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Chain length effect on penetration behavior of mPEG-PCL micelles using multicellular tumor spheroids

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Abstract: **Four amphiphilic diblock copolymers with methoxy poly (ethylene glycol) (mPEG) and different chain lengths of polycaprolactone (PCL), encapsulated anticancer drug doxorubicin (DOX) on the hydropholic cores were fabricated. The 1H nuclear magnetic resonance（1H NMR), Gel Permeation Chromatography (GPC) were characterized to confirm the successful synthesis of the copolymeric micelles. In vitro cytotoxicity verify the micelles was non-toxic to cells and DOX loaded micelles shows efficient anticancer activity. The penetration behavior was might assist the diffusion of DOX, which was confirmed by confocal microscopy images. Results indicate that mPEG-PCL micelles were found to substantially provides an efficient antitumor carrier and reduce drawback of free DOX.**

Keywords: Block copolymer micelle; Tumor spheroids; Penetration behavior

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1. Introduction

Poor penetration of traditional anticarcinogen in solid tumor remains hampers its application to cancer therapy [1,2]. To overcome this limitation, macromolecular drug delivery system has been designed for its high accumulation in tumors via the enhanced permeation and retention effect (EPR) [3]. However, it is not enough to design novel drug delivery system, it is essential to know their effect on penetration solid tumor.

Polymer micelles have found extensive use in drug delivery for its advantages of long circulation and self-assembled function [4-6]. As biocompatible polymers, mPEG-PCL encapsulates anticancer drugs in hydrophobic cores. The various lengths of hydrophilic and hydrophobic segments act an import role in polymer micelles for drug delivery [7-9].

Restricted by experimental and ethical problems, it is difficult to investigate the in vivo itinerary of tumor penetration [10-12]. Therefore, multicellular tumor spheroid (MTS) is one of the most common methods to imitate the vivo environment, which have been used to test drug delivery, drug sensitivity and penetration efficiency due to their cell-cell structure [13-15].

In this paper, four mPEG-PCL diblocks copolymers were fabricated to evaluate chain length effects of drug delivery. Hence, the morphology, drug release profile, in vitro cytotoxicity, antitumor efficacy and penetration behavior, were evaluated in detail.

2. Materials and method

2.1. Materials

α-Methoxy-poly(ethylene glycol) (mPEG, Mw=5 000g/mol), ε-caprolactone (ε-CL), stannous octoat e (Sn(Oct)2) were purchased from Sigma-Aldrich Co. methylbenzenesulfonyl (TsCl) and triethylam ine (TEA) were purchased from Asta Tech Phar maceutical (Chengdu, China) and used as received. Doxorubicin hydrochloride (DOX·HCl, Zhejiang Hisun Pharmaceutical China) was deprotonated acc ording to the method previously reported.The Dul becco's Modification of Eagle's Medium (DMEM), fetal bovine serum (FBS) and cell counting kit-8 (CCK-8) were purchased from HyClone Inc. The penicillin G, The streptomycin sulfate, trypsin an d ethylenediaminetetraacetic acid (EDTA) were p urchased from Solarbio Science Technology Co. Ltd. All the solvents were purchased from Qingda o Shengtianyi Chemical Co. (China) and purified before used.

2.2. Synthesis of mPEG-PCL diblock copolymers

mPEG–PCL diblock copolymers were synthesized by ring-opening polymerization of ε-CL with mPEG homopolymer as macroinitiator and $Sn(Oct)_2$ as the catalyst. Prescribed amount of mPEG, ε-CL and $Sn(Oct)_2$ (ca. 0.1% of ε-CL in molar amount) were mixed in a round-bottom ask connected with a vacuum joint. The mixture was degassed in vacuum. The ask was sealed off and placed in an oil bath and polymerized at 130℃ for 48h. The product was cooled at room temperature, dissolved in chloroform

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and purified by precipitating into large amount of cold diethyl ether. The precipitated product was vacuum-dried at 40℃.

