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Targeted osteosarcoma chemotherapy using RGD peptide-installed doxorubicin-loaded biodegradable polymeric micelle

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ABSTRACT

Osteosarcoma is the most common primary malignant bone tumor in the pediatric age group, and chemotherapy directed by targeted nanoparticulate drug delivery system represents a promising approach for osteosarcoma treatment recently. Here, we designed and developed a novel DOX-loaded targeted polymeric micelle self-assembled from RGD-terminated poly(ethylene glycol)-*block*-poly (trimethylene carbonate) (RGD-PEG-PTMC) amphiphilic biodegradable block copolymer, for high-efficiency targeted chemotherapy of osteosarcoma. Notably, the RGD-installed DOX-loaded biodegradable polymeric micelle (RGD-DOX-PM) with drug loading efficiency of 57%–73% displayed a narrow distribution (PDI = 0.05–0.12) with average sizes ranging from 46 to 73 nm depending on the DOX loading content. The release amount of DOX from RGD-DOX-PM achieved 63% within 60 h under physiological condition. Interestingly, MTT assays in MG-63 and MNNG/HOS osteosarcoma cells exhibited that half-maximal inhibitory concentration (IC_{50}) value of RGD-DOX-PM was much lower than its non-targeted counterpart (DOX-PM), implying RGD decorated nanoparticles had enhanced cell targeting ability and led to more effective anti-tumor effect. Furthermore, the targeting ability of RGD-DOX-PM was confirmed by *in vitro* flow cytometry and confocal laser scanning microscopy (CLSM) imaging assays, where the results showed more RGD-DOX-PM were taken up by MG-63 cells than that of DOX-PM. Therefore, this RGD decorated DOX-loaded polymeric micelle is promising for targeted chemotherapy of osteosarcoma.

1. Introduction

Osteosarcoma, which is the most frequent occurring malignant bone cancer mostly in children and adolescents between 10 and 20 years of age, ranking among the leading causes of cancer-related death in the pediatric age group [1–5]. Osteosarcoma is arising from mesenchymal bone forming tissue and its histological hallmark is the production of malignant osteoid [6]. In clinical practices, surgery followed by chemotherapy regimens of high-dose methotrexate with leucovorin rescue, doxorubicin (DOX), cisplatin, and ifosfamide is the standard treatments for osteosarcoma, which could achieves a 5-year event-free survival of 60–70% in extremity [7]. It should be noted, however, associated toxic side-effects of these chemotherapeutic drugs, have extremely limited its therapeutic indexes [8,9].

Recently, nanoparticulate drug delivery system (NDDS), especially ligand-directed targeted NDDS, has been widely studied as a powerful platform for treatment of cancer [10–12], and achieved great success. Targeted NDDS loaded with chemotherapeutic drugs could not only reduces the toxic side-effects of chemotherapeutic agents by minimizing non-specific normal tissue accumulation, but also strengthens tumor targeting by enhanced permeation and retention (EPR) effect [13–15], and improving affinity between nanoparticles and membranes of treated cells. Taking the merits of targeted NDDS, several ligand-installed nanosystems with loading of chemotherapeutic drugs have been designed and explored for osteosarcoma treatment. For example, Kopecek's group used D-aspartic acid octapeptide as both hydrophilic micellar corona and effective targeting agent, forming targeted pH-sensitive DOX conjugate micelles as potential osteosarcoma therapeutics [16]. Anada et al. explored a novel type of calcium phosphate-binding liposome by conjugation of bisphosphonates as bone-targeting ligand and achieved reduced human osteosarcoma MG-63 cells viability [17]. Ni et al. described a salinomycin-loaded polymeric nanoparticle conjugated with

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CD133 aptamers, demonstrated active cell targeting ability and significantly inhibited the osteosarcoma growth by killing CD133$^+$ osteosarcoma cancer stem cells [18].

Arg-Gly-Asp (RGD), a cell-affinitive peptide is able to interact with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which are widely expressed in osteosarcoma cell lines such as MG-63 and MNNG/HOS cells [19,20]. The affinity of RGD towards osteosarcoma cells brings inspiration to Sun et al. [21] and Grigore et al. [22] incorporated RGD motifs into hydrogels to generate cellular adhesion sites to enhance osteocytes (MG-63 was used as model cell line in their reports) spreading and differentiation for guiding tissue formation, as well as Haubner et al. used ^{18}F-labeled RGD-containing glycopeptide for murine osteosarcomas imaging [23]. However, to the best of our knowledge, few RGD-associated nanosystems were explored for targeted chemotherapy of osteosarcoma. Herein, in this study, we designed and developed DOX-loaded targeted polymeric micelle self-assembled from RGD-terminated poly (ethylene glycol)-*block*-poly (trimethylene carbonate) (RGD-PEG-PTMC) amphiphilic block copolymer, for high-efficiency targeted osteosarcoma chemotherapy (Fig. 1). PEG-PTMC defines as excellent biodegradable and biocompatible biomedical polymer and was successful developed as micellar nanomedicine for a library of cancer chemotherapy [24–26]. Intriguingly, our results in *in vitro* studies showed that this RGD-installed DOX-loaded polymeric micelle (RGD-DOX-PM) displayed enhanced cell uptake ability relative to its non-targeted counterpart (DOX-PM), leading to increased osteosarcoma cells death and demonstrating high potential for targeted chemotherapy of osteosarcoma.

