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Apoptosis induced by paclitaxel-loaded copolymer PLA–TPGS in Hep-G2 cells

Hoai Nam Nguyen[1], Hong Ha Tran Thi[2], Duong Le Quang[1], Toan Nguyen Thi[1], Nhu Hang Tran Thi[2], Mai Huong Le[2] and Phuong Thu Ha[1]

[1] Institute of Materials Science, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam
[2] Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam

E-mail: thuhp@ims.vast.ac.vn and lehuong00@gmail.com

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Abstract
Paclitaxel is an important anticancer drug in clinical use for treatment of a variety of cancers. The clinical application of paclitaxel in cancer treatment is considerably limited due to its serious poor delivery characteristics. In this study paclitaxel-loaded copolymer poly(lactide)–d-α-tocopheryl polyethylene glycol 1000 succinate (PLA–TPGS) nanoparticles were prepared by a modified solvent extraction/evaporation technique. The characteristics of the nanoparticles, such as surface morphology, size distribution, zeta potential, solubility and apoptosis were investigated *in vitro*. The obtained spherical nanoparticles were negatively charged with a zeta potential of about −18 mV with the size around 44 nm and a narrow size distribution. The ability of paclitaxel-loaded PLA–TPGS nanoparticles to induce apoptosis in human hepatocellular carcinoma cell line (Hep-G2) indicates the possibility of developing paclitaxel nanoparticles as a potential universal cancer chemotherapeutic agent.

Keywords: paclitaxel-loaded PLA–TPGS, paclitaxel nanoparticles, apoptosis

Classification numbers: 2.05, 4.02

1. Introduction

Paclitaxel, a natural compound extracted from the Pacific yew tree, is well known as one of the most effective therapeutic agents in cancer treatment. It can be used as a chemotherapy agent against many types of cancer including lung, ovarian, breast cancer or advanced forms of Kaposi's sarcoma [1] and as a preventive agent of restenosis as well [2]. However, its clinical applications are restricted due to its poor solubility, short circulation time and non-selective *in vivo* biodistribution, which causes many adverse effects, especially when using conventional drug delivery systems (DDSs). Therefore, in recent years, many innovative DDSs have been fabricated to maximize the performance and minimize the unwanted side effects of paclitaxel.

Nanotechnology has made a tremendous impact on the development of DDSs which are much more advantageous compared to conventional DDSs. Their small size could lessen body elimination by defense mechanisms and enhance their accumulation at therapeutic sites that help improve the pharmaceutical efficacy and reduce the negative side effects of therapeutic agents [3, 4]. A class of drug delivery nanosystems attracting much attention is polymeric micelles thanks to their small size, highly structural stability and drug loading capacity without chemical interaction. Polymeric micelles are formed by amphiphilic copolymers with hydrophobic and hydrophilic segments making them able to self-assemble to form a core–shell structure. In aqueous environment the hydrophobic segment forms the inner core which plays the role of a nanocontainer for poorly water-soluble drugs, while the outer shell formed by the hydrophilic portion helps to protect drugs inside from chemical attack by body fluids [5–15].

In order to make excellent polymeric micelles encapsulating the poorly water-soluble drug paclitaxel, some highly biocompatible polymers can be used, including

poly(lactide) (PLA) [16–18], poly(dl-lactide-co-glygolide) (PLGA) [19] and poly(caprolactone) (PCL) [20]. However, the disadvantages of these polymers are too high hydrophobicity and too slow degradation, which makes it very difficult to encapsulate drugs by these polymers. To overcome these hindrances, the hydrophobic blocks are encapsulated by a shell of hydrophilic polymers like d-α-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS or simply, TPGS) with high emulsification efficiency. TPGS is a water-soluble derivative of natural vitamin E. The chemical structure and surface characteristics of TPGS make it an excellent emulsifier [21–23].

In this paper we report procedures for the encapsulation of paclitaxel by copolymer PLA–TPGS synthesized from PLA and TPGS in our laboratory for the enhancement of solubility, inhibitory activity of colony formation on tumor cell and apoptotic activity on Hep-G2 cell line *in vitro*.

