbene was 318nm (Figure 2 (D)). When DOTAP-cholesterol and Pterostilbene were combined during liposome synthesis, it gave two discrete lipid and Pterostilbene UV-Vis λ_{max} peaks at 239.5nm and 319nm (Figure 2 (E)) respectively. After centrifugation using the AMICON filter, the top phase contained the liposomes, while the bottom phase contained buffer and free drug (if any). The UV-Vis spectrum of the bottom phase of DOTAP-cholesterol blank liposome gave no peak (Figure 2 (F)), while the UV-Vis spectrum of the top phase of DOTAP-cholesterol blank liposome gave a peak at 217nm (Figure 2 (G)), thus suggesting that the top phase was comprised of the liposomes and the bottom phase only had the buffer. The UV-Vis spectrum of the bottom phase of DOTAP-cholesterol Pterostilbene-loaded liposome gave no major peak (Figure 2 (H)) while the UV-Vis spectrum of the top phase of DOTAP-cholesterol Pterostilbene-loaded liposome gave a peak at 214nm (Figure 2 (I)), but when the DOTAP-cholesterol Pterostilbene-loaded liposome top phase was subjected to probe sonication as described above, it gave UV-Vis λ_{max} at 221nm and at 318.5nm (Figure 2 (J)). This indicates that the top phase contains the liposomes and when the liposomes are subjected to probe sonication, the liposomes are

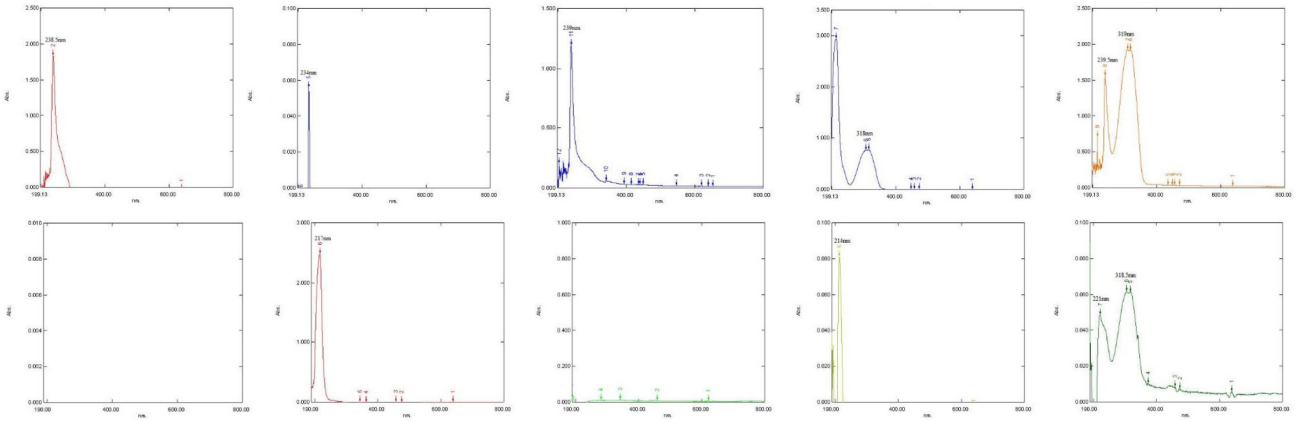

Figure 2: These figures represent UV-Vis spectrophotometer scans of the liposome components viz., DOTAP, Cholesterol as well as of Pterostilbene before liposome preparation, during liposome preparation as well as after liposome preparation as mentioned in the results. Figure 2(A). DOTAP, Figure 2 (B). Cholesterol, Figure 2 (C). DOTAP and cholesterol combined in 2:1 (M ratios), Figure 2 (D). Pterostilbene, Figure2 (E). DOTAP-cholesterol and Pterostilbene in combination, Figure 2 (F). Bottom phase of DOTAP-cholesterol blank liposome, Figure2 (G). Top phase of DOTAP-cholesterol blank liposome, Figure 2 (H). Bottom phase of DOTAP-cholesterol Pterostilbene-loaded liposome, Figure 2 (I). Top phase of DOTAP-cholesterol Pterostilbene-loaded liposome, Figure 2 (J). DOTAP-cholesterol Pterostilbene-loaded liposome top phase after probe sonication.

