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In vitro & *in vivo* targeting behaviors of biotinylated Pluronic F127/poly(lactic acid) nanoparticles through biotin–avidin interaction

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ABSTRACT

Biotinylated Pluronic F127/poly(lactic acid) block copolymers (B-F127–PLA) were successfully synthesized previously by our group. In the present study, the release behaviors of paclitaxel-loaded B-F127–PLA nanoparticles and their targeting properties to human ovarian carcinoma cells were investigated. Paclitaxel (pac) loaded in B-F127–PLA nanoparticles shows an initial burst release in the first 6 h and followed by a slow release. The *in vitro* targeting behaviors of B-F127–PLA nanoparticles against human ovarian cancer cells (OVCAR-3, SKOV-3) were investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) tests and fluorescence microscopy (FM) technique. Targeting was based on a three-step biotin–avidin targeting approach using biotinylated anti-CA125 antibody specific for the CA-125 antigen that is highly expressed on OVCAR-3 cells but not expressed on SKOV-3 cells. MTT results show that the anticancer effect of paclitaxel in B-F127–PLA nanoparticles over OVCAR-3 cells was stronger than that over SKOV-3 cells, indicating that B-F127–PLA nanoparticles were delivered more effectively to OVCAR-3 cells than to SKOV-3 cells. The targeting behaviors of B-F127–PLA nanoparticles were further confirmed by FM technique. The intracellular distribution of B-F127–PLA nanoparticles was also studied using a triple-labeling method. It was observed that B-F127–PLA nanoparticles are mainly localized within the cytoplasm of OVCAR-3 cells. The *in vivo* antitumor efficacy of pac-loaded B-F127–PLA nanoparticles by three-step method as measured by change in tumor volume of OVCAR-3 implanted in Balb/C nude mice was greater than that by one-step method.

1. Introduction

Biocompatible and biodegradable polymeric nanoparticles have attracted increasing attention for their application in drug delivery systems (DDS) because they are able to protect the loaded drug from metabolism or early excretion, and also contain the potential for control of release rate of the drug (Kissel et al., 2002; Otsuka et al., 2003; Alarcon et al., 2005; Savic et al., 2006; Tong and Cheng, 2007). Polymeric nanoparticles with targeting ligands are promising candidates for cancer therapy, which are able to increase the selectivity and efficiency of anticancer drug delivery to the target cells, leading to a better therapeutic efficacy as well as reduced side effects (Torchilin, 2005; Blasi et al., 2007; Nie et al., 2007; Pardridge, 2007; Pulkkinen et al., 2008). A versatile approach to prepare targeting polymeric nanoparticles is to take advantage of the biotin–avidin interaction, which is the strongest known non-covalent biological interaction (association constant $10^{15}\,M^{-1}$) (Gref et al., 2003). The avidin containing four binding sites for biotin can serve as a universal linkage between the biotinylated polymeric nanoparticles and targeting ligands. Targeting of polymeric nanoparticles can then been achieved by the so-called three-step pretargeting technology through biotin–avidin interaction, which has been used *in vivo* and even in phases I–II clinical trials to target radionuclides to cancer cells (Knox et al., 2000; Macor et al., 2007; Urbano et al., 2007; Newton-Northup et al., 2009). The three-step pretargeting system can be achieved by the administration of biotinylated targeting ligands such as monoclonal antibodies (MABs), followed by avidin injection, and finally the injection of biotinylated polymeric nanoparticles.

Ovarian cancer remains the leading rank in cancer deaths among women. Paclitaxel is an effective anticancer agent for treating the ovarian cancer. Paclitaxel has very poor water solubility and is provided clinically in a formulation containing Cremophor EL (polyethoxylated castor oil) and ethanol (Taxol®). However, Cremophor EL can induce a number of side effects such as hypersensitivity, nephrotoxicity and neurotoxicity (Dong and Feng, 2007; Pulkkinen et al., 2008).

Therefore, biotinylated polymeric nanoparticles are believed to be the appropriate delivering carriers of paclitaxel for the treatment of ovarian cancer. Biotinylated Pluronic/poly(lactic acid) block copolymers have been synthesized for the first time by our

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group (Li et al., 2010). To our knowledge, there are only few reports regarding the synthesis of similar block copolymers biotin–poly(ethylene oxide)–PLA (biotin–PEO–PLA) and no study at all on the synthesis of biotin–Pluronic–PLA copolymers (Cannizzaro et al., 1998; Salem et al., 2001; Gref et al., 2003; Pulkkinen et al., 2008). It was reported that poly(ethylene imine)–Pluronic (PEI–Pluronic) block copolymer exhibited higher transfection efficiency toward plasmid DNA than PEI–PEO copolymer due to the amphiphilic property of Pluornic block copolymer (Nguyen et al., 2000). The micelles produced from Pluronic block copolymers have been found to increase the oral and brain bioavailability of drugs (Rösler et al., 2001). These are the reasons for our group to select the PEO–PPO–PEO block instead of hydrophilic PEO in this study. Furthermore, it is well-known that the hydrophobic poly(lactic acid) (PLA) is a kind of polyester with excellent biodegradability and biocompatibility. Research work on the self-assembling behaviors of PLA–Pluronic–PLA block copolymers and their application in drug delivery systems has been studied in detail previously by us (Xiong et al., 2003, 2004, 2005a,b,c; Xiong et al., 2007).

