Docetaxel and curcumin-containing poly(ethylene glycol)-block-poly(ε-caprolactone) polymer micelles

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Abstract
Polymeric nanoparticles (NPs) prepared from poly(ethylene glycol)-block-poly(ε-caprolactone) (PEG–PCL) were fabricated by the modified nanoprecipitation method with and without sonication to entrap docetaxel (Doc) and curcumin (Cur). NPs were characterized in terms of morphology, size distribution, zeta potential, encapsulation efficiency and cytotoxicity. The particles have a ~45–80 nm mean diameter with a spherical shape. The cellular uptake of the NPs was observed after 2 and 4 h of incubation by fluorescence of curcumin loaded with docetaxel. The cell viability was evaluated by an [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay on the Hela cell line. Doc and Doc–Cur NPs had higher cytotoxicity and a much lower IC_{50} value compared with free Doc or Cur after 24 and 48 h of incubation. Doc and Cur incorporated into the PEG–PCL NPs had the highest cytotoxicity in comparison with all other NPs and may be considered as an attractive and promising drug delivery system for cancer treatment.

Keywords: cancer, docetaxel, curcumin, polymeric nanoparticles, extrusion

Classification numbers: 2.04, 2.05

1. Introduction

Many anticancer drugs are hydrophobic and require a solubilizing carrier. Therefore, in recent years, the development of nanomedicines, such as nanoparticles (NPs), liposomes, micelles and dendrimers, for drug delivery systems has been increasing [1]. Among them, polymeric micelles have advantages for preparing efficient anticancer drug carriers, such as their limited toxicity, agent solubility and long circulation time [2, 3]. Amphiphilic block copolymers are assembled to form a micelle with a hydrophobic core and hydrophilic shell [4]. So, their use in drug delivery is a more effective cancer treatment method than conventional chemotherapy.

Recently, polymeric NPs have been extensively used in the delivery of hydrophobic anticancer drugs based on PEG polyesters such as poly(ethylene glycol)-b-poly(ε-caprolactone) (PEG–PCL) for the delivery of doxorubicin and paclitaxel [5]. Poly(caprolactone) (PCL) is a Food and Drug Administration (FDA)-approved biodegradable polymer, which has various unique properties such as excellent biocompatibility and rapid degradability, and good miscibility with a variety of polymers. Moreover, it is not toxic and has great permeability. Therefore, it is used widely as a drug carrier [6]. As it is a combination of PCL and PEG, PEG–PCL is an amphiphilic copolymer with high stability in biological fluids [7].

Docetaxel (taxotere), a member of the taxane family which binds to tubulin, stabilizing spindle microtubules and blocking mitosis [8], is one of the most efficient agents used against a variety of solid tumors such as breast cancer,
oval cancer, small and non-small cell lung cancer, prostate cancer, etc. It is a poorly water-soluble, semi-synthetic taxane and cancer treatment by docetaxel (Doc) results in adverse effects [9]. Therefore, to facilitate its clinical use, alternative Doc formulations are needed, and NP-based drug delivery systems are of special interest in decreasing the major side effects of the drug.

Curcumin, a natural polyphenolic pigment of *Curcuma longa* (Linn.), is a commonly used nutritional supplement [10], and *in vitro* and *in vivo* studies showed that curcumin can have anticancer activity [11–13]. Curcumin in combination with other chemotherapeutic agents has demonstrated synergy in cytotoxicity [14,15]. However, it is less soluble in water or aqueous solution [16].

The purpose of this study is to investigate a combination of Doc and Cur in a PEG–PCL delivery system, and examine the cytotoxicity and cellular uptake mechanism. Doc–Cur-loaded NPs and Doc or Cur-loaded NPs of PEG–PCL copolymer were synthesized by a modified nanopreparation method. These NPs were then characterized by various techniques such as dynamic light scattering for size, size distribution and zeta potential, and by field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM) for morphology. The drug encapsulation efficiency (EE) was measured by a NanoDrop 1000 Spectrophotometer. The cellular uptake and cytotoxicity of the drug were evaluated by using the Hela cell line.

2. Materials and methods

2.1. Materials

PEG–PLC diblock copolymers were purchased from Sigma-Aldrich with a PEG average of Mn ∼5000 and a PLC average of Mn ∼5000. Pure docetaxel anhydrous was purchased from Shanghai Biomax Pharma Co. Ltd, Shanghai, China. Dulbecco’s phosphate-buffered saline (DPBS) was purchased from Sigma. [3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)] was purchased from Promega (USA). Curcumin and tissue culture reagents were purchased from Sigma-Aldrich with a PEG average of Mn 5000 and a PLC average of Mn ∼5000. Pure docetaxel anhydrous was obtained from Puredoc (Canada) and quantified by a UV–vis spectrophotometer at 275 nm for Doc and 450 nm for Cur. Briefly, 10 mg NPs were dissolved in 2 ml of dichloromethane (DCM) and then extracted by milli-Q water. The DCM was evaporated in nitrogen atmosphere and the clear solution was used for analysis by the UV–vis spectrophotometer. The standard curve was linear in the range of 50–1000 µg ml⁻¹ in dimethyl sulfoxide (DMSO). The encapsulation efficiency of Doc and Cur was measured by the amount of Doc or Cur encapsulated compared with the total amount of drug used in the formulation, multiplied by 100.