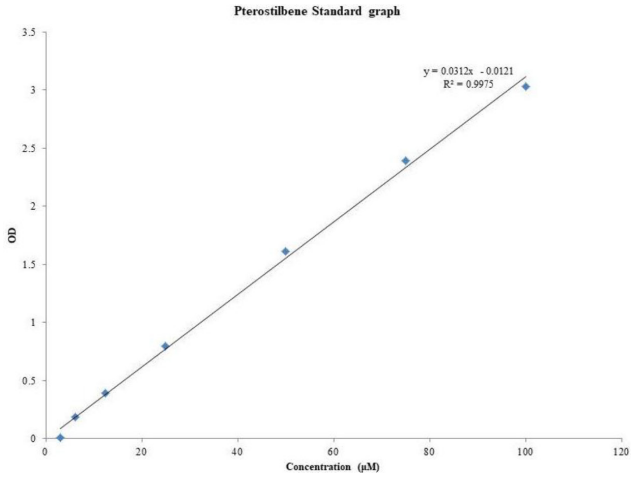

Figure 3: Standard graph of Pterostilbene was plotted at 318 nm. Solvent used was methanol.

lysed and Pterostilbene escapes out. These results show that there is no interaction in the UV-Vis absorbance of various components of the liposomes, viz. DOTAP, Cholesterol and Pterostilbene. The data also revealed that Pterostilbene-loaded DOTAP-Cholesterol liposomes did not display the Pterostilbene UV-Vis absorbance peak, unless subjected to lysis, indicating that Pterostilbene is encapsulated inside the liposomes.

Liposome Encapsulation efficiency

UV-Vis Spectrophotometer was used to determine the encapsulation efficiency of the liposomes. The Pterostilbene content in the top and the bottom phase was quantified by comparing the OD with the standard graph of Pterostilbene. OD of the bottom phase was 0.023 and the concentration of Pterostilbene was 1.12µM, while the OD of the top phase after probe sonication was 1.4 which corresponded to 45.25µM Pterostilbene. The standard graph of Pterostilbene is showed in Figure 3. The encapsulation efficiency was reported to be 97.5±0.8% (n=3).

DISCUSSION

Pterostilbene has been studied earlier for its various properties, including action against various types of cancers such as human gastric carcinoma, leukemia and human colon cancer. Due to its higher log *P* value (log P 3.8), Pterostilbene is not soluble in aqueous media, which can hinder its delivery *in vitro* and *in vivo*. Also, Pterostilbene uptake in Estrogen Receptor-negative cells is a challenge. One of the key pharmacokinetic advantages of Liposome-entrapment of a drug can be possible decrease in the dose required for the therapeutic effect. Further, liposome encapsulation circumvents solubility-related issues of lipid-soluble drugs that will serve to improve the dispersion of our nano-construct in a suitable vehicle as well as contribute to an enhancement in the uptake. Blank and loaded liposomes were synthesized and Pterostilbene was encapsulated within the loaded liposomes. They were characterized for their shape, size, PDI and zeta potential using DLS and TEM. The size observed in the TEM images was fairly similar to that obtained from the DLS data. The average size of ours is similar, barring a slight variation, to the values reported in the literature with the unextruded liposomes being relatively larger.[21,22] Polydispersity of both

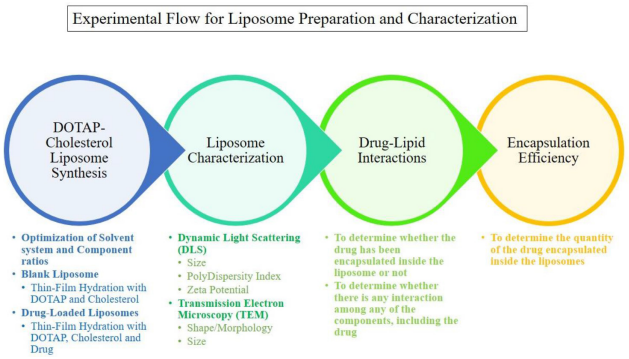

Figure 4: Experimental flow that can be used by educators involved in drug delivery.