2. Materials and methods

2.1. Materials

Lactide (3,6-dimethyl-1,4-dioxane, $C_6H_8O_4$), stannous octotate ($C_{16}H_{30}O_4Sn$), sulforhodamine B, fetal bovine serum (FBS), fetal bovine serum-minimal essential medium (FBS-MEM) were purchased from Sigma-Aldrich. TPGS, toluene, dichloromethane, methanol and dimethyl sulfoxide were obtained from Merck. Paclitaxel was purchased from Dabur Pharma Limited, India. All chemicals were of analytical grade and used without further purification. Antitumor promotion and apoptosis assays *in vitro* on human hepatocellular carcinoma cell line (Hep-G2) (the cell line obtained from National Institute of Hygienic Epidemy—NIHE) have been performed at Experimental Biology Lab—Institute of Natural Products Chemistry of Vietnam Academy of Science and Technology.

2.2. Synthesis of PLA–TPGS copolymer

PLA–TPGS copolymer was synthesized by ring-opening polymerization of PLA and TPGS in the presence of stannous octotate as catalyst. The experimental details and the mechanism of reaction have been described in our previous report [24]. Briefly, a given amount of stannous octotate, PLA and TPGS (50 : 50 w/w) was dissolved in distilled toluene in an ampoule. The reaction was carried out for 10 h at 130 °C under inert gas atmosphere in a silicone oil bath. After the reaction time, the resultant mixture was gently stirred overnight to evaporate the organic solvent. The product was dissolved in dichlomethane and then precipitated in cold methanol in excess to remove unreacted lactide monomers and TPGS. The final product was obtained by filtration.

2.3. Encapsulation of paclitaxel in micelles

A modified solvent extraction/evaporation technique was used to fabricate paclitaxel-loaded PLA–TPGS nanoparticles [25]. In brief, a given amount of copolymer PLA–TPGS was dissolved in 20 ml double distilled water containing a small amount of TPGS as an emulsifier. After that, paclitaxel dissolved in dichloromethane was added dropwise into this mixture under vigorously stirring to form an emulsion. After a period of time, the solvent was evaporated and the resulting mixture was centrifuged at 5000 rpm for 15 min to eliminate un-encapsulated paclitaxel and free surfactants. The supernatant-containing paclitaxel-loaded PLA–TPGS nanoparticles were collected by lyophilization.

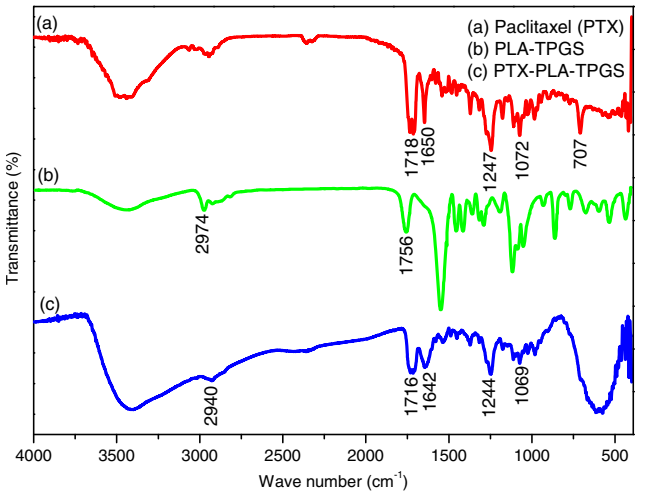


Figure 1. FTIR spectra of pure paclitaxel (a), PLA–TPGS (b) and paclitaxel-loaded PLA–TPGS (c).

2.4. Solubility of paclitaxel

Lyophilized paclitaxel-loaded PLA–TPGS nanoparticles were redispersed into distilled water to saturation level. The amount of entrapped paclitaxel in nanoparticles was determined by the high-performance liquid chromatography (HPLC) method.

2.5. Cell experiment

Cell survival cytotoxicity experiments using sulforhodamine B method were performed in order to determine the maximal doses of the testing materials. Soft agar colony assay antitumor promoting activity was estimated based on the inhibition of soft agar colony induction in the Hep-G2 cell line. The cells were cultured in 10% FBS-MEM medium at 36.5 °C in an incubator with 5% CO_2 and 95% air. Cells growing logarithmically in a monolayer culture were trypsinized and suspended in 0.33% agar medium containing 10% FBS with or without samples at the concentrations of 25 μg ml^{-1}. For antitumor promoting assay, using duplicate 6-well plates, 500 μl of the suspension (1×10^4 cells) was poured onto an agar layer containing the same concentration of sample (10 μg ml^{-1}) in 5% dimethyl sulfoxide solution. Soft agar colonies of cells were investigated after 2 weeks of incubation under an inverted microscope with camera to compare the visual cells in their tumor formation, the tumor size and morphology. The inhibitory activities were the average of triplicated experiments and expressed as a percentage of that of the control.

