

Assessment of a New Ginsenoside Rh2 Nanoniosomal Formulation for Enhanced Antitumor Efficacy on Prostate Cancer: An in vitro Study

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Introduction: Ginsenoside Rh2, purified from the *Panax ginseng* root, has been demonstrated to possess anticancer properties against various cancerous cells including colorectal, breast, skin, ovarian, prostate, and liver cancerous cells. However, the poor bioavailability, low stability on gastrointestinal systems, and fast plasma elimination limit further clinical applications of Ginsenoside Rh2 for cancer treatments. In this study, a novel formulation of niosomal Ginsenoside Rh2 was prepared using the thin film hydration technique.

Methods: The niosomal formulation contained Span 60 and cholesterol, and cationic lipid DOTAP was evaluated by determining particle size distribution, encapsulation efficiency, the polydispersity index (PDI), and surface morphology. The cytotoxic effects of free Ginsenoside Rh2 and Ginsenoside Rh2-loaded niosomes were determined using the MTT method in the PC3 prostate cancer cell line. For the investigation of the in vitro cellular uptake of Ginsenoside Rh2-loaded niosome, two formulations were prepared: the Ginsenoside Rh2-loaded niosomal formula containing 5% DOTAP and the Ginsenoside Rh2-loaded niosomal formula without DOTAP.

Results: The mean size, DPI, zeta potential, and encapsulation efficiency of the Ginsenoside Rh2-loaded nanoniosomal formulation containing DOTAP were 93.5 ± 2.1 nm, 0.203 ± 0.01 , $+4.65 \pm 0.65$, and $98.32\% \pm 2.4$, respectively. The niosomal vesicles were found to be round and have a smooth surface. The release profile of Ginsenoside Rh2 from niosome was biphasic. Furthermore, a two-fold reduction in the Ginsenoside Rh2 concentration was measured when Ginsenoside Rh2 was administered in a nanoniosomal form compared to free Ginsenoside Rh2 solutions in the PC3 prostate cancer cell line. After storage for 90 days, the encapsulation efficiency, vesicle size, PDI, and zeta potential of the optimized formulation did not significantly change compared to the freshly prepared samples. The cellular uptake experiments of the niosomal formulation demonstrated that by adding DOTAP to the niosomal formulation, the cellular uptake was enhanced.

Discussion: The enhanced cellular uptake and cytotoxic activity of the Ginsenoside Rh2 nanoniosomal formulation on the PC3 cell make it an attractive candidate for application as a nano-sized delivery vehicle to transfer Ginsenoside Rh2 to cancer cells.

Keywords: nanoniosomal, Ginsenoside Rh2, chemotherapy, PC3 prostate cancer cell line

Introduction

Prostate cancer is one of the most common cancers and the leading cause of cancer deaths among men worldwide. The best choices of treatment, radiation therapy and chemotherapy, are unsatisfactory due to their detrimental side effects on healthy cells and the problem of drug resistance. Searching for new agents for the treatment of cancer is the goal of numerous studies, and many studies are concentrated on plant-derived

compounds that have curative potential.^{1–3} Ginsenoside Rh2 is one of the ginsenoside agents purified from the *Panax ginseng* root.⁴ This compound has different biological activities, especially an anticancer activity, against various cancerous cells (colorectal, breast, skin, ovarian, prostate, and liver cancer cells).^{5–7} The worst limitation for the application of Ginsenoside Rh2 is its low stability in gastrointestinal and bloodstream systems. Ginsenoside Rh2 has an extremely low oral bioavailability due to its high hydrophobic property. Moreover, it can be quickly eliminated from plasma. Ginsenoside Rh2 can be easily removed by enzymatic reactions in the body.^{8,9} Thus, enhancing the solubility and stability of Ginsenoside Rh2 is a major challenge for its application. For solving this problem, nanotechnology can be used as the best strategy to enhance the stability and efficiency of Ginsenoside Rh2. There are some studies on applications of nanostructures for this purpose.^{8,10} Accordingly, a lipid-based drug delivery system has been considered due to having fewer limitations than conventional formulations. Biocompatibility, biodegradability, efficiency, and large-scale production are the most important properties of lipid nanostructures.^{11,12} Previous studies used liposomes to enhance the efficiency of natural drugs such as Ginsenoside Rh2.^{8,13,14} There are no studies on the application of niosomes for this drug. Niosomes are non-ionic surfactant-based vesicles. Niosomes are formed mostly by non-ionic surfactants and cholesterol. Niosomes are a novel drug delivery system, in which the drug is encapsulated in a vesicle.¹⁵ Both hydrophilic and lipophilic drugs entrap in either the aqueous layer or the vesicular membrane.¹⁶ Niosomes are osmotically active and stable, increase the stability of the entrapped drug, are used for a variety of drugs, have controlled release, biodegradability, and non-immunogenic agents, and are biocompatible.¹⁷ To the best of our knowledge, no study has been performed on niosomes for delivery of Ginsenoside Rh2 to cancer cells, which was examined in the present study for the first time. Moreover, in the current study, the function of Ginsenoside Rh2 encapsulated in niosome was evaluated in comparison with free Ginsenoside Rh2 in terms of in vitro cellular uptake and cytotoxicity using the PC3 prostate cancer cell line.

