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cRGD-functionalized mPEG-PLGA-PLL nanoparticles for imaging and therapy of breast cancer

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ABSTRACT

Cyclic peptide (arginine-glycine-aspartic-glutamic-valine acid, cRGD)-modified monomethoxy (polyethylene glycol)-poly (D,L-lactide-*co*-glycolide)-poly (L-lysine) nanoparticles (mPEG-PLGA-PLL-cRGD NPs) with antitumor drug Mitoxantrone (DHAQ) or fluorescence agent Rhodamine B (Rb) encapsulated in their interior were prepared. The remarkable features of the mPEG-PLGA-PLL-cRGD NPs are the effective improvement for the cytotoxicity and uptake of the cell *in vitro*, and the significant enhancement of delivery ability for DHAQ or Rb *in vivo*. As a consequence, an excellent therapeutic efficiency for cancer is obtained, demonstrating the mPEG-PLGA-PLL-cRGD NPs play a key role in enhancing cancer therapeutic efficiency.

1. Introduction

The NPs for drug delivery have attracted great attention and offer a promising cancer therapy strategy [1], as its properties of increasing drug concentration within the tumor and minimizing the drug-induced toxic side effects on normal tissues, etc. Methods to deliver the drug-loaded NPs to tumor tissues have focused on both passive and active targeting. The NPs can be passively delivered to the tumor site utilize size-dependent properties and the enhanced permeability and retention (EPR) effect [2]. To further enhance targeting efficiency, the NPs are designed to selectively target to tumor through the addition of a targeted moiety, such as a peptide [3] or antibody [4], which actively bind to a receptor that is overexpressed onto tumor cells [5,6].

Despite much work in the application of the NPs for tumor therapy were done, demonstrations of the specific delivery and therapy of MDA-MB-231 breast cancer are few. Furthermore, *in vivo* or *in vitro* studies of NPs has circumvented many challenges, including good biocompatibility [7], stably controlled delivery [8,9] and intracellular tracking [10,11], etc. Solving these problems will be key to enhance the tumor targeting efficiency and promote the application of NPs.

For this, we designed and synthesized the mPEG-PLGA-PLL-cRGD copolymer (Scheme 1), and prepared the mPEG-PLGA-PLsL-cRGD NPs consist of the following integrated advantages: 1) an mPEG molecule that can prolong the circulation of NPs within the body by minimizing their recognition by the reticuloendothelial system (RES) [12]. 2) a PLGA compontent that currently possess US Food and Drug Administration (FDA) approval for use in a variety of biomaterials applications, which can be used as drug carriers for hydrophobic or hydrophilic drugs and has a high drug loading as well as encapsulation efficiency [13,14]. 3) a PLL component that has a number of amino groups in its side chain and can be easily modified by the targeted moiety to adjust and improve the performance of the carrier [15,16]. 4) a cRGD peptide that is a targeted moiety and can effectively and specifically bind with the $\alpha_v\beta_3$ integrins [17–19], which have a high level of expression on tumor cells such as MDA-MB-231 [20,21] and MDA-MB-435 breast cancer cells [22] that are critical for tumor growth [23,24].

Within the construct, DHAQ or Rb is encapsulated into the mPEG-PLGA-PLL-cRGD NPs to form DHAQ-mPEG-PLGA-PLL-cRGD NPs or Rb-mPEG-PLGA-PLL-cRGD NPs for the application of tumor targeted therapy or imaging in MDA-MB-231 breast cancer-bearing mice. The specific internalization into MDA-MB-231 breast cancer cells and improved therapeutic efficiency are then observed and evaluated *in vitro* and *in vivo*. The main purpose is to confirm the above advantages of the constructed mPEG-PLGA-PLL-cRGD NPs.

2. Materials and methods

2.1. Materials

mPEG (M_n = 2000) was obtained from the Sigma–Aldrich Company (Shanghai, China). D,L-lactide and glycolide were purchased from GLACO (Beijing, China), and stannous octoate [$Sn(OCt)_2$] was purchased from the Zhixing Chemical Company (Shanghai, China). The N^{ε}-(carbonylbenzoxy)-L-lysine, N-*t*-butoxycarbonyl-L-phenylalanine (Boc-L-Phe) and cRGD were synthesized by GL Biochem (Shanghai, China). The N^{ε}-(Z)-lysine-N-carboxyanhydride (NCA) was synthesized and purified using the

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method reported by Dorman et al. [25]. N,N-Carbonyldiimidazole (CDI) was purchased from the Acros (China) Co., Ltd. (Shanghai, China). Pluronic™F68 (F68) was obtained from BASF (China) Co., Ltd. (Shanghai, China), and DHAQ (Production code: M-200810002) was purchased from the Chongqing Carelife Pharmaceutical Co., Ltd. (Chongqing, China). MTT, Rb, Paraformaldehyde (PFA) and dihydrochloride 2-(4Amidinophenyl)-6-indolecarbamidine (DAPI) were obtained from the Sigma–Aldrich (China) Co., Ltd. (Shanghai, China). The anti-alpha-tubulin antibody and the anti-mouse FITC antibody were purchased from Earthox LLC (San Francisco, USA).