2. Materials and methods

2.1. Materials

Methoxy poly(ethylene glycol) (MeO-PEG-OH, $M_n = 5.0$ kg/mol, PDI = 1.03, Fluka) and Maleimide activated poly(ethylene glycol) (Mal-PEG-OH, $M_n = 5.0$ kg/mol, PDI = 1.02, Jenkem technology Co., Ltd, China) were dried by azeotropic distillation from anhydrous toluene. Toluene and dichloromethane (DCM) were dried by refluxing over CaH_2 and distilled prior to use. Trimethylene carbonate (TMC, Duoyu biotechnology Co., Ltd., China) was recrystallized by ethyl acetate before polymerization. Diphenyl phosphite (DPP, TCI), RGD peptide (GCGYGRGDSPG, 98%, China Peptides Co., Ltd.), Amberlyst A21 macroporous resin (Aladdin), doxorubicin hydrochloride (DOX·HCl, >99%, Beijing Zhongshuo Pharmaceutical Technology Development) were used without any disposing after received.

2.2. Characterization

The chemical structures of polymers were characterized using ^{1}H NMR on a Unity Inova 400 spectrometer operating at 400 MHz or Agilent DD2 operating at 400 MHz. Pre-saturation pulse sequence (zgpr), spectral width of 4795.4 Hz, 16,384 data-points, number of scans of 98 and relaxation delay of 1 s were utilized for spectra acquisitions. The chemical shifts were calibrated against residual solvent signal. The molecular weight and polydispersity of copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC) instrument equipped with two linear PLgel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector. The measurements were performed using DMF as an eluent at a flow rate of 1.0 mL/min at 30 °C and a series of narrow polystyrene standards for the calibration of the columns. The thermal properties of the copolymers were determined by differential scanning calorimetry (DSC, Q20, TA). The samples were heated from 80 °C to 120 °C at a rate of 20 °C/min, kept at 120 °C for 2 min, cooled to −80 °C at a rate of 300 °C/min, kept at −80 °C for 2 min, and then a second heating scan from −80 °C to 120 °C at a rate of 20 °C/min was recorded. The second heating curves were used to determine the T_g. The size and size distribution of micelles were determined at 25 °C using dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern Instruments) equipped with a 633 nm He-Ne laser using back-scattering detection. The measurements were performed in triplicate. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 120 kV. The samples were prepared by dropping 10 μL of 0.5 mg/mL polymeric micelle suspension on the copper grid followed by staining with phosphotungstic acid (1 *wt.*%). The CLSM images were taken on a confocal laser scanning microscope (TCS SP5).

2.3. Synthesis of PEG-PTMC and RGD-PEG-PTMC block copolymers

PEG-PTMC was synthesized by ring-opening polymerization of TMC using MeO-PEG-OH as a macro-initiator and diphenyl phosphite as a catalyst. Briefly, in a glove-box under a nitrogen atmosphere, diphenyl phosphite (250 mg, 1.07 mmol) was quickly added to a stirred solution of MeO-PEG-OH ($M_n = 5.0$ kg/mol, 0.5 g, 100 μmol) and TMC (0.5 g, 4.9 mmol) in DCM (6.0 mL). The reaction vessel was sealed, placed into an oil-bath thermostated at 30 °C and stirred for 4 days. PEG-PTMC copolymer was absorbed by macroporous resin to remove excess catalyst and then twice precipitation from cold diethyl ether, filtrated and dried in vacuo at room temperature (r.t.). Yield: 89.4%.

RGD-PEG-PTMC diblock copolymer was synthesized *via* two steps. Firstly, Mal-PEG-PTMC was acquired as described above except that Mal-PEG-OH ($M_n = 5.0$ kg/mol) was used as a macro-initiator. Yield: 87.9%. Secondly, Mal-PEG-PTMC reacted with RGD peptide. In brief, Mal-PEG-PTMC (60 mg, 6.06 μmol) and RGD (13 mg, 12 μmol) were dissolved in 3 mL of DMF and the reaction was proceeded for 24 h at 30 °C. Finally, RGD-PEG-PTMC was isolated through dialysis (Spectra/Pore, MWCO 7000) against DMF

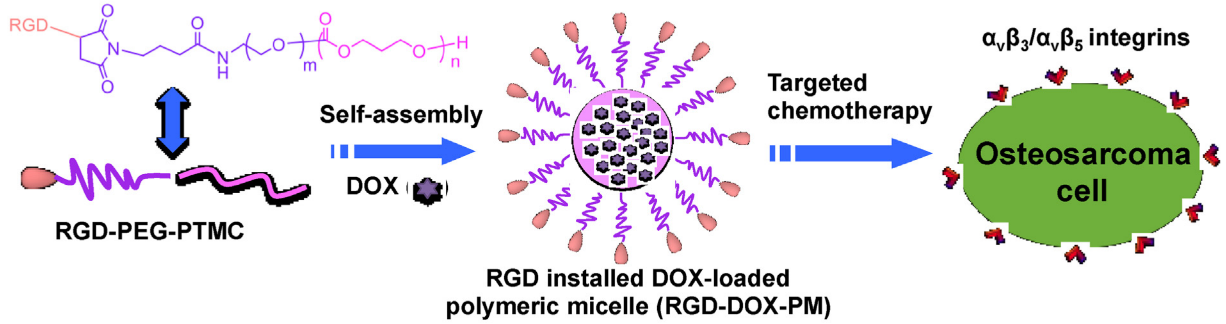

Fig. 1. Schematic illustration of RGD-DOX-PM formation process and RGD-DOX-PM used for targeted osteosarcoma chemotherapy.