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In Vitro Apoptosis Enhancement of Hep-G2 Cells by PLA–TPGS and PLA–PEG Block Copolymer Encapsulated Curcumin Nanoparticles

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Nanodrug systems containing curcumin (Cur) encapsulated with the block copolymers poly(lactide)-*d*-α-tocopheryl poly(ethylene glycol) 1000 succinate (PLA–TPGS) and poly(lactide)–poly(ethylene glycol) (PLA–PEG) have been prepared and characterized by infrared and fluorescence spectroscopy, field-emission scanning electron microscopy (FE-SEM), and dynamic light scattering (DLS). Upon encapsulation, the highest solubility of Cur–PLA–TPGS and Cur–PLA–PGE dried powder was calculated as high as 2.40 and 2.20 mg mL^{-1}, respectively, an increase of about 350-fold compared to that of Cur (6.79 μg mL^{-1}). The antitumor assays (cytotoxic and antitumor-promoting assays) on Hep-G2 cellsof copolymer-encapsulated Cur nanoparticles showed the apoptotic activity due to the remarkable changes in size, morphology, and angiogenesis ability of tumor cells in all cases of the tested samples as compared with the control.

Nanomedicine, application of nanotechnology studying prophylaxis, diagnosis, and therapeutics, applies nanomaterials to manipulate biological systems at cellular or molecular levels. Contemporary trends in nanomedicine are surging in fields of drug delivery (DDs) and diagnosis of abnormal diseases such as diabetes or cancers, which has attracted attention of many researchers. However, the effect of anticancer drugs is limited because of their poor aqueous solubility, easy elimination by the body, undesirable selectivity, or unwanted side-effects. Most present commercial anticancer drugs do not have a highly selective orientation, killing both malignant and healthy, causing several serious side effects or damaging organs such as the heart, kidneys, liver, and nervous system. In order to utilize drugs for various clinical applications, a variety of methods including the incorporation of drugs into polymers, liposomes, and lipid emulsions have been studied to reduce size to nanoscale, increase solubility, prolong circulation and reduce adverse effects.[1–8] However, there have been some disadvantages in these systems such as low drug capacity and fast elimination from the blood. More recently, a promising approach of polymeric micelle nanoparticles has been developed,[8–14] offering therapeutic performance of anticancer drugs by the improvement in terms of pharmacokinetics and pharmacodynamics[15] to their bioavailability, solubility, drug content, and retention time.[16]

Curcumin, a natural compound extracted from *curcuma longa*, has attracted great attention due to its anticancer activity by suppressing transformation, proliferation, and metastasis of tumors.[17] However, like other hydrophobic drugs, there is a need for suitable carriers to load and deliver Cur effectively. As a part of our continuing interest in the preparation of polymer-encapsulated Cur nanoparticles,[2,3] we report procedures for a high yield encapsulation of Cur by block copolymers PLA–TPGS and PLA–PEG synthesized from PLA, TPGS, and PEG for the enhancement in solubility, inhibitory activity of colony formation of tumor cells, and apoptotic activity of Hep-G2 cell line in vitro.

Two block copolymers PLA–TPGS and PLA–PEG were synthesized by ring-opening polymerization. Briefly, for the synthesis of PLA–TPGS at the given ratio PLA and TPGS were dissolved in toluene and stirred for 10 h at 130 °C in the presence of stannous octoate (tin(II) bis(2-ethylhexanoate)) as catalyst. The solvent was then naturally evaporated overnight to get a gel before precipitation in cold ethanol. The final copolymers were obtained through filtration. The synthesis of PLA–PEG was similar to that of PLA–TPGS.

Next, to dichloromethane solutions of PLA–TPGS or PLA–PEG, Cur dissolved in ethanol at different concentrations was added with gentle stirring. After vacuum evaporation of the solvent, the gelatin mixtures were then dispersed in PBS solution. The final Cur solution was obtained by centrifugation at 3000 rpm to remove nonencapsulated Cur.