2.3. Preparation of DOX- loaded micelles

Micelles were prepared from mPEG-PCL diblock copolymers by hydration of thin films. The mPEG–PCL amphiphile (10mg) and DOX (4.3mg) were dissolved in 1ml of DMSO with ultrasound for 0.5h. The solution was dropped into 10ml deionized water under magnetic stirring for 12h. The crude product was dialyzed against distilled water at 4℃ overnight in a dialysis membrane tubing (Spectra/Por MWCO 3500). After the completion of reaction, the obtained primary micelles were removed from the dialysis tubing with centrifugation and freezedried.

2.4. DOX release experiment

DOX loaded micelles were dispersed in PBS (1ml, pH =7.4). The release experiments were employed under sink conditions in order to ensure the good solubility of DOX. The mixture was put in dialysis membrane bags (Spectra/Por MWCO=1000). The tubings were immersed in vials containing 25ml of phosphate buffered saline (PBS) solution and put in a shaking bed at 37℃. 1ml of PBS solution was taken out and the same volume PBS was added to the vials at prescribed time intervals. Spectrofluorophotometer was used to test the in vitro released DOX with excitation wave-length at 485 nm and emission wavelength at 550nm [16]. The release experiments were conducted in triplicate, the results were demonstrated as mean±SD.

2.5. Cytotoxicity study in vitro

To evaluate the cytotoxicity induced by blank micelles, CCK8 assay was used. The HUVEC and HepG2 cells were separately inoculated into 96-well plates with 5×10^3 cells per well in 100ml of medium. After 24h incubation, the medium was removed and replaced with 100ml of medium containing different concentrations of blank micelles. The micelles were incubated with cells for 48h. The medium was removed and the wells were washed with PBS (pH =7.4). 10ml of 5mg/ml CCK8 solution in PBS (pH=7.4) was added to each well. A er incubated for 4h, the medium containing unreacted CCK8 was removed carefully and the absorbance was measured.

2.6. In vitro anticancer activity study

HepG2 cells were separately inoculated into 96-well plates with 5×10^3 cells per well in 100 ml of medium for 24 h. DOX·HCl and DOX loaded micelles solutions in DMEM were added to the plates and incubated for 48 h. The cell viability was measured by CCK8 assay.

2.7. Stacking culture method for tumor spheroids

HepG2 (hepatocytes) cells were cultured in DMEM medium containing 1% penicillin streptomycin solution supplemented with 10% FBS at 37℃ in a 5%CO_2 humidity atmosphere. An agarose solution (1%, w/v) was prepared as a layer coated on the bottom of 96-well plates. In order to ensure sterile, the culture flasks must be exposes to ultraviolet light for 1h before use. HepG2 cells were trypsinized and count the cell numbers. 3000 cells in fresh DMEM medium was seeded in processed 96-well. After cellular aggregation and growth, each well contained a single tumor spheroid. Half of the culture medium was replaced every other day.

2.8. Penetration behavior on tumor sphere

The tumor spheroids were used to evaluate penetration behavior, which treated with different types of drug loaded mPEG-PCL micelles, then transferred to confocal microscopy (Nikon, Japan). Images were acquired at a magnification of 40× using a CCD camera with fixed settings of expore and contrast after the tumor spheroids were treated with DOX loaded micelles for 4h. Z-stack images were captured by scanning the tumor spheroid step by step, that was started from the top to the bottom.

3. Results and discussion

3.1. Synthesis and identification of mPEG-PCL micelles

As described in the method section, Figure 1 shows that mPEG5k-PCL1k, mPEG5k-PCL3k, mPEG5k-PCL5k, mPEG5k-PCL8k were successfully synthesized,which was evidenced by 1H NMR spectrum.The sharp peaks at 3.35 to 4.5ppm were attributed to the protons in mPEG chain, the proton signal in PCL blocks were 3, 4, 5 and 6.