2.2. Methods

2.2.1. Preparation of drug-loaded PEG–PCL NPs. Doc-loaded, Cur-loaded and Doc–Cur-loaded PEG–PCL NPs were prepared by modified nanoprecipitation with and without sonication. Briefly, an amount of Doc (5 mg ml⁻¹) in methanol and Cur (5 mg ml⁻¹) in acetone were added into 50 mg of PEG–PCL copolymer solution in acetone. The mixtures were poured into Millipore water solution with a solvent:water ratio of 1:5 under gentle stirring at 25 °C for 3–6 h. Methanol and acetone were completely removed by evaporation at 4 °C overnight. The drug-loaded NPs were homogenized by extrusion through the EmulsiFlex™ C5 High Pressure Homogenizer (INVESTIN, Ottawa, Canada) with 100 nm nanoporous membranes (Whatman).

2.2.2. Morphology of the NPs. The drug-loaded PEG–PCL NPs were imaged by a FE-SEM system (Hitachi S-4800 FE-SEM) and a TEM system (Jeol JEM-1010, USA). A Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) equipped with a 60× objective lens was used to observe the fluorescence of the NPs.

2.2.3. Particle size and zeta potential. The average size and polydispersity of drug-loaded PEG–PCL NPs were analyzed by dynamic light scattering (DLS). The Zeta potential of the drug-loaded PEG–PCL NPs was evaluated in deionized water (∼1 mg ml⁻¹) using the electrophoretic mode of Zetasizer 3000 HS (Malvern instruments Ltd, UK) at 25 °C. Each sample was measured in triplicate.

2.2.4. Drug encapsulation efficiency. The Doc and Cur entrapped in the drug-loaded PEG–PCL NPs were measured by a NanoDrop 1000 spectrophotometer (Canada) and quantified by a UV–vis spectrophotometer at 275 nm for Doc and 450 nm for Cur. Briefly, 10 mg NPs were dissolved in 2 ml of dichloromethane (DCM) and then extracted by milli-Q water. The DCM was evaporated in nitrogen atmosphere and the clear solution was used for analysis by the UV–vis spectrophotometer. The standard curve was linear in the range of 50–1000 µg ml⁻¹ in dimethyl sulfoxide (DMSO). The encapsulation efficiency of Doc and Cur was measured by the amount of Doc or Cur encapsulated compared with the total amount of drug used in the formulation, multiplied by 100.

2.2.5. In vitro cell uptake assays. Hela cells were seeded in 30 mm Petri dishes for 2 and 4 h at 37 °C with 2 ml of medium containing Doc–Cur loaded NPs (2 µg ml⁻¹ calculated by Doc and 4 µg ml⁻¹ calculated by Cur). Then the cells were washed twice with cold phosphate buffered saline (PBS) to eliminate traces of the NPs. The fluorescent signals of the cell monolayer were observed using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan).

2.2.6. Cytotoxicity assay. The *in vitro* cytotoxicity study of drug-free and drug-loaded NPs and the free drug was carried out on Hela cells using the MTS assay. Hela cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 100 IU ml⁻¹ of penicillin G sodium and 100 µg ml⁻¹ of streptomycin sulfate at 37 °C in a humidified environment of 5% CO₂. The cells were seeded in a 96-well plate for 24 h to allow attachment, then the medium was replaced by 100 µl with various concentrations of Doc-free, Doc-loaded, Cur-loaded and Doc–Cur-loaded NPs (ranging from 0.025, 0.25, 2.5, 12.5 and 25 µg ml⁻¹ of Doc or Cur concentration) and free Doc and Cur.

Free Doc was prepared in DMSO and the DMSO concentration in the medium was lower than 0.1% (no effect on cell proliferation). After incubation for 24 or 48 h, the cells were incubated with 20 µl per well of MTS and phenazine methosulfate (PMS) solution (MTS/PMS ratio: 20/1) for 1–4 h at 37 °C in a humidified, 5% CO₂ atmosphere. Absorbance was measured at 490 nm using
Table 1. The physicochemical characteristics of NPs.

| Drug encapsulation efficiency (%) | Nanoprecipitation | Sonication | Extrusion |
|-----------------------------------|--------------------|------------|-----------|
| Doc (%)                           | 16 ± 1.2           | 19 ± 1.7   |           |
| Cur (%)                           | 50 ± 4.8           | 48 ± 5.2   |           |
| Average size of peaks (nm)        | 45, 80, 231, 1294  | 45, 80, 419| 45, 80    |
| PI                               | 0.43 ± 0.02        | 0.31 ± 0.01| 0.1 ± 0.03|
| ζ potential (mV)                 | −6.97              |           |           |
| ζ deviation (mV)                 | 12.6               |           |           |

Figure 1. FE-SEM (a) and TEM (b) images of drug-loaded PEG–PCL NPs after extrusion by the EmulsiFlex™-C5 high pressure homogenizer.