In the ^{1}H NMR spectra of the PLA, TPGS, and PLA–TPGS (see Supporting Information 1[18]), the signals at 5.043 and 1.630 ppm were assigned to the protons of –CH and –CH$_3$ groups, respectively, of PLA. The signal at 3.641 ppm was assigned to –CH$_2$CH$_2$– protons in TPGS. These three signals were, correspondingly, shifted to 5.166, 1.562 and 3.639 ppm in the spectrum of PLA–TPGS. In the case of PLA–PEG, respective signals of PLA were shifted to 5.145 and 1.598 ppm while respective peak of PEG were shifted from 3.668 to 3.747 ppm. Moreover, within the spectra of two copolymers, trace signals of monomers were not recorded individually.

FT-IR spectra of PLA, PLA–TPGS, and PLA–PEG were recorded (see Supporting Information 2a[18]). The CH stretching bands of PLA was observed at 2930 cm^{-1} and it changed to 2974 and 2918 cm^{-1} in PLA–TPGS and PLA–PEG, respectively. The peak at 1768 cm^{-1} was assigned as the carbonyl stretching band in the –CO–O– group of PLA which was observed to shift respectively to 1745 and 1750 cm^{-1} in PLA–TPGS and PLA–PEG.

The encapsulation efficiency (EE (%)) of Cur in micelle was calculated by the following formulation:

$$\mathrm{EE}(\%) = \frac{\text{Total amount of Cur} - \text{nonencapsulated amount of Cur}}{\text{Total amount of Cur}} \times 100$$

It was found that the encapsulation efficiency of Cur in the micelles was approximately 80% for both cases (PLA–TPGS and PLA–PEG micelle).

In order to demonstrate the formation of copolymer-encapsulated Cur nanoparticles, spectra of IR, UV–vis, and fluorescence for Cur and encapsulated Cur solutions were recorded.

Compared with pure Cur, the IR spectrum of Cur–PLA–TPGS (see Supporting Information 2b[18]) shows band shifts from 1502 (C=O and C–C vibrations) to 1499 cm^{-1} and 1285 (aromatic C–O stretching vibration) to 1280 cm^{-1}. Especially, when comparing PLA–TPGS and Cur–PLA–TPGS, peak shifts were observed from 2974 to 2964 cm^{-1} and from 1756 to 1746 cm^{-1} due to C–H stretching and ester stretching, respectively. The IR data showed that Cur was encapsulated in the hydrophobic core of micelles of PLA–TPGS and PLA–PEG.

The fluorescence emission bands were observed at 530 and 535 nm for Cur–PLA–TPGS and Cur–PLA–PEG, respectively, which were shifted from 540 nm of free Cur (see Supporting Information 3[18]). Similarly, Cur in ethanolic solution exhibited an absorption band at 428 nm, while an aqueous solution of Cur–PLA–TPGS and Cur–PLA–PEG showed the same band at 415 nm (data not shown). The blue shifts in both fluorescence and UV–vis spectra were likely due to the formation of hydrophobic interaction between Cur and hydrophobic segments PLA for both PLA–TPGS and PLA–PEG. This hydrophobic bonding was also supported by the obtained IR data of lyophilized powders Cur–PLA–TPGS, Cur–PLA–PEG, and Cur. Besides, the fluorescence intensity of encapsulated Cur solution was higher than that of nonencapsulated Cur solution. The increase in emission intensity probably came from the interaction of Cur with copolymers.

The size and morphology of copolymers without Cur and copolymer-encapsulated Cur nanoparticles were determined by FE-SEM and DLS. As can be seen in Figure 1, all nanoparticles were round-shaped. It is clear that empty PLA–TPGS micelles in the range of 50 to 100 nm (Figure 1a) are much smaller than empty PLA–PEG micelles in the range of 200 to 300 nm (Figure 1c). The difference in size of these nanoparticles was due to the different ratio of hydrophobic segment (PLA) and hydrophilic segment (TPGS and PEG) that were applied to synthesize copolymers (1:1 w/w for PLA–TPGS and 4:1 w/w for PLA–PEG). The higher this ratio is, the larger the micelles are. The sizes of copolymer-encapsulated Cur nanoparticles (Cur–PLA–TPGS in Figure 1b and Cur–PLA–PEG in Figure 1d) were rather similar to those of empty micelles. This could be explained by the core–shell structure of polymeric micelles. Curcumin was trapped in the inner core of polymeric micelles.

