Shell Cross-Linked Nanoparticle Based on Poly(ε-caprolactone)-Poly(ethylene glycol) for Photosensitizer Delivery

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Abstract

Polymeric micelles as drug carriers attract highly concern recent years for their ability to incorporate hydrophobic drugs. Photodynamic therapy (PDT) is a promising new cancer treatment that involves the combination of visible light and a photosensitizer. In this study, protoporphyrin IX (PpIX) was encapsulated into shell crosslinked nanoparticles (SCKs) for photosensitizer delivery to reduce the side effects of PDT.

The particle size of the SCKs without drug was about 140 nm, and the size of the SCKs with PpIX were about 240 nm. The cytotoxicity reveals that SCKs/PpIX have some toxicity to normal cell line. As shown in the results of fluorescence microscopy and flow cytometry, the uptake of SCKs with PpIX reach the maximum at the incubation time of 12-24 hours. The PDT effects of SCKs/PpIX show no significant differences with free PpIX.

In summary, *in vitro* characterization and *in vitro* PDT effect of SCKs encapsulated with PpIX were studies. These results may lead to more successful development on the application of SCKs for photodynamic therapy.

1. Introduction

Polymeric micelles are nanoparticles self-assembled from amphiphilic block copolymers in aquaeous media. The micelles have core-shell structure where their shells consist of hydrophilic blocks, and theirs cores consist of hydrophobic segment. The cores could serve as nonaqueous reservoirs for poorly soluble drugs, and the shells could conjugate with some specific antibodies. Micelles as drug carriers are able to provide various advantages such as solubilizing lipophilic drugs and increasing their bioavailability, accumulating in body regions with leaky vasculature and avoiding renal clearance and non-specific reticuloendothelial uptake because of the nano size, protecting the drug from inactivation under the effect of biological surroundings, and being targeted by attachment of some specific ligands. [Kwon et al., 1996; Torchilin, 2001; Lee et al., 2003; Shuai et al., 2004]

Micelles as drug carriers are studied popularly recent days. It's easily to fine that there are many anticancer drugs used as model drugs for the experiments. That is because many anticancer drugs are lipophilic, such as doxorubicin, cisplatin and taxol. To incorporate this kind of hydrophobic drug with micelle could increase the bioavailability and decrease the toxicity to normal cell. [Torchilin, 2001]

Allen et al. proposed three possible mechanisms of drug releasing from micelles: (1) micelles remain outside the cell and the drug is released into the external medium following which it enters the cell; (2) the micelles enter the cell and release the drug into the cytoplasm after which it is transported into the nucleus; (3) the micelles enter the nucleus where the drug is then released. [Allen et al., 2000]

Crosslinking self-assembled nanoparticles impart greater stability by providing protective layer of covalent linkages between the self-assembled polymer chains. This strategy has been applied to create core crosslinked nanoparticles, shell crosslinked nanoparticles, shell crosslinked rods, nanocages, nanotubes, and nanoporous films. Recently, the shell crosslinked nanoparticles(SCKs) have demonstrated potential for targeted drug delivery, sequestration of metabolites, entrapment of environmental pollutants, and similar encapsulation/release capabilities. Since therapeutics, imaging agents and targeting ligands can be employed for the covalent attachment of these molecules to the SCKs. The potential uses of SCKs are expanded by their stability toward more environments than their non-crosslinked precursors. [Murthy et al., 2001; Becker et al., 2001; Joralemon et al., 2005]

Photodynamic therapy (PDT) is a new treatment utilizing the combined action of photosensitizers and visible light for the treatment of various cancers (lung, esophagus, gastric, cervix, bladder, etc.), and the treatment of neovascularization-related disorders such as choroidal neovascularization (CNV) secondary to age-related macular degeneration (AMD). PDT for cancer involves the administration of photosensitizers to solid tumor and local illumination with light of a specific wavelength, depending on the photosensitizers we choose. Photochemical destruction of cancer cells via generation of singlet oxygen or superoxide from molecular oxygen. Most photosensitizers are porphyrin-like macrocycles and include porphyrins, chlorins, and bacteriochlorins. An ideal photosensitizer should be high potential of light-activated tissue damage and no dark toxicity, activate at wavelengths that penetrate deep into tissues, fast accumulate in target tissues and rapid clearance and have tumor selectivity. One of the problems limiting the use of many photosensitizers is the difficulty in preparing pharmaceutical formulations because of their low water solubility. Suitable carriers for photosensitizers could provide more accumulation for tumor tissues, higher bioavailability after systemic administration and lower side effects. [Jori, 1996; Yan et al., 2003; Zhang et al., 2003; Derycke et al., 2004; Vargas et al., 2004; Jang et al., 2005]

