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Anti-tumor activity of paclitaxel through dual-targeting carrier of cyclic RGD and transferrin conjugated hyperbranched copolymer nanoparticles

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ABSTRACT

Targeted delivery strategies are becoming increasingly important. Herein, a novel hyperbranched amphiphilic poly[(amine-ester)-co-(D,L-lactide)]/1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine copolymer (HPAE-co-PLA/DPPE) with RGD peptide (cRGDfK) and transferrin (Tf) on the periphery was synthesized and used to prepare paclitaxel-loaded nanoparticles (NPs) for dual-targeting chemotherapy. These NPs show satisfactory size distribution, high encapsulated efficiency and a pH-dependent release profile. The intrinsic fluorescence of the hyperbranched copolymer renders the detection and tracking of NPs *in vitro* and *in vivo* conveniently. *In vitro* cytotoxicity studies proved that the presence of cRGDfK enhanced the cytotoxic efficiency by 10 folds in $\alpha_v\beta_3$ integrin over-expressed human umbilical vein endothelial cells, while Tf improved cytotoxicity by 2 folds in Tf receptor over-expressed human cervical carcinoma cells. The drug-loaded NPs can be efficiently transported into the vascular endothelial cells and the target tumor cells. These results indicate that the cRGDfK and Tf decorated HPAE-co-PLA/DPPE could deliver chemotherapies specifically inside the cell via receptor-mediated endocytosis with greater efficacy. Therefore, such a fluorescent nanocarrier prepared from non-cytotoxic and biodegradable polymers is promising for drug delivery in tumor therapy.

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1. Introduction

Chemotherapy is essential in the treatment of malignant tumors, however, its efficacy is usually limited due to the poor physiochemical properties, low stability, short circulating half-life, and the toxicity to normal tissues associated with the anti-tumor drugs. Paclitaxel (PTX), a major chemotherapeutic drug extracted from the bark of *Taxus brevifolia*, is widely used to treat patients with lung, ovarian, breast cancer and advanced forms of Kaposi's sarcoma [1,2]. However, its high hydrophobicity and serious side effects greatly limit its clinical application [3]. Drug delivery systems have been investigated for many years and among them, encapsulating drugs into the hydrophobic core of self-assembled polymer micelles in an appropriate size range is a promising alternative. Polymeric micelles are thermodynamically stable, and the hydrophilic shell enhances its stable dispersion in aqueous solution by a steric stabilization effect, which assists its long-term blood duration following intravenous injection [4]. Moreover, polymeric nanoparticles (NPs) can target tumors by either a passive or active process. Passive targeting indicates that NPs can enter the leaky endothelial tissue that surrounds the tumor and accumulate in certain solid tumors by the well-known enhanced permeation and retention (EPR) effect [5]. Active targeting is delivering drugs to a specific site in terms of molecular recognition with a suitable ligand which can recognize its receptor on the targeting site [6]. Among all the neoplastic targeting ligands that are presently under investigation, the RGD sequence and transferrin (Tf) are two popular ones used in drug delivery systems [6].

The RGD sequence, an arginine-glycine-aspartic acid (RGD) tripeptide, has been proved to be an efficient binding motif to assist interactions between drug delivery systems including NPs and some integrins which mediate binding between cells and proteins of the extracellular matrix [7]. Among these integrins, integrin $\alpha_v\beta_3$ was proved to be over-expressed on the angiogenic endothelium in malignant or diseased tissues [8]. Histological analysis of breast cancer biopsy tissue also exhibited that $\alpha_v\beta_3$ was an important marker of blood vessels in the most malignant tumors, making it an attractive target for anti-angiogenesis strategy [9–11]. Various peptides containing RGD sequence have been developed to be ligands of $\alpha_v\beta_3$ integrin for therapeutical applications. cRGDfK

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peptide, cyclic (arginine-glycine-aspartic acid-phenylalanine-lysine), is usually chosen as it can alternatively bind to the $\alpha_v\beta_3$ integrin receptors with high affinity [12].

Transferrin (Tf) is a human serum glycoprotein (~80 kDa) involved in the delivery of ferric ions throughout the body. Tf is transported into cells through a receptor-mediated endocytosis via the transferrin receptor (TfR) [13]. Many studies have indicated that the expression level of TfR on tumor cells is much higher than that on normal cells [14,15], which has been widely utilized for targeting drug delivery systems as a novel potential approach [6,16,17].