The size of nanoparticles measured by the DLS (see Supporting Information 4[18]) was in good agreement with the results depicted from FE-SEM images shown in Figure 1. The mean sizes of Cur–PLA–TPGS and Cur–PLA–PEG nanoparticles were 50.75 and 297.6 nm, respectively. The narrow size distribution of Cur–PLA–TPGS could be advantageous for passive targeting of tumor cells thanks to the enhanced permeation and retention (EPR) effect. As a result, the drug would kill directly cancer cells.

Besides, as shown in Figure 2, after being capped with the block copolymers, Cur–PLA–TPGS and Cur–PLA–PEG nanoparticles also displayed photoluminescence, to a lesser extent than free Cur. Thus, the nanoparticles could be considered novel labeling materials for monitoring drug delivery processes.

To verify practical pharmaceutical use, the solubility of Cur before and after the encapsulation was tested. The highest solubility of Cur–PLA–TPGS and Cur–PLA–PEG dried powder was calculated as high as 2.40 and 2.20 mg mL^{-1}, respectively, an increase of about 350-fold compared to free Cur (6.79 μg mL^{-1}). The results suggested that Cur was indeed trapped in the copolymeric micelles and improved significantly its aqueous solubility. Subsequently, PLA–TPGS and PLA–PEG are very promising candidates for drug delivery systems.

To investigate chemoprevention and chemotherapeutic potential of Cur–PLA–PEG and Cur–PLA–PEG, their proliferation inhibition and apoptosis enhancement to Hep-G2 in vitro was studied. Cell survival cytotoxicity experiments using Sulforhodamine B 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

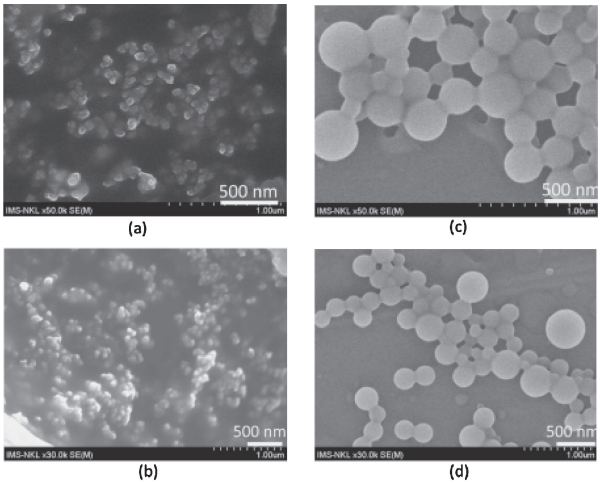

Figure 1. FE-SEM images of (a) PLA–TPGS, (b) Cur–PLA–TPGS, (c) PLA–PEG, and (d) Cur–PLA–PEG.

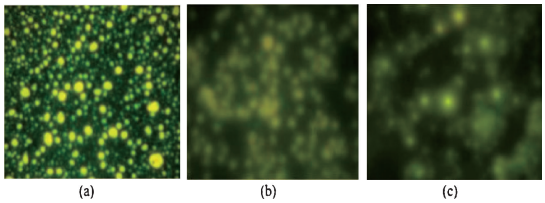

Figure 2. Photoluminescence images of (a) Cur, (b) Cur–PLA–TPGS, and (c) Cur–PLA–PEG.

without samples at the concentration 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 (DMSO) 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, tumor size, and morphology. The inhibitory activities were the average of triplicated experiments and expressed as a percentage of that of the control.

The results of cytotoxicity assays show that there was no distinct difference of cell survival between tested samples (Cur, PLA–TPGS, and PLA–PEG) and the control. However, in the antitumor promoting assay, there were remarkable changes in size and morphology of tumors in all the samples tested as compared with the control. Especially, the tumor size decreased significantly in the case treated with encapsulated Cur (17 ± 0.1 and 19 ± 0.1 μm for PLA–TPGS and PLA–PEG, respectively). Those are much better than results given by unencapsulated Cur (22.5 ± 0.5 μm) and the control (25.5 ± 0.3 μm) (see Supporting Information 5[18]). Thus, it can be concluded that encapsulated Cur has positive effects on tumor promotion of Hep-G2 cell line in vitro.