Materials and Methods

Preparation of Ginsenoside Rh2 Niosome

A thin-film hydration method was employed for preparing Ginsenoside Rh2-loaded niosome.^{18–20} We dissolved Cholesterol (Sigma-Aldrich, USA), cationic lipid DOTAP (1,2-dioleoyl-3-trimethylammonium-propane, Sigma-

Aldrich, USA) (66.5:28.5: 5 molar ratios), and Span 60 (Sigma-Aldrich, USA) in chloroform as an oil phase (organic solution). Ginsenoside Rh2 (Sigma-Aldrich, USA) was then dissolved in one mL chloroform, and it was mixed with lipids/surfactant. Cellular uptake was assessed by the addition of fluorescent label Dil (0.1 mol%, Sigma, USA) to the lipid phase for lipid staining. The solvent (chloroform) was evaporated (organic phase removal) using a rotary evaporator (Heidolph, Germany) at 40°C as long as a thin-layered film was obtained. After preparation, we hydrated dry lipid film with phosphate-buffered saline (PBS, pH= 7.4) for 30 minutes at 50°C so that the niosomal suspensions are obtained. Once hydration was completed, sonication of vesicles was done for 20 minutes by a microtip probe sonicator for reducing the mean size of vesicles. When hydration was completed, a microtip probe sonicator was used for 45 minutes (15 s on – 10 s off, amplitude of 60 at 100 watts) for reducing the mean size of the vesicles. In the end, separation of free drugs (unloaded) from niosomal vesicles was done using dialysis tubing cellulose membrane (molecular weight cutoff = 12,000, Sigma-Aldrich, USA) against PBS for one hour.²¹ Similarly, empty niosomes were developed, but Ginsenoside Rh2 was not added. The Ginsenoside Rh2 dosage was 0.2 mg/mL. We prepared two formulations in order to investigate the in vitro cellular uptake in Ginsenoside Rh2-loaded niosome: a Ginsenoside Rh2-loaded niosomal formula that contained 5% DOTAP and a Ginsenoside Rh2-loaded niosomal formula with no DOTAP.¹⁹

Encapsulation Efficiency Analysis

The entrapment efficiency was determined using spectroscopic measurement.^{18,19} A UV spectrophotometer was used at 232 nm (λ_{max}) for estimating amounts of Ginsenoside Rh2 loaded into niosomes within the preparation.²² We mixed Ginsenoside Rh2-loaded niosome with isopropyl alcohol for lysing the membranes and allowing quick shedding of entrapped Ginsenoside Rh2 so that encapsulation efficiency can be estimated. In the end, the following relation was used for determining encapsulation efficiency:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{The amount of Ginsenoside Rh2 encapsulated within niosome}}{\text{Total amount of Ginsenoside Rh2 added}} \times 100$$

Determining Polydispersity Index, Size, and Zeta Potential of Niosomal Vesicles

The polydispersity index (PDI) and particle size distribution of the obtained niosome were determined using the

dynamic light scattering (DLS) method. To this end, a particle size analyzer was employed. The surface charge of the niosome was measured by the ZetaPALS zeta potential analyzer. A scanning electron microscope (SEM) was used for evaluating niosome surface morphology.

In vitro Release Investigation of Ginsenoside Rh2 from Niosome

One mL samples (formulations of Ginsenoside Rh2-loaded niosome) were prepared in dialysis tubes soaking in 10 mL PBS (at pH = 7.4, 37°C) constantly stirring for 72 hours. Then, the profile of Ginsenoside Rh2 release from niosome was evaluated. Extraction of one mL of PBS directly surrounding the dialysis tube was done at pre-specified intervals, and UV/visible spectroscopy at 232 nm was used for reading the absorbance intensity. The release amount of Ginsenoside Rh2 was measured by the division of the amount of Ginsenoside Rh2 in the medium by its primary amount.^{18,19,23}

Examination of Physical Stability

The changes in zeta potential, particle size, PDI, and surplus amount of drug in the vesicle were examined at various periods for determining the physical stability of the optimal formulation of Ginsenoside Rh2-loaded niosome (formula with 5% DOTAP) during storage. The niosome samples were sealed and kept in a glass vial for 90 days at 4°C under light protection. During 14 and 28 days, two and three-month intervals, stability analysis was done.^{18,19,24,25}

Culture Conditions and Cell Lines

We cultured human prostate cancer PC-3 cell (Iranian Biological Resource Center, Tehran, Iran) in DMEM with penicillin/streptomycin (Gibco, USA) and 10% FBS (Fetal Bovine Serum, Gibco, USA) supplementation.

Cytotoxicity Assays

The MTT test was conducted for determining cell viability and comparing the inhibitory impact of free Ginsenoside Rh2 and Ginsenoside Rh2-loaded niosome on cell proliferation in test cells.^{26–28} 96-well plates with 200 μ L of DMEM were used for seeding PC-3 cells, with 10% FBS supplementation. The cells were seeded for one day, so that cell attachment happens. Then, 200 μ L fresh medium with series dilutions of Ginsenoside Rh-loaded niosome and free Ginsenoside Rh2 solution replaced the cell

medium. Following one-day incubation, we added 20 μ L of MTT solution (5 mg/mL) to the wells, and incubation of cells was done for 4 h. The wells' supernatant was eliminated, followed by the addition of 180 μ L of DMSO to each well. An EPOCH Microplate Spectrophotometer at 570 nm was used for recording absorbance of each well. The cytotoxicity of Ginsenoside Rh2 as a niosomal drug or single drug was given as an IC₅₀ value, which the concentration of drug needed for cell growth inhibition by 50% relative to an untreated control cell without treatment with Ginsenoside Rh2 as a niosomal drug or single drug. GraphPad Prism was used for calculating IC₅₀ values.