2.6. Characterization methods

Molecular structure of synthesized copolymer was characterized by Fourier transform infrared spectroscopy

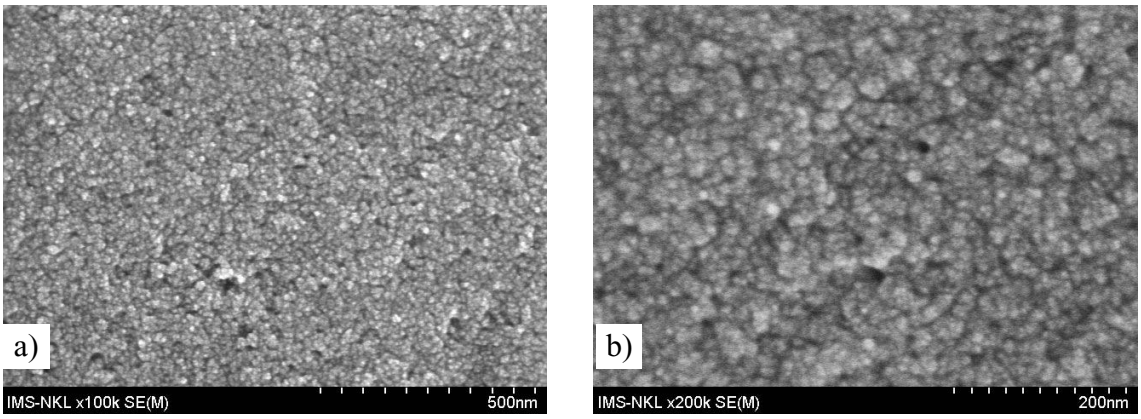


Figure 2. FE-SEM images of paclitaxel-loaded PLA–TPGS nanoparticles.

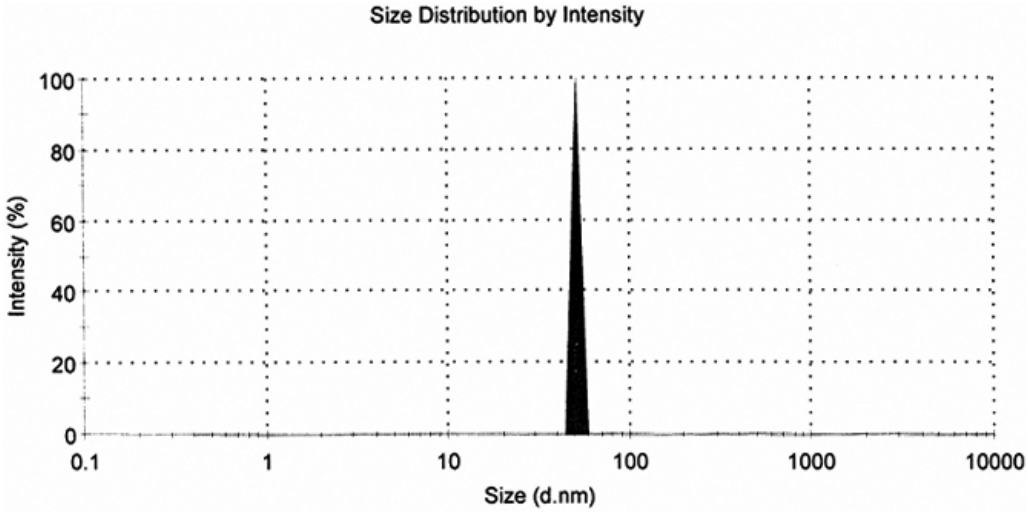


Figure 3. Size distribution of paclitaxel-loaded PLA–TPGS nanoparticles.

(FTIR, SHIMADZU spectrophotometer) using KBr pellets in the wave number region of 400–4000 cm^{-1}. Surface morphology of paclitaxel-loaded PLA–TPGS nanoparticles was investigated by field-emission scanning electron microscopy (FE-SEM) on a Hitachi S-4800 system while size distribution and Zeta potential were determined by dynamic light scattering (DLS) method on a Malvern's Zetasizer–NanoSeries.