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.[19]

Under the phase-contrast microscope, the cells exposed to 1, 2, 4, and 5 μg mL^{-1} of Cur–PLA–PEG and Cur–PLA–TPGS exhibited obvious changes in morphologic characteristics. There were more shrinking cells and floating cells that had lost their adhibit's ability in the copolymer-encapsulated Cur group than in the controls (Figure 3). With lower concentration of Cur–PLA–TPGS (1 μg mL^{-1}), the cell morphology was changed and the cells began to separate from each other. There seemed to be a loss of surface receptors of the cell, therefore, losing their adherence onto the bottom of wells or losing their cell–cell connective signals needed for angiogenesis. The complex of Cur–PLA–PEG also caused similar changes in morphology to the cells with a lower level. When 1 μg mL^{-1} Cur–PLA–PEG was employed, the cells began to change slightly in their connection, and their apoptosis increased following the increase of tested complex concentration (2, 4, and 5 μg mL^{-1}). Besides the apoptosis pathway, with small size ranging from 50 to 100 nm, Cur–PLA–TPGS nanoparticles could penetrate through cell membranes and interfere in the metabolite actions of cells and at last cause cell death, whereas the larger size of Cur–PLA–PEG (200–300 nm) made it difficult to penetrate through the cell membrane and could not interfere in metabolism of the cells (Figure 3).

The crosslinking of the antitumor promotion and apoptotic signals caused by copolymer-encapsulated Cur may suggest an ultimate determination for further research at the molecular level.

In conclusion, our work revealed that copolymer-encapsulated Cur nanoparticles could significantly inhibit the proliferation and induce apoptosis in Hep-G2 cells. The ability of Cur nanoparticles to induce apoptosis in Hep-G2 and other cancer cells offers the possibility to develop copolymer-encapsulated Cur nanoparticles as a promising cancer chemoprevention and chemotherapeutic agent.

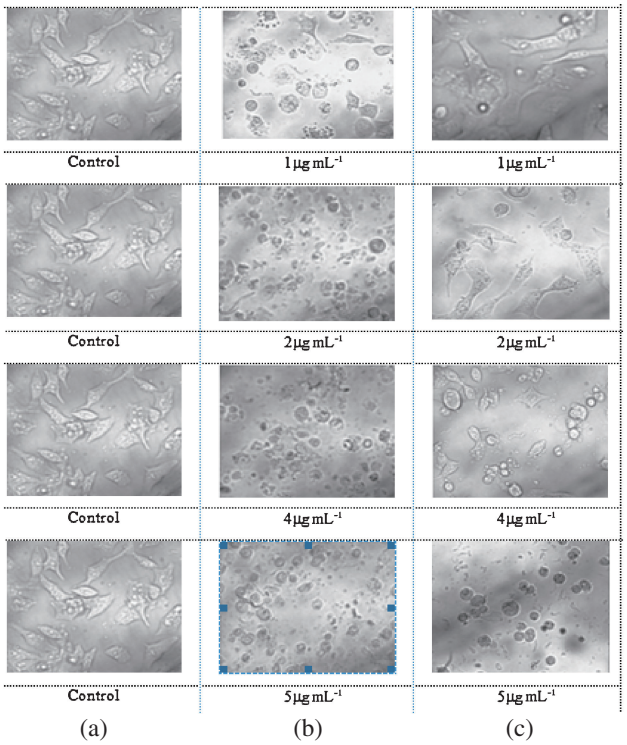

Figure 3. Morphological changes of Hep-G2 cells upon their exposure to Cur–PLA–TPGS and Cur–PLA–PEG monitorized by phase-contrast microscope: (a) Control, (b) Cur–PLA–TPGS, and (c) Cur–PLA–PEG.

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