Cellular Uptake of Ginsenoside Rh2-Loaded Nanoniosome

We prepared two formulations for analyzing in vitro cellular uptake of Ginsenoside Rh2-loaded niosome: the Ginsenoside Rh-loaded niosomal formula without DOTAP and Ginsenoside Rh2-loaded niosomal formula with 5% DOTAP. The in vitro cellular uptake of Ginsenoside Rh2-loaded niosome was analyzed on PC-3 cells in 6-well plates at a density of 150,000 cells/well. Cell incubation was done for one day for cell attachment. Then, the treatment of cells with two formulations of Ginsenoside Rh2-loaded niosomes was performed during incubation for 4 h at 37°C. Following a four-hour incubation process, the cells were rinsed three times using cold PBS, and then they were fixed with 4% paraformaldehyde solution (Sigma, USA). Later, the DAPI solution was used for cell staining and a fluorescence microscope (Olympus, Japan) was used for imaging.^{21,26,28}

Statistical Analysis

The tests were performed three times, and the data were provided as mean \pm SD. Student's *t*-test was conducted as a statistical analysis to compare two independent groups. Data were considered significant at *p*-value <0.05.

Results

Characterization of Ginsenoside Rh2-Loaded Nanoniosome

The zeta potential, mean size, PDI, and encapsulation efficiency of the formulation of Ginsenoside Rh2-loaded nanoniosomes with DOTAP were $+4.65 \pm 0.65$, 93.5 ± 2.1 nm, 0.203 ± 0.01 , and $98.32\% \pm 2.4$ (Figure 1A and B). The zeta potential, mean size, PDI, and encapsulation efficiency of the formulation of Ginsenoside Rh2-loaded nanoniosome