3. Results and discussion

3.1. Characterization of drug-loaded NPs

The characteristics of the drug-loaded NPs are shown in table 1. Drugs were successfully entrapped in PEG–PCL NPs. In this work we investigated the effect of nanoprecipitation and sonication methods on drug entrapment efficiency. When the Doc/polymer ratio was equal to 1/5, the encapsulation efficiency of Doc was 16 ± 1.2%, and the encapsulation efficiency of Cur was 50 ± 3.5% when the Cur/polymer ratio was equal to 1/10 (by the nanoprecipitation method). But Doc entrapped by nanoprecipitation combined with sonication had an increase of encapsulation efficiency of about 19 ± 1.7% in just 30 min.

Particle size influences the kinetic parameters of drugs, for example drug biodistribution and clearance. The accumulation of drugs with a particle size ranging from 20 to 100 nm in tumor tissues is significantly increased due to the enhanced permeability and retention (EPR) effect and the escape of NPs from the reticulo-endothelial system (RES) [3,17]. Therefore, we investigated the effect on particle size of forming NPs using a combination of nanoprecipitation with sonication for about 30 min. NPs were fabricated with narrower size, lower polydispersity index (PI) and steady encapsulation efficiency. To homogenize the size of NPs, formulated NPs were extruded through a EmulsiFlex™-C5 High Pressure Homogenizer (INVESTIN, Ottawa, Canada) with 100 nm nanoporous membranes (Whatman). Thus we obtained drug-loaded NPs with a narrower mean diameter and size distribution than is shown in table 1.

There was no significant effect on the encapsulation efficiency of Doc and Cur as they were incorporated into PEG–PCL NPs. Furthermore, the size and size distribution of formulated NPs were not influenced by the presence of the drug for drug-unloaded NPs and these characteristics were similar to other drug-loaded NPs (data not shown).

Zeta potential can greatly influence particle stability in suspension and avoid the aggregation of particles. The zeta potential of drug-loaded NPs detected by dynamic light scattering was −6.97 mV.

3.2. Surface morphology

For surface morphology, the NPs were evaluated by FE-SEM and TEM analysis. As shown in figure 1, the NPs had a spherical shape and a smooth surface with uniform size distribution.

3.3. Cellular uptake of drug-loaded PEG–PCL NPs

The feasibility of the drug-loaded PEG–PCL NPs was evaluated by cellular uptake of fluorescent Doc–Cur-loaded PEG–PCL NPs on the Hela cell line. Curcumin has been widely used due to its biocompatibility and high fluorescence activity. Fluorescent images of Doc–Cur-loaded PEG–PCL NPs (figure 2(a)) and cellular uptake images on Hela cells (figures 2(b) and (c)) were taken using the Nikon Eclipse 80i microscope (Japan) with a 40× objective lens, and Cur in PEG–PCL NPs was used as a probe for marking NPs in the
cellular uptake experiment to visualize and measure cellular uptake of polymeric NPs.

Cellular uptake images of Doc–Cur-loaded NPs on Hela cells after 2 h (figure 2(b)) and 4 h (figure 2(c)) incubation with Doc–Cur-loaded PEG–PCL NPs at 2 μg ml⁻¹ (calculated by Doc) and 4 μg ml⁻¹ (calculated by Cur) are shown in figure 2. We find that the fluorescence of the Doc–Cur-loaded NPs (green) is located in cells, which means that the NPs have been internalized by the cell. The fluorescence intensity of Hela cells increased after 2 h and decreased after 4 h of incubation. So, cellular uptake was dependent on the incubation time. These results are consistent with published data [18].

3.4. In vitro cytotoxicity

The cytotoxicity of Doc-loaded NPs with and without Cur and the free Doc and Cur was evaluated on the Hela cell line. Table 2 shows the IC₅₀ values of Hela cells after 24 and 48 h at various Doc concentrations of 0.025, 0.25, 2.5, 12.5 and 25 μg ml⁻¹ (Cur concentration in NPs was 0.05, 0.5, 5.0, 25 and 50 μg ml⁻¹).