the liposomes suggested a uniform size distribution.[16,23] The zeta potential, however, was negative, despite the presence of DOTAP, a cationic lipid. One possibility of this cationic lipid acquiring a negative charge might be due to the presence of Cholesterol, which is consistent with certain reports in the literature.[15] There might also be a formation of a protein adsorption layer or protein corona around the liposomes, which can modulate the uptake mechanism[16,24] thereby circumventing the negative charge-mediated barriers for the uptake processes.[25,26] Taken together, our results conclusively demonstrate that Pterostilbene can be encaspsulated in DOTAP-liposomes fairly reproducibly. This synthesis and characterization has provided a basis for their testing in cell culture systems for possible improvements in cytotoxicity and cell death potential. Also, this work can be extended in model systems *in vivo* as well.

CONCLUSION

This work reports the hitherto undocumented synthesis and preliminary characterization of Pterostilbene-encapsulated DOTAP liposomes. This approach would pave the way for evaluating the *in vitro* and *in vivo* cancer cell death potential of Pterostilbene in the DOTAP liposome-based delivery vehicle. Validation of our experimental flow has paved the way to encapsulate other molecules in the stilbenoid class, followed by their subsequent characterization. Also, this design can be an iterative experimental tool (Figure 4 -experimental flow-chart for the educators) for the demonstration of the encapsulation, characterization and subsequent enhancements in bioavailability by pharmaceutical educators as well.

Authors' Contributions

The lead author was primarily responsible for executing the project in terms of standardization, trouble-shooting and performing experiments (at the bench). He was also responsible for writing the first draft of the manuscript. The second and corresponding author was involved with the conceptualization, data interpretation, trouble-shooting as well as editing and critical evaluation of the manuscript. He is the mentor of the first author of this manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

ABBREVIATIONS

DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; **TEM:** Transmission Electron Microscopy; **kHz:** Kilo Hertz; **PDI:** Polydispersity Index; **DLS:** Dynamic Light Scattering; **EE:** Encapsulation Efficiency; **kDa:** Kilo Dalton; **UV-Vis:** Ultraviolet –Visible; **OD:** Optical Density.

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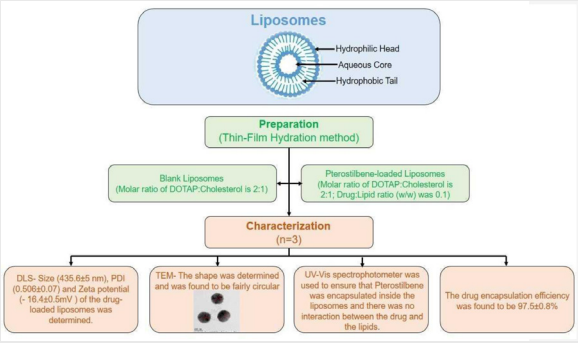
PICTORIAL ABSTRACT



SUMMARY

We have demonstrated reproducibly the synthesis and characterization of Blank & Pterostilbene-encapsulated DOTAP-cholesterol liposomes. Data obtained was in consonance with published data.

About Authors

Hiray K.S: He has finished his Bachelor of Technology in Biotechnology from VIT Vellore. He is currently pursuing his Master of Technology by Research and is working on the effects of drugs (purified natural compounds) on cancer cells and formulation of an effective nano-derived drug delivery system.

P.K.Suresh: Professor Higher Academic Grade (PHAG) in the Department of Biomedical Sciences, School of Biosciences and Technology. He has approximately 21 years of teaching, research and administrative experience (post-Ph.D.). He received his second masters and Ph.D. in SIUE, IL, USA and the University of Cincinnati, Ohio, USA respectively. He was a Post-doctoral fellow at the University of Texas at Austin, TX, USA as well as Rutgers University, Piscataway, USA. P.K.Suresh has authored/co-authored over 45 publications in SCOPUS-indexed journals with an *h*-index of 10 and a cumulative citation index of 407. He has been a resource person and/or coordinator in several Faculty Development Programs as well as in International Conferences in India and overseas. He has mentored students at several levels including those pursuing their doctoral degree. Apart from *in silico* and *in vitro* Chemical Biology/Technology, he is also involved in drug development and delivery systems.

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