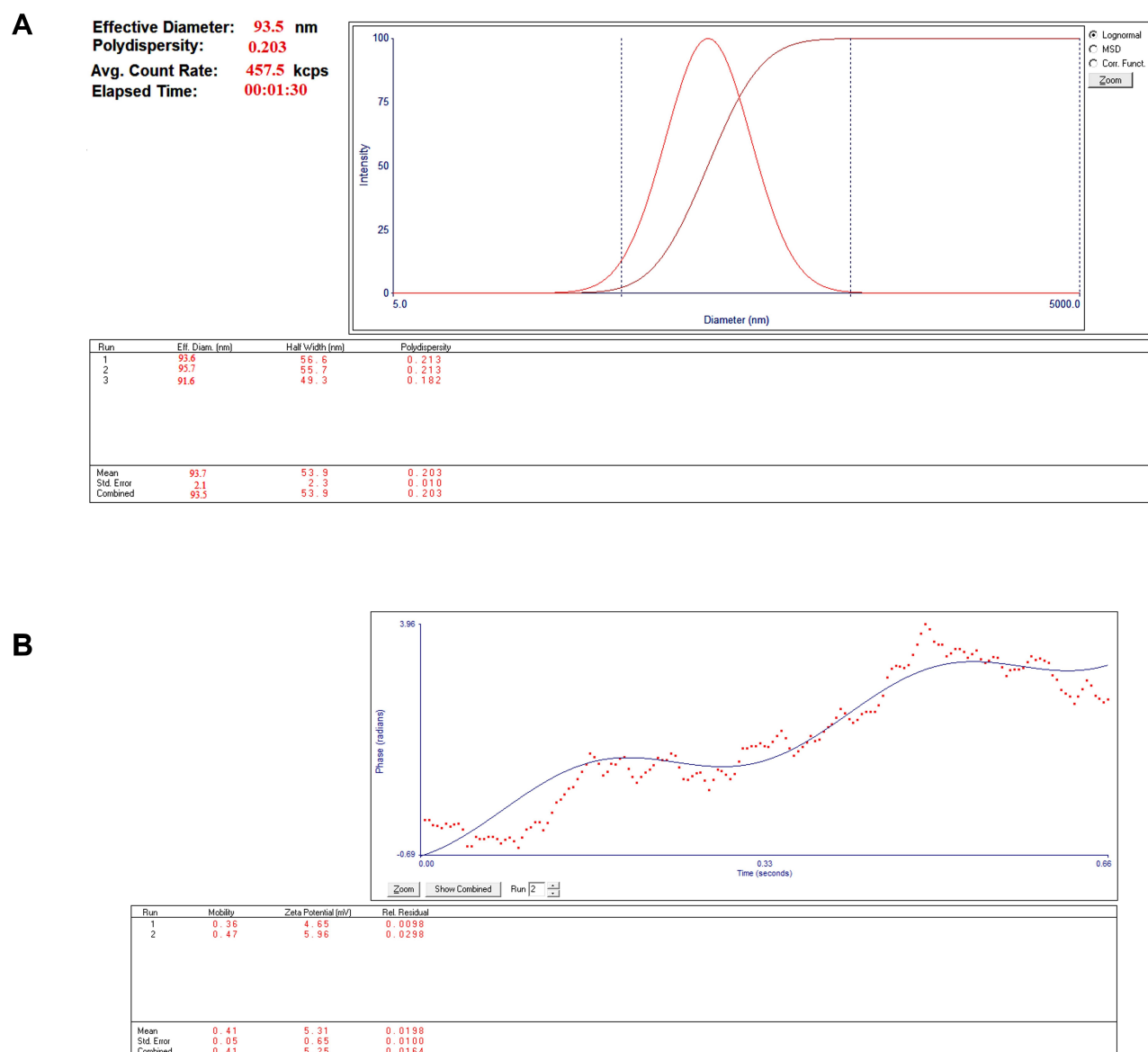


Figure 1 Physicochemical characteristics of formulation of Ginsenoside Rh2-loaded nanoniosome with DOTAP. **(A)** The size distribution and PDI formulation of Ginsenoside Rh2-loaded nanoniosome with DOTAP. **(B)** The zeta potential formulation of Ginsenoside Rh2-loaded nanoniosome with DOTAP.

without DOTAP were -16.5 ± 3.96 , 113.4 ± 0.7 nm, 0.247 ± 0.011 , and $81.27\% \pm 2.1$ (Figure 2A and B). An SEM micrograph indicated that the formulations of Ginsenoside Rh2-loaded nanoniosome are in round shape with a smooth surface (Figure 3A and B).

Investigation of in vitro Drug Release

The dialysis method and dialysis tubing cellulose membrane were used for assessing in vitro release of the drug. Figure 4 indicates the profile of Ginsenoside Rh2 release from niosomal formulations at 37°C . Following 72 hours, there were 33.75% and 42.09% release of loaded

Ginsenoside Rh2 from nanoniosome with DOTAP and Ginsenoside Rh2-loaded nanoniosome without DOTAP, respectively (Figure 4A and B). The Ginsenoside Rh2 had a biphasic release profile with a quick primary release, and then a slower release phase appeared.

Examination of Physical Stability

For determining the physical stability of the optimal formulation of Ginsenoside Rh2-loaded niosome (formulation with 5% DOTAP) during storage, regarding encapsulation efficiency, PDI, vesicle size, and zeta potential were examined by storage at 4°C . Following being