The MDA-MB-231 breast cancer cells obtained from the Shanghai Cancer Institute were grown in DMEM medium (Paisley, UK) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified environment containing 5% CO_2.

Nude female Balb/c mice (5 weeks old, body weight: 14.71 ± 1.82 g) were supplied by the Shanghai Cancer Institute (Shanghai, China). All animal procedures were performed according to the research protocol approved by the Animal Care and Use Committee at the Shanghai Cancer Institute.

2.2. Synthesis of the mPEG-PLGA-PLL-cRGD copolymer

The mPEG-PLGA-PLL-cRGD copolymer (M_n = 11,000, mPEG-PLGA-PLL : cRGD = 1:1, mol:mol) were synthesized [26] according to the following steps: 1) synthesis of the hydroxyl-terminated mPEG-PLGA through ring-opening polymerization (ROP) of D,L-lactide and glycolide, which was initiated by mPEG and catalyzed by $Sn(OCt)_2$. 2) synthesis of Boc-L-Phe end-capped mPEG-PLGA through the hydroxyl end-group of the mPEG-PLGA copolymer converted to Boc-L-Phe. 3) synthesis of amino-terminated mPEG-PLGA through the removal of the *t*-Butoxycarbonyl end-group from the Boc-L-Phe end-capped mPEG-PLGA. 4) synthesis of mPEG-PLGA-poly(N^{ε}-(Z)-L-lysine) through ROP of the initiated NCA by the amino-terminated mPEG-PLGA. 5) synthesis of amino-terminated mPEG-PLGA-PLL through the removal of the N^{ε}-(carbonylbenzoxy) end-group of the mPEG-PLGA-poly(N^{ε}-(Z)-L-lysine) block copolymer. 6) synthesis of mPEG-PLGA-PLL-cRGD by conjugating cRGD onto the amino-terminated mPEG-PLGA-PLL using CDI.

2.3. Preparation and physicochemical characteristics of the NPs

The DHAQ-mPEG-PLGA-PLL-cRGD NPs were prepared using the emulsion-evaporation method [27]. Briefly, DHAQ solution (0.2 mg in 20 μL aqueous solution) was emulsified in mPEG-PLGA-PLL-cRGD solution (4 mg in 200 μL dichloromethane solution) by sonicating (400 W, 10 s × 4). Subsequently, 2.2 mL F68 aqueous solution (1 mg/mL) was rapidly added to the first emulsion and sonicated (400 W, 10 s × 4). The resultant emulsions were stirred to evaporate the dichloromethane and were lyophilized.

The size of the NPs was measured using an H-7000 transmission electron microscope (TEM) from Hitachi, Ltd. (Tokyo, Japan) operated at an acceleration voltage of 200 kV, and the size distribution and zeta potential of the NPs in aqueous solutions were determined using a Nicomp™-380ZLS zeta potential analyzer from Particle Sizing System, Inc. (Florida, USA).

The encapsulation efficiency was defined by the ratio of the amount of DHAQ encapsulated in the NPs to the total amount of DHAQ initially used. The drug loading efficiency was expressed as the ration of the amount of DHAQ encapsulated in NPs to the total amount NPs initially used. Briefly, the 2 mL NPs solution were centrifuged (14,000 rpm/min × 30 min) at 4 °C, and the supernatants were then assayed spectrophotometrically at 610 nm using a TU-1901 ultraviolet–visible spectrophotometer (Beijing, China).

2.4. Drug release

The drug release of the NPs was evaluated using the dialysis method. Briefly, 3 mg DHAQ-mPEG-PLGA-PLL NPs and DHAQ-mPEG-PLGA-PLL-cRGD NPs solution (3 mg in 2 mL pH 7.4 PBS) were separately suspended in a dialysis bag from Spectrum Laboratories, Inc. (Georgia, USA) (molecular weight cutoff: 7000) and was then immersed into PBS (18 mL) and was kept at 37 °C under horizontal shaking (120 rpm/min). At predetermined intervals, the aliquots (2 mL) were withdrawn and replaced by the same amount of PBS. The amount of DHAQ released at different time point was measured at 610 nm with an ultraviolet–visible spectrophotometer. This experiment was carried out under the conditions of protection from light.