2. Materials and methods

2.1. Preparation of SCKs

Dissolve PEG-PCL copolymer 40mg with or without PpIX 2mg with THF 1mL. Mix well and add drop wise (10mL/hr) into ddH_2O 20 mL. Apply sonication (Decon F5300b) for 10 mins. Add 1,4-diaminobutane 700 μL and react for 3 days. After 3 days, apply sonication for 10 mins again, and then perform dialysis for another 3 days. Centrifuge the micelle solution for 10 minutes under 3500 r.p.m. and remove the aggregates.

2.2 Determination of particle size

The measurement of the particle size distribution was carried out by Zetasizer (Malvern-zetasizer 3000hs, Malvern, UK). All measurements were performed at 25 ◦C at a measurement angle of 90 ◦C, in triplicate. The measurement yields a hydrodynamic diameter for the particles.

2.3 Cell culture and incubation conditions

Human cervical epithelioid carcinoma (HeLa) cells were maintained in Minimum essential medium (MEM) containing 10% fetal bovine serum and 1% antibiotics (Antibiotic-Antimycotic), and were cultured in a 5 % CO_2 incubator at 37℃. Subculture HeLa cells 2~3 times every week.

2.4 Cytotoxicity assay

HeLa cells were plated into 96-well plates (5×10^3 cells/well) with 100 μL MEM medium and were cultivated overnight. The cells were then incubated respectively with micelle only (no PpIX), micelle which encapsulated with PpIX, and free PpIX for 24hrs and 48hrs in MEM. The concentration of PpIX is 20 μg/mL. After the incubation, the cells were rinsed with phosphate-buffered saline(PBS), and incubate for 3 hours at 37℃ with 0.4 mg /mL MTT MEM solution 50 μL. After 3 hours, dissolve formazan crystals with DMSO 100μL and read the OD value by ELISA reader at 550 nm.

2.5 Fluorescence microscopy

Cells were seeded on glass cover slips in 35 mm dishes and incubated at 37℃. After 18 hrs, the medium in each dish was replaced with micelle-PpIX and free PpIX solution in MEM for 1, 3, 6, 12, 24 hrs. The concentration of PpIX is 20 μg/mL. Then, the cells were washed three times with PBS and fixed with 10% formalin for 10 mins. After thorough wash, the cells were mounted and processed for fluorescence microscopy (Zeiss , Axiophot 2 Microscope).

2.7. Flow cytometry

Cells (1×10^6) were plated in 60mm dishes the day before experiments. To quantify the total fluorescence intensity of PpIX taken by the cells, cells were incubate with micelle solution (encapsulated with PpIX) for different time points (1, 3, 6, 12, 24 hrs). The concentration of PpIX is 20 μg/mL . After the incubation, washed with PBS, trypsinized, and transferred to centrifuge tubes. The cells were fixed in 10% formalin for 15 min on ice, washed and resuspended with FACSFlow™ PBS, and then analyzed by using the FacsCalibur (Becton-Dickinson, USA).

2.8. Photodynamic effects

In this study we used PpIX as the photosensitizer for PDT. The light source for activating PpIX consisted of an array of light emitting diodes (LED) that divided the light at 635 nm wavelength. HeLa cells were incubated at 37℃ with MEM containing SCKs/PpIX solution (PpIX concentration:20 μg/mL) for 12 hrs. The cells were irradiated by the light source with 100 μL PBS per well for 50 and 150 seconds, respectively. Incubate the cells again at 37℃ for 24 hrs, and then analyze the cell survival with MTT assay . The cells were rinsed with phosphate-buffered saline, and incubate for 3 hours at 37 ℃ with 0.4 mg /mL MTT MEM solution 50 μL. After 3 hours, dissolve formazan crystals with DMSO 100 μL and read the OD value by ELISA reader at 550 nm.

3. Results and discussion

3.1 Particle size of SCKs

The particle size of the SCKs without drug was about 140 nm, and the size of the SCKs/PpIX was about 240 nm.