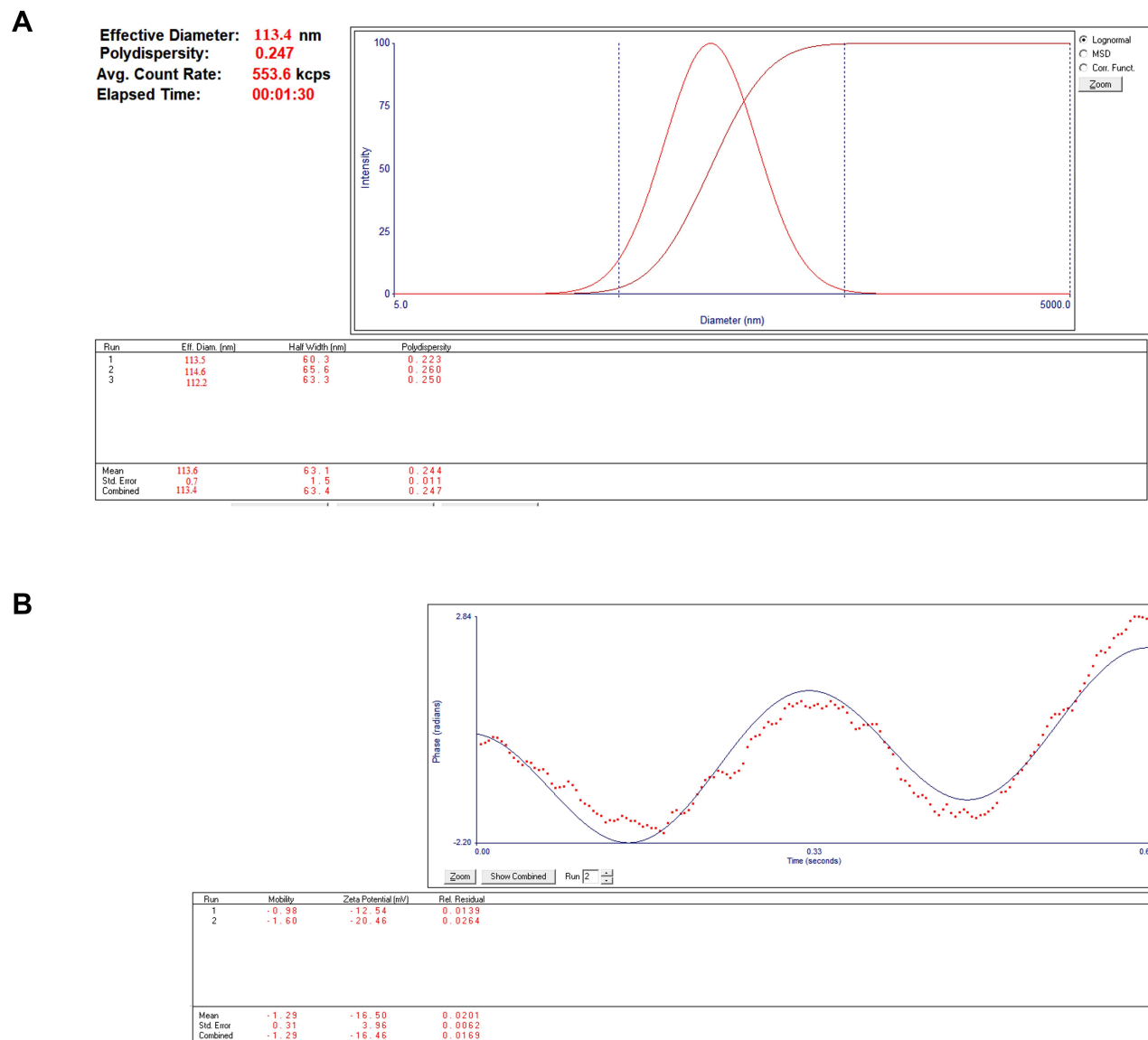


Figure 2 Physicochemical characteristics of formulation of Ginsenoside Rh2-loaded nanoniosome without DOTAP. **(A)** The size distribution and PDI formulation of Ginsenoside Rh2-loaded nanoniosome without DOTAP. **(B)** The zeta potential formulation of Ginsenoside Rh2-loaded nanoniosome without DOTAP.

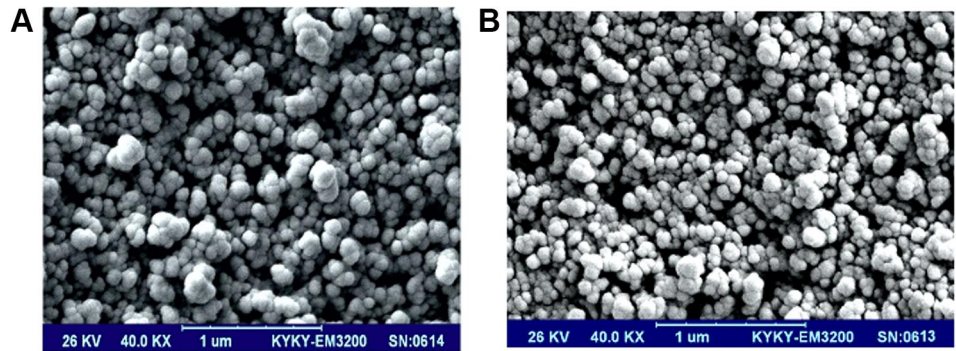


Figure 3 Morphological study of formulations of Ginsenoside Rh2-loaded nanoniosome. **(A)** Scanning electron microscopy of Ginsenoside Rh2-loaded nanoniosome with DOTAP. **(B)** Scanning electron microscopy of Ginsenoside Rh2-loaded nanoniosome without DOTAP.

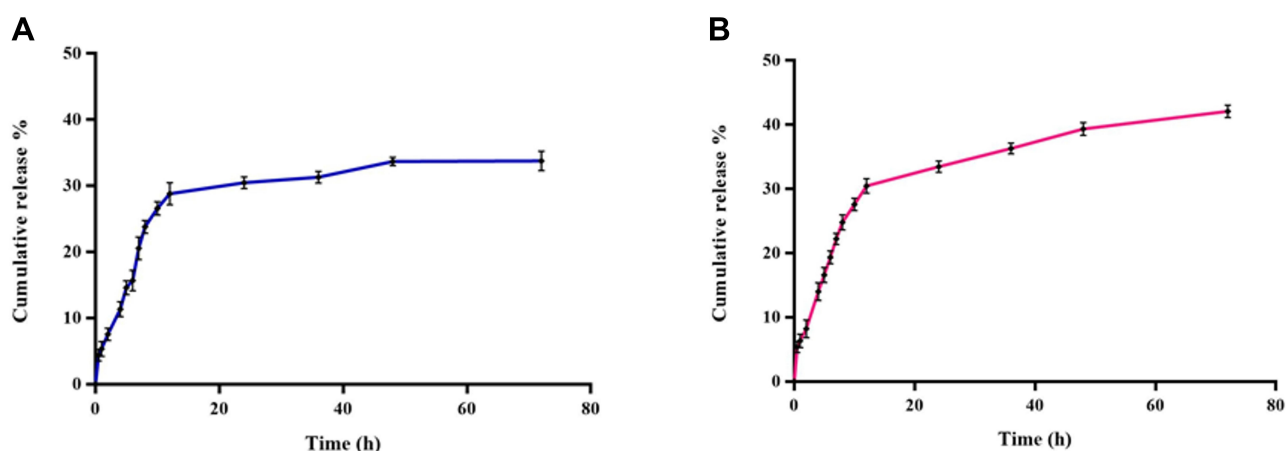


Figure 4 The in vitro release profile of Ginsenoside Rh2 from nanoniosome formulations. **(A)** The in vitro release profile of Ginsenoside Rh2 from nanoniosome formulation with DOTAP. **(B)** The in vitro release profile of Ginsenoside Rh2 from nanoniosome formulation without DOTAP.

stored for 90 days, a significant change was not observed in the PDI, vesicle size, encapsulation efficiency, and zeta potential of the optimized formula compared to the samples that were freshly generated (p -value >0.05). Thus, the stability of the Ginsenoside Rh2-loaded niosome formula with 5% DOTAP was confirmed.