The number-average molecular weight of the four synthesized copolymers determined by GPC (Figure 2), only one peak was observed and no unreacted mPEG was found in the spectra. The results suggesting that the obtained had welled-controlled Mn and narrow distribution of molecular weight. The PDI of mPEG5k-PCL1k, mPEG5k-PCL3k, mPEG5k-PCL5k, mPEG5k-PCL8k copolymers were 1.037, 1.023, 1.065 and 1.054.

mPEG-PCL could self assemble into micelles,which was confirmed by DLS measurement. Four micelles were monodisperse and the calculated mean diameter mPEG5k-PCL1k, mPEG5k-PCL3k, mPEG5k-PCL5k, mPEG5k-PCL8k were 34.34, 60.08, 85.74 and 95.38 nanometers (Figure 3).

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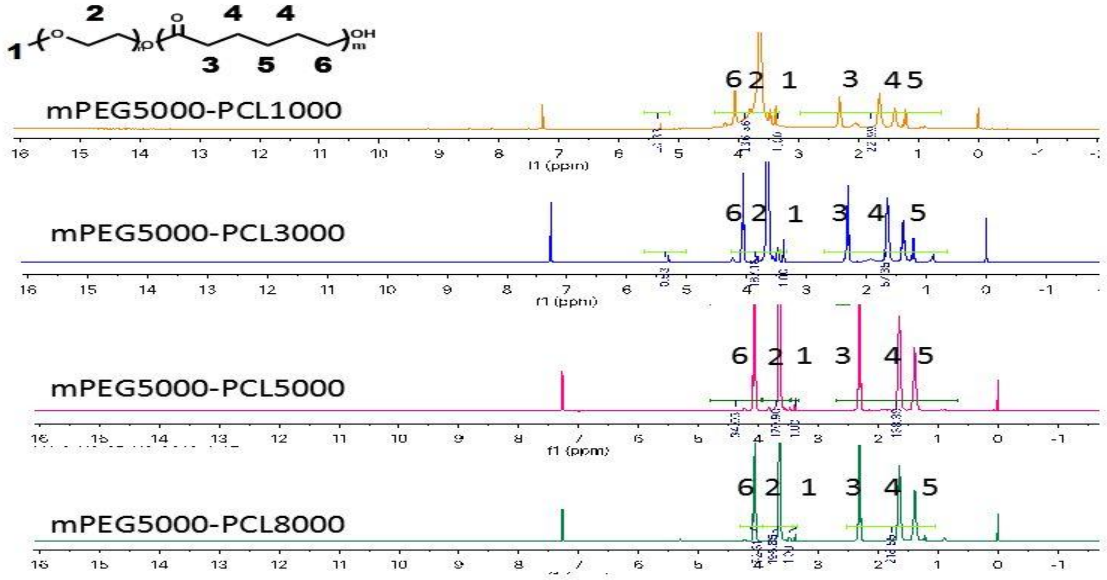


Figure 1. The ^{1}H NMR spectra of mPEG-PCL copolymers.

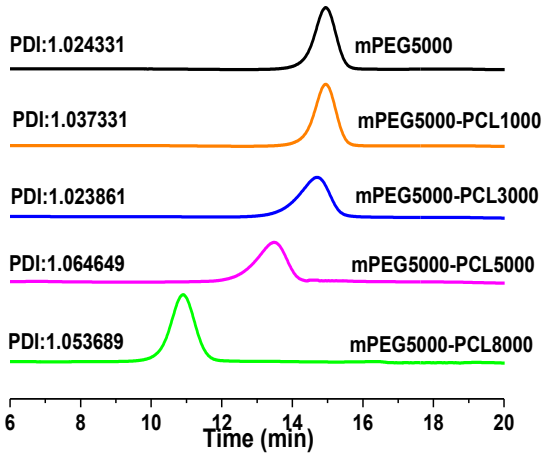


Figure 2. The GPC spectra of mPEG-PCL block copolymers.