Versatile polymeric vehicles have been widely used for drug delivery. Dendritic polymers, including dendrimers and hyperbranched polymers, have a highly branched structure, intramolecular voids, small rheological volumes and lower viscosity in solution, providing a high density of functional groups at the periphery [18,19]. Previously we first synthesized a series of amphiphilic hyperbranched poly[(amine-ester)-co-(D,L-lactide)] (HPAE-co-PLA) and HPAE-co-PLA/DPPE copolymers that were used as carriers for both hydrophilic and hydrophobic drugs, achieving improved water solubility, high entrapment efficiency and controlled release *in vitro* [20,21]. As DPPE is a phospholipid with both high biocompatibility and high flexibility in the lipid bialyer of cell membrane, the HPAE-co-PLA/DPPE copolymers show better absorbability than HPAE-co-PLA [21–23].

In present work, we aim to construct a dual-targeting PTX-loaded NPs based on HPAE-co-PLA/DPPE copolymer, which was modified with two targeting ligands, RGDfK and Tf. RGDfK has high affinity with the $\alpha_v\beta_3$ integrin, which is a primary marker of tumor vessels and Tf is specific ligand for TfR, which is over-expressed on tumor cells. Thus, these dual-targeting NPs may achieve more accumulation and improved lethality of the PTX-loaded NPs in tumors (Scheme 1C). Passive targeting is achieved by extravasation of NPs through enhanced permeability of the tumor vasculature. Active tumors targeting can be achieved in two steps: the ligand RGD enhances the targeting migration and accumulation of NPs to the $\alpha_v\beta_3$ integrin-expressing tumor vasculature and Tf then improves the cellular uptake of NPs by TfR-expressing tumor cells. In addition, a heterobifunctional cross-linker, p-maleimidophenyl isocyanate (PMPI), used for hydroxyl to sulfhydryl coupling should be introduced to the HPAE-co-PLA/DPPE copolymer for the successful modification of targeting ligands [24,25].

In present paper, we firstly constructed the PMPI-HPAE-co-PLA/DPPE copolymer, and then two target ligands, RGDfK and Tf, were linked to form the tumor-targeting drug nanocarrier. NPs loaded with PTX were prepared by the emulsion/solvent evaporation method and characterized for surface morphology, size distribution, drug encapsulation efficiency, and *in vitro* release. Finally, the cytotoxicity and cellular uptake of PTX-loaded NPs against human umbilical vein endothelial cells (HUVECs) and human cervical carcinoma (HeLa) cells were evaluated for their tumor-targeting effects using the CCK-8 assay, confocal laser scanning microscopy (CLSM), and flow cytometry.

2. Materials and methods

2.1. Materials

1, 1, 1-Trimethylolpropane and methyl acrylate purified by vacuum distillation and diethanolamine were purchased from National Medicines Chemical Reagent Co. Ltd (Shanghai, China). Titanium tetraisopropoxide (Ti(OiPr)$_4$), benzoic anhydride and imidazole were purchased from Beijing Reagent Factory (Beijing, China). D, L-Lactide (DLLA), Transferrin (Tf), 2-iminothiolane hydrochloride (Traut's reagent), 5, 5-Dithiobis (2-nitrobenzoic acid) (Ellmann's reagent) was obtained from Alfa Aesar (Ward Hill, MA, USA). 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) was purchased from Avanti Polar Lipids, Inc (Alabaster, Alabama, USA). Sn(Oct)$_2$, 4-nitrophenyl chloroformate (pNP) (97%), 4-dimethylaminopyridine (DMAP) (99%), p-maleimidophenyl isocyanate (PMPI) and dibutylin dilaurate (DBTDL) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Paclitaxel (PTX) was purchased from Beijing HuaFeng Unite Co, Ltd (Beijing, China). Commercial Cell Counting-8 (CCK-8) Kits were purchased from Dojindo Laboratories (Japan). RGDfK-SH was purchased from ChinaPeptides Co, Ltd (Shanghai, China). All other reagents and solvents were of analytical grade.

Human Umbilical Vein Endothelial cells (HUVECs) and human cervical carcinoma (HeLa) cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL) and streptomycin (100 U/mL).

2.2. Synthesis and characterization of dual-targeting and fluorescent copolymers

The fluorescent PMPI-HPAE polymer was firstly obtained by reacting partial surface hydroxyl groups of HPAE (G4) with isocyanate groups from PMPI, a heterobifunctional cross-linker used for hydroxyl to sulfhydryl coupling. Then, PMPI-HPAE-co-PLA copolymers were prepared with a ring-opening polymerization method. The PMPI-HPAE-co-PLA/DPPE copolymers were afterward synthesized by the reaction of activated PMPI-HPAE-co-PLA-pNP and DPPE. Finally, the targeting copolymers were obtained by conjugating either Tf or RGDfK to the PMPI-HPAE-co-PLA/DPPE copolymers (Scheme 1A).