Cytotoxicity Assays

Dose-response tests were conducted for determining the inhibitory impact of Ginsenoside Rh2-loaded nanoniosome and drug-free form on PC-3 cells. Figure 5 indicates that treating with drug niosomal form and drug-free form increased inhibition of PC-3 cells in a dose-dependent model. The IC_{50} values of drug-free form and drug

niosomal form were 62.66 ± 0.84 and 31.24 ± 0.75 $\mu\text{g}/\text{mL}$ against PC-3 cells (Figure 5A and B). Figure 5B depicts that the niosomal formulation of Ginsenoside Rh2 had higher efficiency to deliver the Ginsenoside Rh2 to PC-3 cells. After administration of Ginsenoside Rh2, a two-fold decrease in the concentration of Ginsenoside Rh2 was found in the niosomal formula in comparison with the drug-free form.

Cellular Uptake of Ginsenoside Rh2-Loaded Nanoniosome

The cellular uptake behavior of Ginsenoside Rh2-loaded nanoniosome in PC-3 cells is indicated in Figure 6.

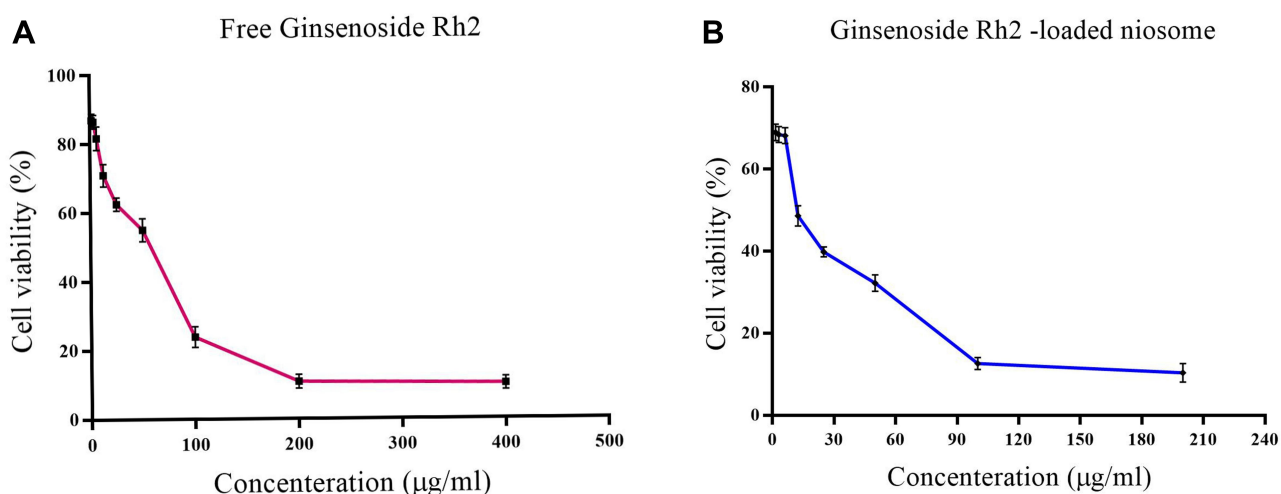


Figure 5 Cell viability assay on PC-3 cell line by Ginsenoside Rh2. **(A)** Cell viability on PC-3 cell after 48 hours of treatment with various concentrations of free Ginsenoside Rh2. **(B)** Cell viability on PC-3 cell after 48 hours of treatment with various concentrations of the optimal formulation of Ginsenoside Rh2-loaded nanoniosome.