2.5. Cellular cytotoxicity

The cellular cytotoxicity of the NPs was determined using the MTT assay. Briefly, the MDA-MB-231 breast cancer cells (1×10^5/well) were cultured in 96-well plates and incubated for 24 h. The mPEG-PLGA-PLL NPs, mPEG-PLGA-PLL-cRGD NPs, DHAQ, DHAQ-mPEG-PLGA-PLL NPs and DHAQ-mPEG-PLGA-PLL-cRGD NPs of different concentrations were added and incubated for 24 h, 48 h or 72 h at 37 °C. Subsequently, 0.1 mL MTT (0.5 mg/mL) was added and incubated for an additional 4 h at 37 °C. The culture medium was then removed from the wells and replaced with DMSO (0.1 mL). The absorbance was measured at the wavelength of 490 nm using a 680 model microplate reader from Bio-Rad Laboratories (California, USA).

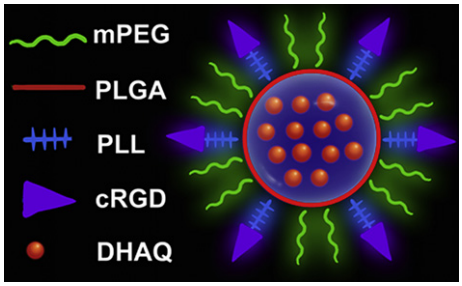

Fig. 1. Structure model diagram of mPEG-PLGA-PLL-cRGD NPs.

2.6. Cellular uptake

DHAQ was used as a fluorescence probe to assess the cellular uptake of the NPs. MDA-MB-231 breast cancer cells (2×10^5/well) were seeded in 24-well plates and incubated for 24 h. The DHAQ-mPEG-PLGA-PLL NPs and DHAQ-mPEG-PLGA-PLL-cRGD NPs were added to the wells at equivalent DHAQ concentration, and the cells were incubated for an additional 2 h. After removing the supernatant, the cells were fixed with 4% PFA for 20 min and then DAPI was added for a 10-min incubation. Subsequently, the cells were washed 3 times with PBS and sealed with glycerine. The cellular uptake of the NPs was determined using a FV1000 confocal laser scanning microscope (CLSM) from Olympus Corpoartion (Tokyo, Japan).

2.7. Cellular localization

To further investigate the cellular localization of the NPs, MDA-MB-231 breast cancer cells (2×10^5/well) were seeded in 24-well plates and incubated for 24 h. The DHAQ-mPEG-PLGA-PLL-cRGD NPs were added, and the cells were incubated for an additional 2 h. The cells were then washed 3 times with PBS and fixed for 20 min with 4% PFA. Subsequently, the cells were stained with DAPI for 10 min, with the alpha-tubulin antibody for 60 min and the anti-mouse FITC antibody for 60 min. Lastly, the cells were washed and sealed with glycerine. The cellular localization of the NPs was observed using CLSM.

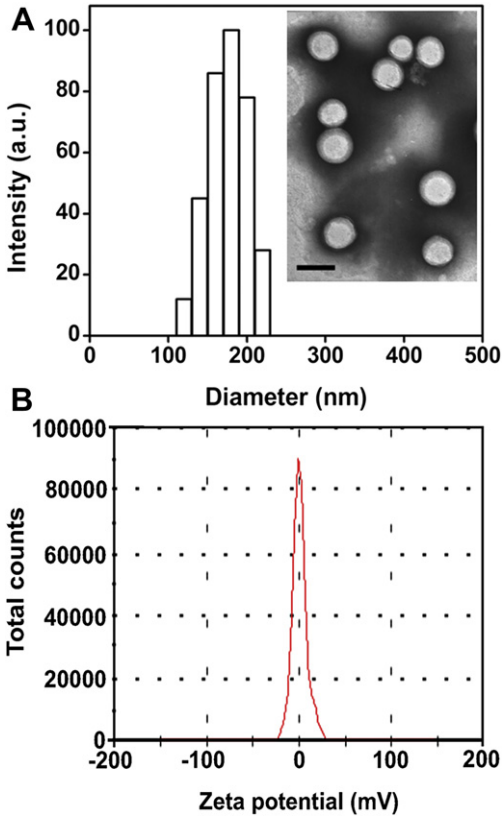

Fig. 2. TEM (inset image), particle size distribution (A) and zeta potential (B) images of the DHAQ-mPEG-PLGA-PLL-cRGD NPs. Scale bar is 200 nm.