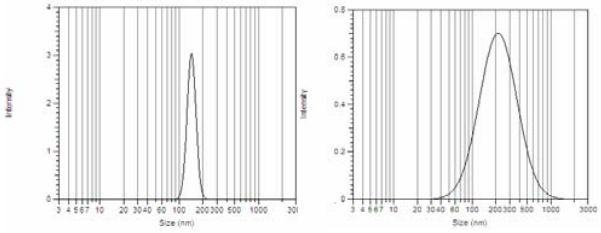

Fig. 1 Size distribution of PCL-PEG SCKs w/o and w/ PpIX.

3.2 Cytotoxicity assay

The cytotoxicity of the PCL-PEG SCKs (with or without PpIX) on HeLa cells compared with the free PpIX under the same concentration were shown in Fig. 2. The viability of both HeLa cells incubated with PCL-PEG micelles (with or without PpIX) after 24 and 48 hrs showed no significant differences compared with the control.

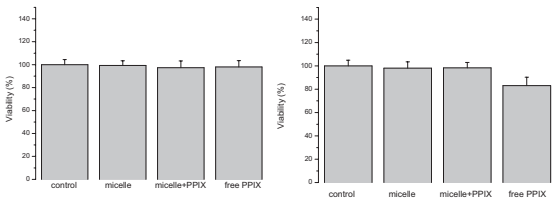

Fig. 2 Cytotoxicity study of SCKs, SCKS/PpIX and free PpIX on HeLa cells after incubation for 24 and 48 hrs.

3.4 Intracellular distribution of SCKs/PpIX

To assess the cellular uptake of PpIX encapsulated in PCL-PEG micelles, we implemented time-sequencing studies using fluorescence microscopy on HeLa cells. As shown in Fig. 3, the fluorescence of PpIX encapsulated in PCL-PEG SCKs gradually increased with the incubation time, and the most strong fluorescence intensity appeared at 12~24 hrs incubation.

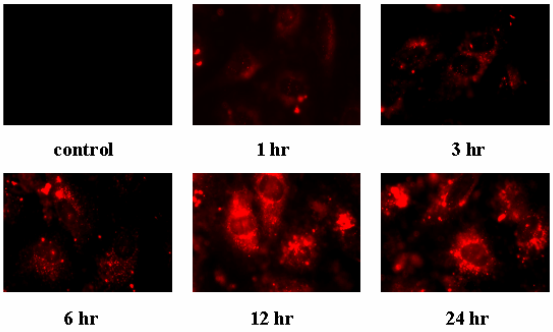

Fig. 3 Fluorescence microscopy images of HeLa cells incubated with micelle-PpIX for different incubation time.

3.4 Cellular uptake of SCKs

To quantify the uptake of PpIX encapsulated in PCL-PEG micelles and free PpIX by HeLa cells, the fluorescence intensity of cellular PpIX was analyzed by flow cytometry. As shown in Fig.4, the fluorescence intensities of PCL-PEG SCKs/PpIX increased with time, and the maximums appeared at 12 hrs time point. The fluorescence intensities of free PpIX were also increased with time, and reached the peak after 24 hrs. The results of flow cytometry were consentient with the observations of fluorescence microscopy.

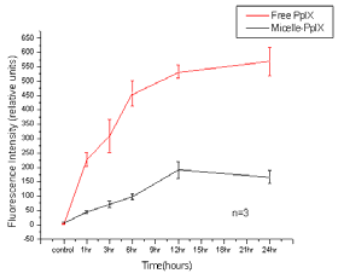

Fig. 4 Time-dependence of SCKs/PpIX and free PpIX uptake by HeLa cells for 24h.

3.5. PDT effects of SCKs/PpIX

Fig. 5 showed the results of different illumination time with SCKs/PpIX of HeLa cells. After 50s illumination, the viability of HeLa cells with SCKs/PpIX incubation reduced about 60% that was equal to the PDT effect of free PpIX. After 150s illumination, the photodynamic effect of SCKs/PpIX was better than free PpIX on HeLa cells.

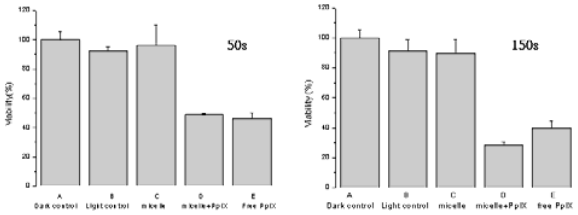

Fig.5. Photodynamic effect of SCKs, SCKs/PpIX and free PpIX with 50s or 150s illumination on HeLa cells.

Acknowledgments

This research was supported by the fund provided by Industrial Technology Research Institute of the Republic of China.

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