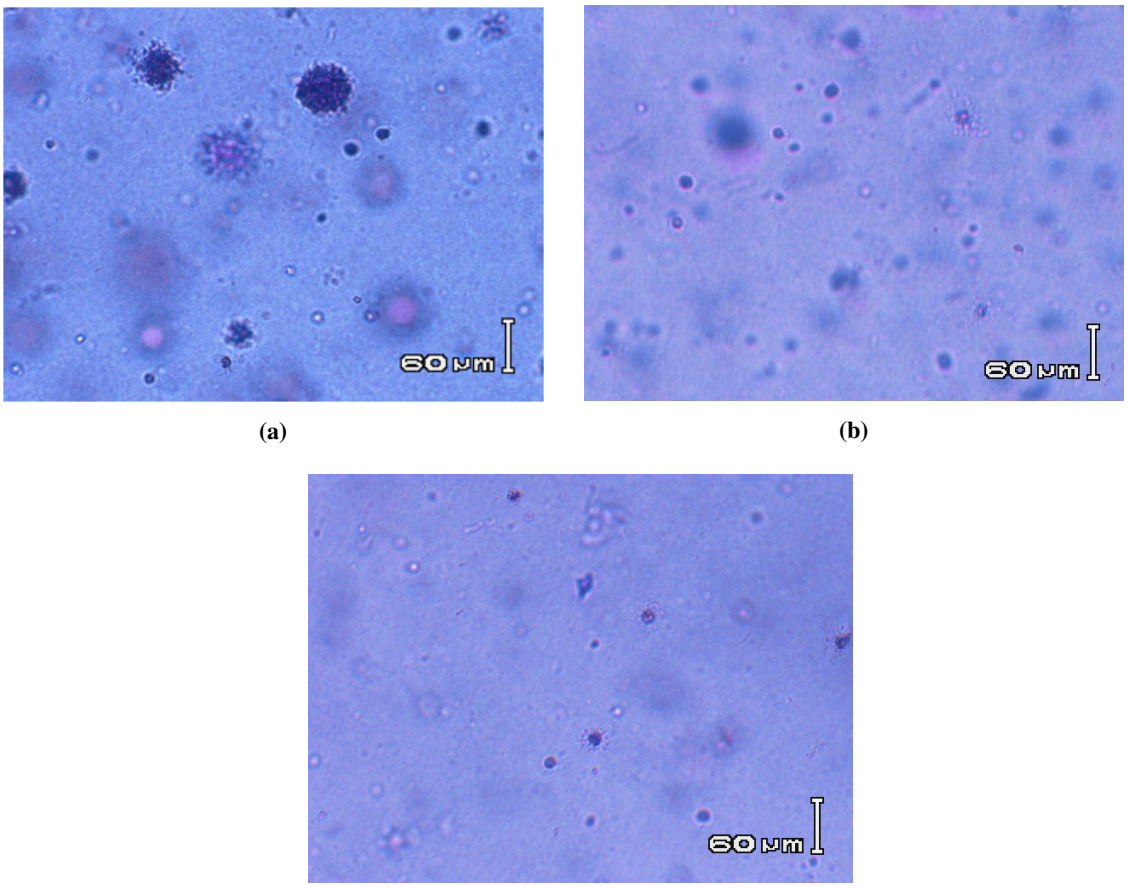
3. Result and discussion

3.1. FTIR spectrum

Figure 1 shows FTIR spectra of pure paclitaxel, copolymer PLA–TPGS and paclitaxel-loaded nanoparticles. It can be seen from the FTIR spectra that C–H and C═O stretching bands of PLA–TPGS obtained at 2974 and 1756 cm^{-1} were observed to shift to 2940 and 1716 cm^{-1}, respectively, in paclitaxel loaded PLA–TPGS. Compared with pure paclitaxel, in IR spectrum of paclitaxel-loaded copolymer nanoparticles there were band shifts from 1072 (C–O stretching of ester bond) to 1069 cm^{-1}, from 1247 (CO–O– stretching vibration) to 1244 cm^{-1} and from 1650 (amide stretching band of C═O) to 1642 cm^{-1}. The peak at 1718 cm^{-1}, assigned to carbonyl stretching vibration of ester bond in TPGS, changed to 1716 cm^{-1} in paclitaxel-loaded copolymer. Especially, there was a substantial change in the region of 400–950 cm^{-1}. This may be due to the direct interaction between the hydrophobic core (paclitaxel) and the hydrophobic segment of copolymer (PLA) which created changes in vibrational frequency of C–H out-of-plane deformation (or C–C═O deformation) (689 cm^{-1}) and C–H in-plane deformation (803–941 cm^{-1}). All of these confirmed the success in formation of polymeric micelles loaded with paclitaxel.