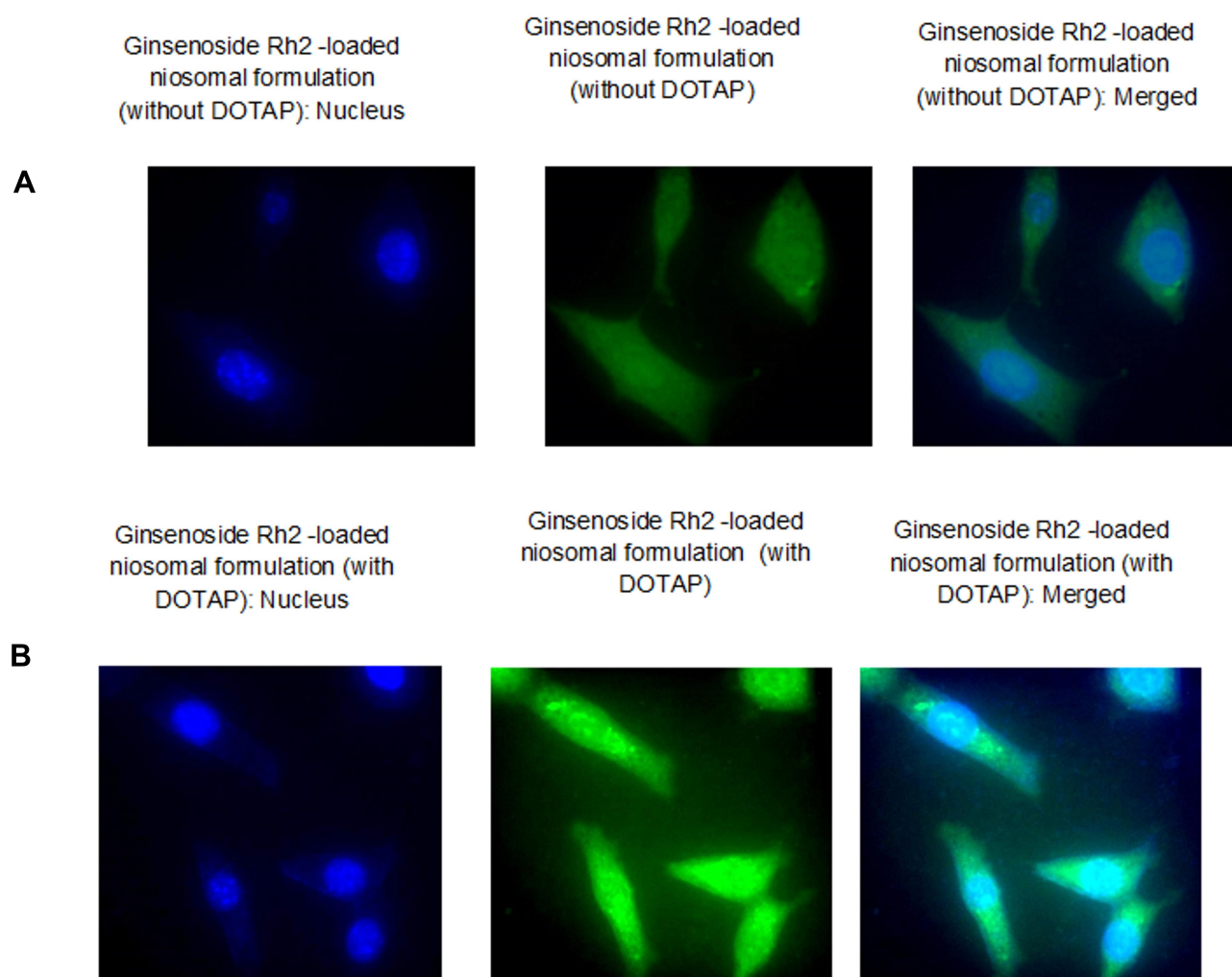


Figure 6 Cellular uptake of Ginsenoside Rh2-loaded niosome on PC-3 line. **(A)** Ginsenoside Rh2-loaded niosomal formulation (without DOTAP). **(B)** Ginsenoside Rh2-loaded niosomal formulation (with DOTAP).

Figure 6B shows that the PC-3 cells with Ginsenoside Rh2-loaded niosomal treatment containing 5% DOTAP had more green and cyan (blue-green) color intensity in comparison with PC-3 cells treated with Ginsenoside Rh2-loaded niosome formula without DOTAP (Figure 6A). Cellular uptake was improved with the addition of DOTAP to niosomal formulation. In addition, the behavior of Ginsenoside Rh2-loaded nanoniosome cellular uptake presented a higher distribution of Ginsenoside Rh2 in the nuclear area in comparison with the cytoplasm.

Discussion

In this study, a novel nanoniosomal formulation was synthesized to encapsulate Ginsenoside Rh2. The niosomal vesicular systems were prepared from the nonionic surfactant Span 60, as a commercial surfactant. The

encapsulation efficiency of Ginsenoside Rh2-loaded nanoniosomal formulation containing DOTAP was 98.32%. RationalThis high encapsulation efficiency may be speculated to be due mainly to the structural affinity between Ginsenoside Rh2 (as a hydrophobic agent) and the non-polar stearate moiety (hydrophobic C-H chain) of Span 60.²³ Several other studies have attempted to determine a new formulation to obtain effective encapsulation for Ginsenoside Rh2. Chen et al synthesized novel double pH-sensitive mixed micelles to fabricate multicore niosomes for drug delivery. The double pH-sensitive mixed micelles (PMM) were prepared with different pH-sensitive polymers, mPEG2000-Hz-CHEMS and mPEG2000-IS (2:1 w/w). Ginsenoside Rh2-loaded DPMM was mixed with Pluronic F-68 in the aqueous medium, and multicore niosomes were fabricated. The size of multicore niosomes