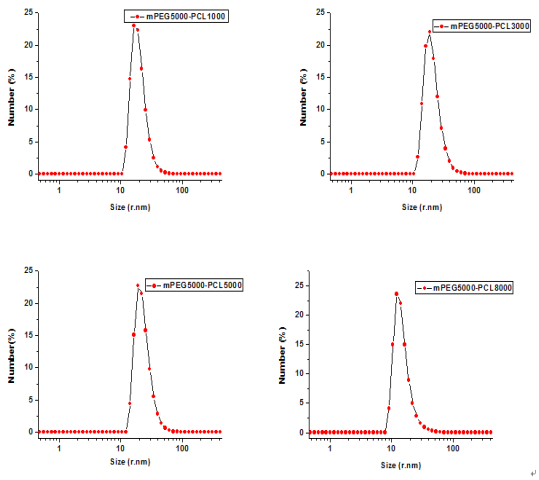


Figure 3. Size tested by DLS of mPEG-PCL micelles.

3.2. The release of DOX from micelles

The in vitro release profiles of DOX loaded micelles were carried out in physiological condition (pH=7.4) at 37 ℃. As shown in Figure 4, DOX-loaded conjugate micelles showed a remarkably burst release in the first 10h, the extent of DOX released from DOX-loaded conjugate micellesn were different, and then the DOX release profile reached a plateau. In comparison, the release rate decreased with the sequence of mPEG5k-PCL1k, mPEG5k-PCL3k, mPEG5k-PCL5k and mPEG5k-PCL8k. The drug release mechanism in these polymeric micelles was diffusion control. The drug diffused from the hydrophobic cores of micelles to the medium. The burst release was attributed to the drug absorbed in the hydrophilic PEG layer, which dispersed easier and faster to the medium.

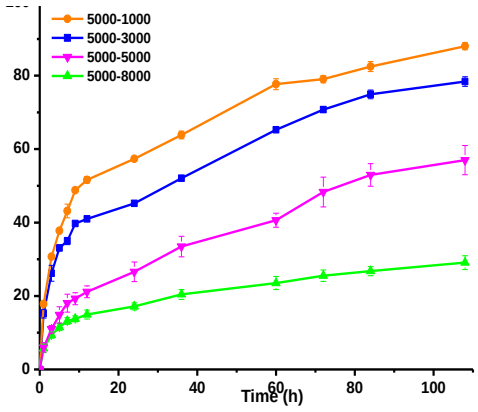


Figure 4. The release profiles of DOX loaded mPEG-PCL micelles in physiological condition (PH=7.4).

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3.3. In vitro toxicity of mPEG-PCL micelles

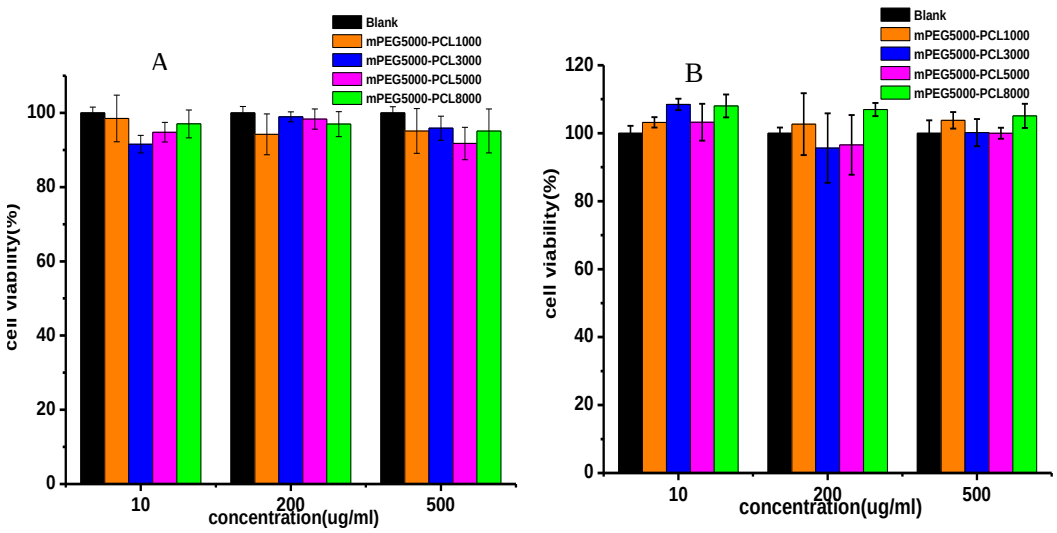


Figure 5. In intro cytotoxicity of mPEG-PCL against HUVEC cells (A) and HepG2 cells (B).