2.2.1. Synthesis of fluorescent PMPI-HPAE-co-PLA/DPPE copolymers

Hyper-branched poly(amine-ester) polymers (HPAE-OHs) were synthesized at 120 °C by alcoholysis through a pseudo-one-step process using 1,1,1-trimethylol propane (as a molecular core) and N,N-diethylol-3-amine methylpropionate (as an AB_2 monomer) with Ti(OiPr)$_4$ as the catalyst, according to our previous study [20,26]. The generation of HPAE-OHs was increased by repeatedly adding *N,N*-diethylol-3-amine methylpropionate monomer to the former reaction product. The fourth-generation product (*i.e.* HPAE-OHs4) was prepared by repeating the process three times.

The PMPI-HPAE polymer was synthesized by reacting partial surface hydroxyl groups of HPAE-OHs4 with isocyanate groups of PMPI [25]. Briefly, 500 mg of HPAE-OHs4 was dissolved in 4 mL dimethyl sulfoxide (DMSO), then 40 μL DBTDL and 400 μL triethylamine (TEA) were added. 20 mg of PMPI was dissolved in 1 mL DMSO and added to the HPAE-OHs4 solution. The mixture was stirred at 45 °C, under nitrogen flow, in darkness for 24 h. The obtained solution was precipitated in 150 mL cold ethyl acetate (EA), washed further with EA three times to remove the residual DMSO and catalyst, and dried at room temperature in a vacuum oven, and stored at −20 °C.

PMPI-HPAE-co-PLA copolymer at a molar ratio of 4:1 (DLLA/PMPI-HPAE-OHs4) was synthesized as previously described [26]. DLLA and PMPI-HPAE-OHs4 were put into a flask and Sn(Oct)$_2$ was added at about 0.1% (w/w). After 1 h of evacuation by a vacuum pump at 30 °C, the flask was sealed and heated to 140 °C in an oil bath. 13 h later, the reaction solution was cooled down to room temperature. The obtained viscous product was dissolved in methylene chloride (DCM) and precipitated with petroleum, and further washed three times with petroleum. After that, the petroleum was evaporated and the product was then dissolved in acetone and precipitated with deionized water. Finally, the purified PMPI-HPAE-co-PLA was dried in a vacuum oven at room temperature for 48 h.

PMPI-HPAE-co-PLA/DPPE copolymer was synthesized in two steps according to our previous study [20,27] with small changes. Firstly, the PMPI-HPAE-co-PLA was activated with pNP. 500 mg of PMPI-HPAE-co-PLA was dissolved in 5 mL chloroform by magnetic stirring and was pre-cooled in an ice bath for 20 min. 200 mg of pNP, 20 mg of DMAP and 0.5 mL pyridine were then added to the solution. The mixture was determined to react for 6 h at 0 °C and 10h at 25 °C under magnetic stirring. The obtained product was evaporated to partly remove the chloroform and precipitated with diethyl ether/petroleum ether (1:1, v/v). The precipitate was washed with the diethyl ether/petroleum ether for three times and dried in a vacuum oven at room temperature for 48 h. Secondly, PMPI-HPAE-co-PLA/DPPE copolymer was synthesized by reaction of DPPE and the above activation of PMPI-HPAE-co-PLA copolymer (PMPI-HPAE-co-PLA-pNP) with mass ratio of 1:15 (DPPE/PMPI-HPAE-co-PLA-pNP). The dehydrated PMPI-HPAE-co-PLA-pNP was dissolved in 5 mL chloroform and then DPPE solution containing 0.1 mol TEA was added dropwise. The reaction continued with magnetic stirring at room temperature under nitrogen in the absence of light for 24 h. After this time, the resulting product was evaporated to remove part of chloroform and precipitated with diethyl ether/petroleum ether (1:1, v/v). The precipitated PMPI-HPAE-co-PLA/DPPE was then washed three times with the diethyl ether/petroleum, dried in a vacuum oven at room temperature for 48 h and eventually stored at −20 °C as a powder.

The chemical structures of the polymers were detected with Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) spectral analysis. FT-IR spectra of the polymers were recorded on a spectrophotometer (Perkin–Elmer, Fremont, CA, USA) using KBr as a reference. ^1H NMR, ^{13}C NMR and ^{31}P NMR spectra of the polymers were obtained by a Bruker AVANCE 400 NMR spectrometer (Billerica, MA, USA). Molecular weight and molecular weight distribution were detected with permeation chromatography apparatus (Waters 2410, Milford, MA, USA). The fluorescence spectra of PMPI-HPAE-co-PLA/DPPE were detected by an LS 55 Fluorescence Spectrometer (Perkin–Elmer, Fremont, CA, USA)

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