3.2. Surface morphology and size distribution

Surface morphology of paclitaxel-loaded PLA–TPGS nanoparticles is shown in figure 2. It can be seen clearly from FE-SEM images that paclitaxel-load PLA–TPGS nanoparticles were round-shape with mean diameter about 10 nm. This result is quite compatible with the size distribution revealed in figure 3. The size distribution is rather narrow and the average size of nanoparticles is about 44 nm. This size is much smaller in comparison with 300 nm of nanoparticles which were fabricated by Zhiping Zhang and Si-Shen Feng [26]. The difference in size between the results of FE-SEM and DLS was because of the different states. Data from FE-SEM showed the dried state of nanoparticles, while



(a) **(b)** **(c)**

Figure 4. Antitumor promoting effects of the paclitaxel-loaded PLA–TPGS in Hep-G2 cell lines after 2 weeks of cell growth on agar: control (a) paclitaxel-free (b) and paclitaxel-loaded PLA–TPGS (c) under inverted microscope ×100.

DLS method measured the size of nanoparticles suspended in aqueous environment.

3.3. Zeta potential

Zeta potential is the electrostatic potential on the slip surface of a particle dispersed in liquid environment. It is a very important index to assess the stability of nanoparticle suspensions. The higher the absolute value of Zeta potential is, the more stable the system will be. The Zeta potential of our paclitaxel-loaded PLA–TPGS nanoparticles was found to be about −18 mV.

3.4. Solubility of paclitaxel

The poor solubility of paclitaxel in water is very low ($0.4\,\mu g\,ml^{-1}$). After being entrapped in polymeric micelles, the solubility of paclitaxel was found to be $0.2\,mg\,ml^{-1}$ increasing 500-fold compared to pour paclitaxel.

3.5. Cell assay

To investigate chemotherapeutic potent of paclitaxel-loaded PLA–TPGS nanoparticles, their proliferation inhibition and apoptosis enhancement to Hep-G2 *in vitro* was studied.

The results in antitumor promoting assay show that there were remarkable changes in size and morphology of tumor in all the samples tested (paclitaxel-free and paclitaxel-loaded PLA–TPGS) as compared with the control. Especially, the tumor size decreased significantly in the case treated with paclitaxel-loaded PLA–TPGS (figure 4). Thus, it can be concluded that paclitaxel-loaded PLA–TPGS has positive effects on tumor promotion of Hep-G2 cell line *in vitro*.

Because proliferation and apoptosis are the most important manners of cancer cells, when the proliferation is in a dysregulated condition, cancer cells may become resistant to antiproliferative factors and uncontrollable in their proliferation rate. The apoptosis pathway in many cases can regulate the cell proliferation [27, 28].

Under the phase-contrast microscope, the cells exposed to 0.1, 0.2, 0.4, 0.5 and $1\,\mu g\,ml^{-1}$ of paclitaxel-loaded PLA–TPGS exhibited obvious changes in morphologic characteristics. There were more shrinking cells and floating cells that had lost their adhibit ability in the paclitaxel-loaded PLA–TPGS than in the controls (figure 5). With lower concentration of paclitaxel-loaded PLA–TPGS ($0.1\,\mu g\,ml^{-1}$), the cell morphologic had been changed and the cells began to separate from each other. It seems that there was a loss of surface receptors of the cell, resulting in their losing their adherence onto the bottom of wells or losing their cell–cell connective signals needed for their angiogenic forming. From figure 5 we can see that their apoptosis increased with increasing of tested complex concentration (0.2, 0.4, 0.5