The four mPEG-PCL micelles were expected as carriers to deliver anticancer drugs, whether it are toxic or not should be first tested before its futher development. Here, CCK8 assay was first performed to investigate the cytotoxicity of mPEG-PCL micelles. Figure 5 shows the effect of the micelles at different concentration on the proliferation of HUVEC and HepG2. Results demonstrated that no significant decrease in cell viability was detected even at the high concentration of 500μg mol-1, which was much higher than the concentration applied in vivo. The results revealed that the four mPEG-PCL micelles did not render detectable toxicity.

3.4. In vitro anticancer activity of DOX loaded micelles

To further determine the anticancer activity of different micelles against HepG2 cells, CCK8 assay was investigated (Figure 6). The in vitro anticancer efficiency of drug loaded micelles was dosage-dependent. The IC50s (the concentration of anti-drug that killed 50% of cells of free DOX·HCl, DOX-mPEG5k-PCL1k, DOX-mPEG5k-PCL3k, DOX-mPEG5k-PCL5k and DOX-mPEG5k-PCL8k micelles were 0.10, 0.26, 0.35, 0.68and 1.90 mg ml^{-1}, respectively. Noticeably, free DOX·HCL exhibited the highest cytotoxicity owing to its easier diffution to cells and accumulation at DNA backbone. The HepG2 cells were more sensitive to lower drug rlease rate micelles, the anticancer activity was enhanced after encapsulated in mPEG-PCL micelles.

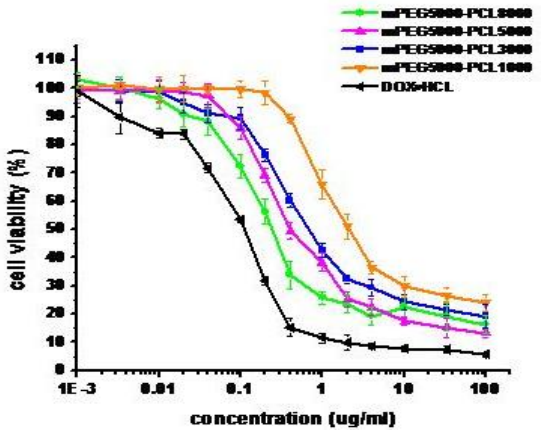


Figure 6. In vitro anticancer of DOX loaded mPEG-PCL micelles against HepG2 liver cancer cells, the incubation time was 48h.

3.5. Penetration on cells and tumor sphere

DOX was not only used as anticancer drug, but also as the marker of four DOX loaded mPEG-PCL micelles to exhibition their penetration processes (Figure 7). In order to make an easy confocal scanning, we chose logarithmic growth phase cells and tumor spheroids with an average diameter of 300 μm since the spheroids had good status. We compared penetration behaviors of mPEG5k-PCL1k, mPEG5k-PCL3k, mPEG5k-PCL5k, mPEG5k-PCL8k in HepG2 tumor spheroids in vitro, mPEG5k-PCL3k and mPEG5k-PCL5k displayed better penetration ability due to its size and biodegradable.