In this paper, paclitaxel was loaded into biotinylated Pluronic F127/PLA (B-F127–PLA) nanoparticles and their *in vitro* release behaviors were examined. The cytotoxicity tests using human ovarian cancer cells (OVCAR-3, SKOV-3) were performed to study the targeting properties of pac-loaded B-F127-PLA nanoparticles. Targeting was based on a three-step biotin–avidin targeting approach using biotinylated anti-CA125 antibody specific for the CA-125 antigen that is highly expressed on OVCAR-3 cells but not expressed on SKOV-3 cells. The targeting behaviors of B-F127–PLA nanoparticles were further confirmed by Fluorescence Microscopy technique through labeling a fluorescence dye to the PLA end of B-F127–PLA block copolymers. The intracellular fate of B-F127–PLA nanoparticles was also investigated by FM. The *in vivo* antitumor behaviors of pac-loaded B-F127–PLA nanoparticles were also explored so as to further prove the feasibility of B-F127–PLA nanoparticles as targeting drug carriers.

2. Materials and methods

2.1. Materials

Pluronic F127 was kindly supplied by BASF Corporation. Paclitaxel was kindly supplied by Fujian Nanfang Biotechnology Inc. L-lactide was purchased from Sigma–Aldrich and recrystallized twice from ethyl acetate (EtAc). The purified L-lactide was stored at 4–5 °C under argon environment. D-biotin, stannous octoate ($Sn(Oct)_2$), sodium phosphotungstate and avidin was purchased from Sigma–Aldrich and used as received. *N,N′*-Dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP) was purchased from J&K Chemica and used as received. Tetramethylrhodamine-5-carbonyl azide (TMRCA) was purchased from Molecular Probes. Hoechst 33342 and $DiOC_{18}(3)$ (DiO) were purchased from Beyotime institute of Biotechnology (Haimen, China). Biotinylated anti-CA125 antibody, MAB X306 were purchased from Hytest (Turku, Finland) and used as received. MTT was purchase from Solarbio and used as received. Dulbecco's Modified Eagle's Medium (DMEM) was purchase from Gibco and used as received. All other chemicals were of reagent grade. Human ovarian cancer cells OVCAR-3 and SKOV-3 were purchased from CICAMS, Beijing.

2.2. Synthesis of B-F127–PLA block copolymers

The detailed synthesis procedures of B-F127–PLA block copolymers have been described in detail elsewhere (Li et al., 2010). Briefly, B-F127–PLA block copolymers were synthesized by two steps. F127 was firstly modified by biotin. B-F127–PLA block copolymers were then synthesized by ring opening polymerization of the monomer L-lactide using B-F127-OH as the initiator and $Sn(Oct)_2$ as the catalyst. PLA segment was attached to one end of F127 by ring-opening polymerization to obtain amphiphilic block copolymers B-F127–PLA.

2.3. Paclitaxel loading studies

B-F127–PLA copolymer (24 mg) and hydrophobic drug pac (1.5 mg) were dissolved in THF. The solution with polymer and pac was added drop-wise to distilled water (45 g) under gentle stirring and THF was removed under reduced pressure. The drug loaded polymer aggregates in water were dialyzed against distilled water using a dialysis membrane (molecular weight cut-off, MWCO 12,000–14,000 Da) to remove excess drug molecules outside the particles.

In order to determine the loading efficiency and loading capacity, a part of the pac-loaded B-F127–PLA nanoparticles solution in the dialysis membrane was lyophilized and then dissolved in THF. The amount of pac entrapped was determined by measuring the UV absorbance at 252 nm using the UV–visible spectrophotometer (Perkin Elmer UV–vis Spectrophotometer Lambda 35, USA). A calibration curve was obtained using THF as the solvent. The loading efficiency and loading capacity of pac into B-F127–PLA nanoparticles was calculated as follows:

$$\text{Loading efficiency} = \frac{\text{Pac in nanoparticles (mg)}}{\text{Total pac in fomulation (mg)}} \quad (1)$$

$$\text{Loading capacity} = \frac{\text{Pac in nanoparticles (mg)}}{\text{Nanoparticles recovered (mg)}} \quad (2)$$

2.4. In vitro release studies

The pac-loaded B-F127–PLA nanoparticles solution (0.5 mg/ml, 10 g) were added into the dialysis membrane and then placed in phosphate buffered saline (PBS, pH 7.4, 0.01 M) aqueous solution (100 g). The whole solution was then placed in a shaking water bath at 37 °C for the drug release study. At predetermined time intervals, released pac solution (5 ml) outside the dialysis membrane was withdrawn and measured by HPLC to determine the concentration of pac. The fresh PBS solution (5 ml) was added to replenish the sample that was removed in order to maintain a constant volume. A Agilent HPLC Series 1100 equipped with a UV–vis detector set at 227 nm, connected with a Zorbax Eclipse XDB-C18 column, acetonitrile/PBS aqueous solution (50/50 v/v) as the mobile phase, and a flow rate of 1 ml/min was used.

2.5. In vivo antitumor efficacy

2.5.1. Cell cultures

OVCAR-3 and SKOV-3 cells were cultured in DMEM supplemented with 2.0 mmol/l glutamine, 10% fetal bovine serum (FBS), 100 μg/ml streptomycin sulfate and 100 U/ml penicillin at 37 °C in humidified 5% CO_2.

2.5.2. Three-step targeting to ovarian cancer cells and in vitro cytotoxicity studies

OVCAR-3 cells were seeded on 96-well plates with a cell density of 3×10^4 cells/ml for 8 h. Following this, the medium was removed and the following compounds were added by three steps. Firstly, cells were incubated with biotinylated MAB X306 (50 μg/ml) for 1 h; secondly, cells were incubated with avidin (50 μg/ml) for 20 min; thirdly, cells were incubated with pac-loaded B-F127–PLA nanoparticles at pac concentrations between 0.01 and