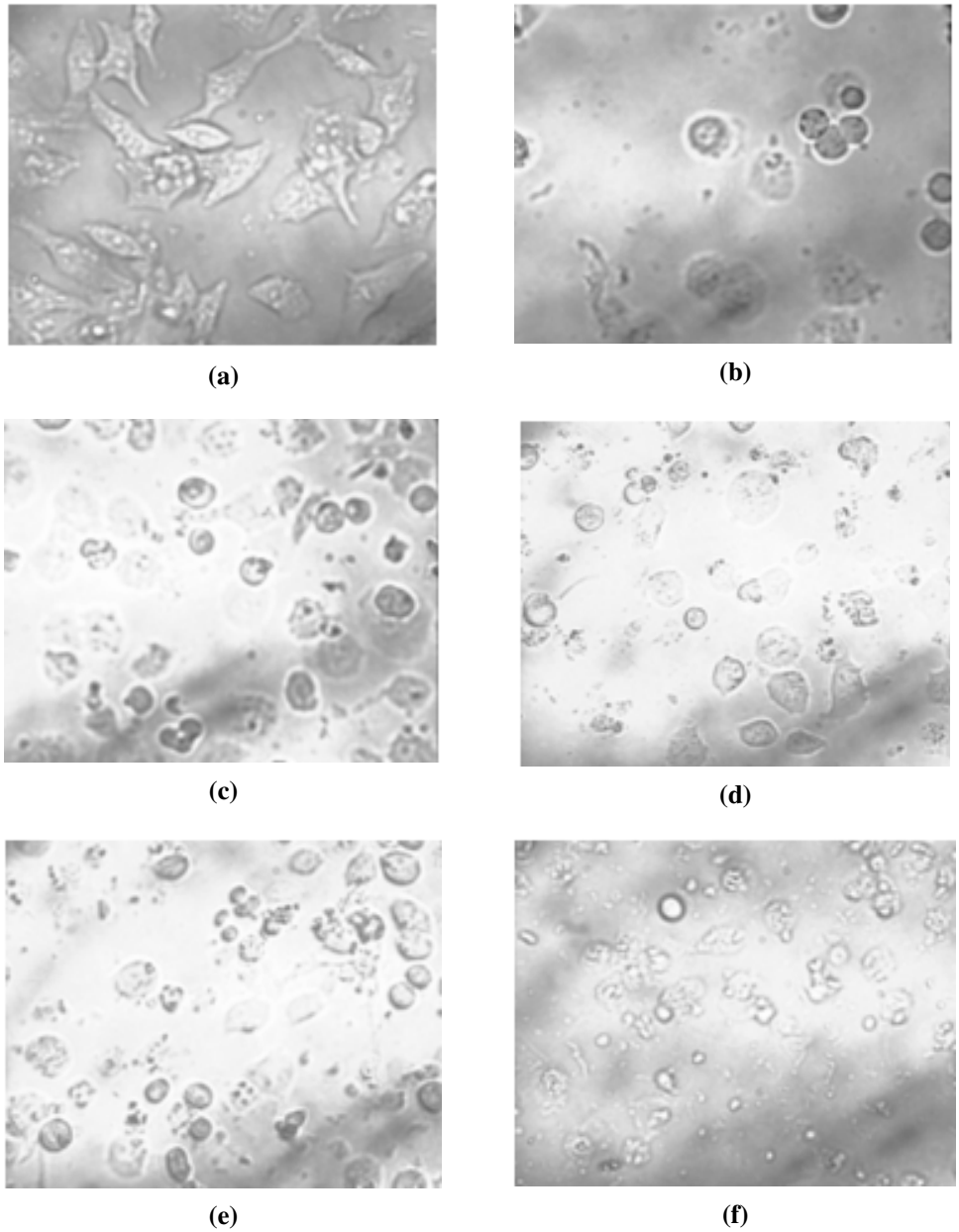


Figure 5. Apoptosis induced by paclitaxel-loaded PLA–TPGS in different concentrations: (a) control, (b) 0.1 μg ml^{-1}, (c) 0.2 μg ml^{-1}, (d) 0.4 μg ml^{-1}, (e) 0.5 μg ml^{-1} and (f) 1 μg ml^{-1}.

and 1 μg ml^{-1}). Besides the apoptosis pathway with small size ranging from 40 to 50 nm, paclitaxel-loaded PLA–TPGS nanoparticles may penetrate through cell membrane and may interfere in the metabolite actions of cells, and at last cause cell death. This should also be one of the most typical apoptotic pathways while the larger size of paclitaxel-free PLA–TPGS makes it hard to penetrate through the cell membrane and it cannot interfere in metabolism of the cells. In this case, the cross-talking of the antitumor promotion and pro-apoptotic signaling pathways caused by paclitaxel-loaded PLA–TPGS may suggest an ultimate determination for further research at the molecular level. These pro-apoptotic or anti-apoptotic factors are spotlighted in both the clinical treatments of malignancy and cancer chemoprevention strategy as well.

4. Conclusion

In conclusion, our work reveals that paclitaxel-loaded PLA–TPGS nanoparticles could significantly inhibit the proliferation and may induce apoptosis in Hep-G2 cells. The ability of paclitaxel nanoparticles to induce apoptosis in Hep-G2 and other cancer cells indicates the possibility of developing paclitaxel nanoparticles as a potential universal cancer chemotherapeutic agent.

Acknowledgments

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