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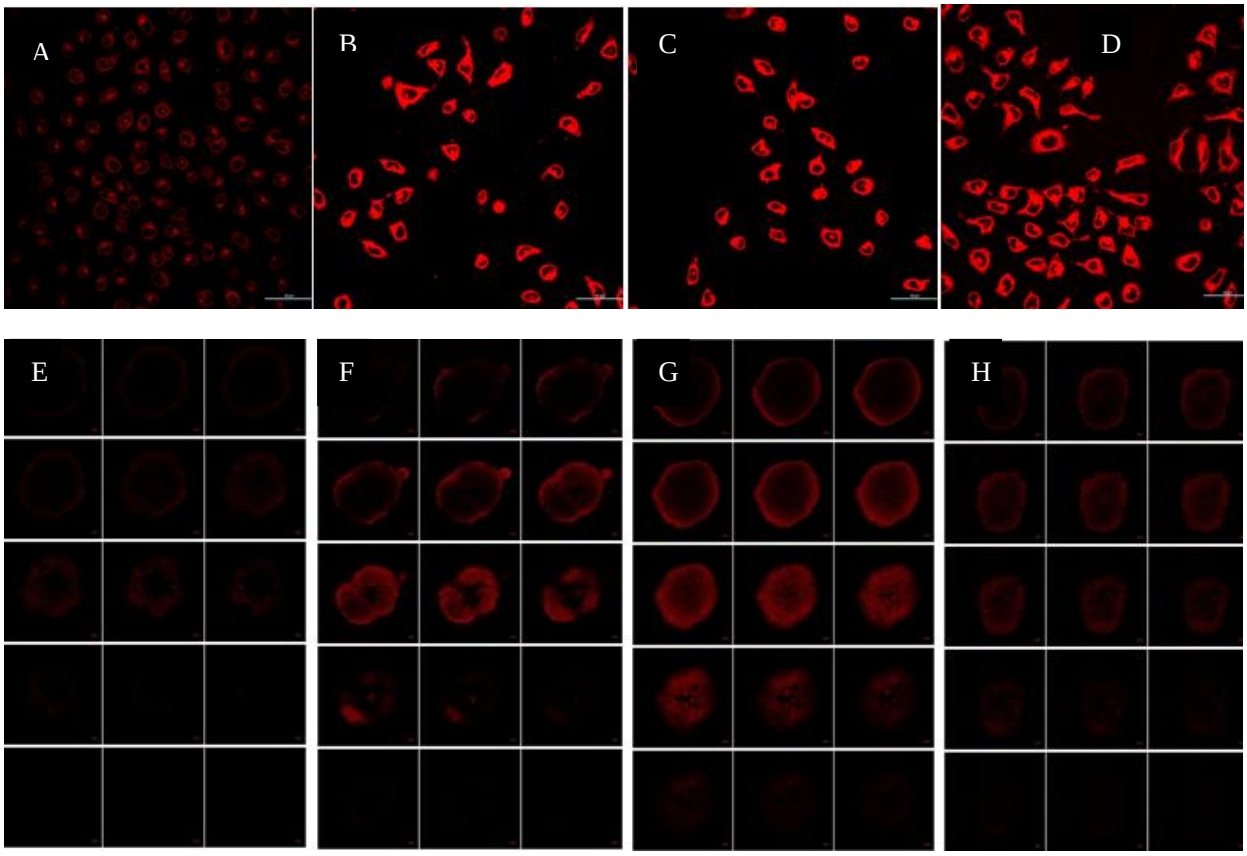


Figure 7. Confocal images of HepG2 cells and tumor spheroids incubated with DOX-mPEG5k-PCL1k (A,E), DOX-mPEG5k-PCL3k (B,F), DOX-mPEG5k-PCL5k (C,G) and DOX-mPEG5k-PCL8k (D,H) for 4h. Radius is normalized starting from top to the bottom of tumor 3-D model (E, F, G, H).

4. Conclusions

Four amphiphilic mPEG-PCL copolymers with different chain length were synthesized via ring-opening polymerization of ε-CL with mPEG as macroinitiator, the terminal group of PCL segment was interaction with anticancer drug doxorubicin. The chain length effects of mPEG-PCL diblock copolymers on the self-assembly of micelles, drug loading, release profiles, in vitro anticancer activity of drug loaded micelles were investigated. This study suggested that penetration behavior played an significant role in enhancing chemotherapeutic agents into tumor tissues.

Acknowledgment

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