was around 100–200 nm with the encapsulation efficiency around 93%.²⁹ The cumulative release profile of Ginsenoside Rh2 from the nanoniosomal formulation was apparently biphasic with an initial rapid release period extended up to almost 12 hours followed by a slower release phase. The initial fast rate in the release occurring at the beginning phase can be regulated by the combination of diffuse mechanisms in the outside layers of niosomes and the emancipation of drugs located on the outer surface. In such situations, it was expected to transfer drug agents from the successional inner layer to the adjacent outer layers, and then, to the surface of the vesicle with delay.^{18,23,30} After storage for 90 days, the optimum formulation of Ginsenoside Rh2-loaded niosome (containing 5% DOTAP) demonstrated no significant changes when compared to the freshly prepared samples in terms of encapsulation efficiency, vesicle size, polydispersity index (PDI), and zeta potential. This implies that the optimum formulation of Ginsenoside Rh2-loaded niosome could minimize problems associated with niosome instability, including aggregation, fusion, and drug leakage.^{18,19,24,25} Ginsenoside Rh2-loaded nanoniosome was synthesized and evaluated in terms of anticancer activities. The results indicated that treatment with the drug-free form and the drug niosomal form resulted in the growth inhibition of PC-3 cells in a dose-dependent pattern. Similar studies acquired identical data.^{31–33} Ginsenosides, especially Ginsenoside Rh2, are attractive candidates for anticancer drug development as they are relatively non-toxic to normal cells. Due to rapid proliferation, cancerous cells lose appropriate apoptotic control. Ginsenosides induce apoptosis in cancerous cells using the mitochondrial-dependent intrinsic apoptotic pathway and the death receptor-dependent extrinsic pathway.^{34–36} Thus, it is concluded that Ginsenoside Rh2 can lead to apoptosis induction in cancerous cells. Based on the data, a two-fold reduction was measured in the Ginsenoside Rh2 concentration, when Ginsenoside Rh2 was administered in the nanoniosomal form compared to free Ginsenoside Rh2 in PC-3 cells. Thus, the encapsulation of Ginsenoside Rh2 in the nanoniosomal form can lead to the reduction of the essential drug, but with similar effects as higher concentrations of free drug. The status of Ginsenoside Rh2 can reduce the cost of the therapeutic index of the used drug. The main challenge for the application of Ginsenoside Rh2 as a drug is its low stability in blood and GI systems. Encapsulation in delivery systems such as niosome can solve this challenge. Xin et al prepared a cocktail of

betulinic acid, parthenolide, honokiol, and Ginsenoside Rh2 in liposome systems for lung cancer treatment by direct hydration of a lipid film. The average hydrodynamic size of the cocktail liposome system was 115.7 nm with a zeta potential of -18.31 mV. The EE was 88.2%, 90.3%, 89.5%, and 91.4% for Ginsenoside Rh2, parthenolide, betulinic acid, and honokiol, respectively, and the total loading efficiency was 15.3%. In vitro studies indicated that the combination of betulinic acid, parthenolide, honokiol, and Ginsenoside Rh2 exhibited a synergistic action. An increased effect was observed when these four natural products were loaded into liposome systems.³⁷

The cellular uptake behavior of Ginsenoside Rh2-loaded nanoniosome in PC-3 cells was investigated by adding fluorescent label Dil to the lipid phase. The Ginsenoside Rh2-loaded niosomal formula containing cationic lipid DOTAP demonstrated greater green and cyan color intensity compared to the Ginsenoside Rh2-loaded niosome formula without DOTAP. By adding DOTAP to niosomal formulation, cellular uptake was enhanced. On the other hand, the Ginsenoside Rh2-loaded nanoniosomal cellular uptake behavior showed more Ginsenoside Rh2 distribution in the nuclear region compared to the cytoplasm. The large molecular size of Ginsenoside Rh2 leads to low membrane permeability.^{38,39} Due to its adsorption, endocytosis, and fusion, niosomes can penetrate into cells with high efficiency.^{18,19,40,41} Therefore, the cationic niosomal form of Ginsenoside Rh2 can enter cells with a higher quality compared to the niosomal form of Ginsenoside Rh2. Similar studies (eg, Han et al) showed the enhanced membrane permeability of ginsenosides with microemulsions.⁴²

Xu et al optimized and prepared three types of Rh2-loaded liposomes, including Rh2-loaded normal liposome (Rh2-LP), Rh2-loaded cationic liposome (Rh2-CLP), and Rh2-loaded methoxy poly(ethylene glycol)-poly(lactide) (mPEG-PLA) liposome (Rh2-PLP), with the mean particle size of 80–125 nm. Rh2-LP presented better tumor accumulation of the fluorescent cyanine dye (DiR) in HepG2-xenografted nude mice than CLP (1.3-fold) or LP (1.6-fold) and prolonged the resident time of DiR in tumor and organs (more than 24 h). The in vivo anticancer efficacy assessments indicate that Rh2-PLP presents the highest activity on suppressing tumor growth in HepG2-xenografted mice, compared to Rh2-LP and Rh2-CLP, without any significant toxicity.

Conclusions

In conclusion, this study suggested a novel formulation of cationic niosomal Ginsenoside Rh2 to deliver drugs to the PC3 prostate cancer cell line. Ginsenoside Rh2 loading up to 93.5% was incorporated in the nanoniosomal bilayer with the formulation of Span 60, cholesterol, and DOTAP. The drug's release profile showed a burst release followed by a sustained drug release for Ginsenoside Rh2. After storage for 90 days, the optimum formulation of Ginsenoside Rh2-loaded niosome (containing 5% DOTAP) demonstrated no significant changes when compared to freshly prepared samples in terms of encapsulation efficiency, vesicle size, PDI, and zeta potential. The Ginsenoside Rh2-loaded niosomal formula containing cationic lipid DOTAP demonstrated greater green and cyan color intensity compared to the Ginsenoside Rh2-loaded niosome formula without DOTAP. By adding DOTAP to niosomal formulation, cellular uptake was enhanced. The enhanced cellular uptake and cytotoxic activity (two-fold) of Ginsenoside Rh2 nanoniosomal formulation with PC3 cells make it an attractive candidate for application as a nano-sized delivery vehicle to transfer Ginsenoside Rh2 to cancer cells.

Disclosure

The authors report no conflicts of interest in this work.

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