The IC₅₀ value for Hela cells decreased from 3.5 μg ml⁻¹ for free Doc to 2.01 μg ml⁻¹ for Doc–NPs and 1.52 μg ml⁻¹ Doc–Cur–NPs after 24 h of incubation. After 48 h of incubation, the significant difference of IC₅₀ values between free Doc, Doc–NPs and Doc–Cur–NPs was 1.16, 0.58 and 0.35 μg ml⁻¹, respectively. No cytotoxic activity was observed for the drug-free NPs. These results suggest that the incorporation of Doc and Cur into NPs enhanced its anti-tumoral activity compared to Doc-NPs and free Doc.

Thus, the higher cytotoxicity of the drug-loaded NPs can be attributed to different mechanisms between free drug molecules and drug molecules entrapped into NPs. The NPs were adsorbed on the cell surface, therefore the drug concentration near the cell membrane significantly increases, resulting in a concentration gradient and promoting penetration of the drug into the cell [19]. However, for free drug molecules, part of these molecules were transported into the cytoplasm by passive diffusion and transported out by P-glycoprotein pumps. Moreover, drug-loaded NPs penetrate into cells through endocytosis and they can escape from the influence of P-glycoprotein pumps, thus leading to a higher cellular uptake compared to free drug molecules [20].

Thus, the combination of Doc and Cur into PEG–PCL NPs is able to increase the synergistic effect on cancer cells. Moreover, fluorescent signals in cancer cells can be observed by the presence of Cur in NPs for further studies in vitro and in vivo.

4. Conclusion

In this study poly(ethylene glycol)-block-poly(ε-caprolactone) polymers were successfully employed for the nanoencapsulation of Doc and Cur, by using a modified nanoprecipitation method and sonication. NPs with small size and a more uniform distribution were fabricated by extrusion through an EmulsiFlex™-CS High Pressure Homogenizer. Among the investigated NPs, the in vitro studies showed an increased antiproliferative efficacy of Doc–Cur-loaded PEG–PCL NPs compared to other PEG–PCL NPs and to free Doc or Cur on Hela cells.

In conclusion, the enhanced bioavailability of Doc–Cur-loaded PEG–PCL NPs can be considered as alternative and promising drug delivery system for cancer chemotherapy and this hypothesis needs to be verified in vivo.

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References

[1] Cho K, Wang X, Nie S, Chen Z and Shin D M 2008 Clin. Cancer Res. 14 1310
[2] Ai H, Flask C, Weinberg B, Shuai X, Pagel M D, Farrell D, Duerk J and Gao J 2005 Adv. Mater. 17 1949
[3] Kataoka K, Harada A and Nagasaki Y 2001 Adv. Drug Deliv. Res. 47 113
[4] Kwangjae C, Xu W, Shuming N, Zhuo (Georgia) C and Dong M S 2008 Clin. Cancer Res. 14 1310
[5] Zhang X, Jackson J K and Burt H M 1996 Int. J. Pharm. 132 195
[6] Hiljanen V M, Karjalainen T and Seppälä J 1996 J. Appl. Polym. Sci. 59 1281
[7] Vila A, Gill H, McCallion O and Alonso M J 2004 J. Control. Release 98 231
[8] Petrylak D P 2006 Rev. Urol. 8 S48
[9] Schiff P B and Horowitz S B 1980 Proc. Natl. Acad. Sci. USA 77 1561
[10] Kawanishi S, Oikawa S and Murata M 2005 Antioxidants Redox Signaling 7 1728
[11] Burgos-Moron E, Calderon-Montano J M, Salvador J, Robles A and Lopez-Larazaro M 2010 Int. J. Cancer 126 1771
[12] Dance-Barnes S T, Knock N D, Moore J E, Lin E Y, Mosley L J, D’Agostino R B Jr, McCoy T P, Townsend A J and Miller M S 2009 Carcinogenesis 30 1016
[13] Lekha Nair K, Thulasidasan A K T, Deepa G, Anto R J and Vinod Kumar G S 2012 Int. J. Pharm. 425 44
[14] Yin H, Guo R, Xu Y, Zheng Y, Hou Z, Dai X, Zhang Z, Zheng D and Xu H 2012 Acta Biochim. Biophys. Sin. 44 147
[15] Altenburg J D, Bieberich A A, Terry C, Harvey K A, VanHorn J F, Xu Z, Davisson V J and Siddiqui R A 2012 BMC Cancer 11 149
[16] Yan C, Qingqing W, Zhenghai Z, Ling Y, Xuan L and Lei Z 2012 Molecules 17 5972
[17] Brigger I, Dubernet C and Couvreur P 2002 Adv. Drug Deliv. Rev. 54 631
[18] Davda J and Labhasetwar V 2002 Int. J. Pharm. 233 51
[19] Fonseca C, Simoes S and Gaspar R 2002 J. Control. Release 83 273
[20] Panyam J and Labhasetwar V 2003 Pharm. Res. 20 212