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Protocols

Amino-functionalized nano-vesicles for enhanced anticancer efficacy and reduced myelotoxicity of carboplatin

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ABSTRACT

Carboplatin is one of the few platinum-based antitumor drugs used worldwide. Unfortunately, systemic carboplatin therapy is limited by its dose-related myelosuppression. In this study, a viable way to reduce the serious myelotoxicity of carboplatin while improving its therapeutic efficacy using amino-functionalized polyphosphazene vesicles is reported. First, three polyphosphazenes with different amino content were synthesized by amidating hydrophobic side groups; these polyphosphazenes could self-assemble into polymersomes with similar particle size and morphology. Based on FTIR analysis, hydrogen bonding interactions between the polymeric carriers and carboplatin were validated and shown to predominantly contribute to the significant improvement of drug loading in polymersomes. Therefore, it turned out that the amino level of polymers played a substantial role in carboplatin encapsulation. Then, PEAP-2-C polymersomes were optimized for further experimental verification to achieve depressed carboplatin-induced myelotoxicity as well as promote curative effects against CT-26 colon adenocarcinoma. These results *in vitro* and *in vivo* proved that amino-functionalized polyphosphazene vesicles have great potential for carboplatin delivery in the application of cancer therapy.

1. Introduction

Carboplatin (cis-diamine (1,1-cyclobutanedicarboxylato)-platinum (II)), as a second-generation antineoplastic drug, is widely recommended for chemotherapy against ovarian, head, neck, lung, colon, breast, cervix, testis, and digestive system cancers [1–6]. However, severe carboplatin-induced myelotoxicity can hardly be avoided [7,8], potentially overshadowing its prominent treatment effect [9]. So far, however, only a few attempts have been made to achieve the goal of reducing myelosuppression. The most commonly used method for reducing myelosuppression has been coadministration of carboplatin with possible protective agents, such as corticosteroids [10], norepinephrine [11], and amifostine [12]. Unfortunately, these adjuvants might introduce other non-ignorable toxicities such as masked septicemia [13], emesis and hypotension [12]. In addition, the major limitation on the curative effect of carboplatin is strongly related to its low uptake by tumor cells because of its molecular structure [14]. Therefore, finding a better avenue that could improve the efficacy of carboplatin and simultaneously depress its myelotoxicity *in vivo* is of great significance.

In recent decades, nanoparticles in various forms have been extensively explored as vehicles for chemotherapeutic delivery by taking advantage of their efficient protection of drugs, prolonged circulation time, tumor targeting, and better endocytosis by tumor cells [15–17]. Based on this concept, we assumed that encapsulating carboplatin in nanoparticles not only might boost its clinical effects but also reduce its myelosuppression especially in virtue of tumor targeting. Several papers have constructed micelles composed of polymers containing inherent carboxylate groups such as polyamine acids [18,19] or terminal carboxylate groups. For example, poly(amidoamine) dendrimers (PAMAM) [20,21] were conjugated with cisplatin, which resulted in relatively high drug loading content (LC). For carboplatin, however, there is no literature about forming complexes with polymers. It is even quite difficult to fabricate any carboplatin-loaded formulations because it is nearly insoluble in any common organic solvent and sparingly soluble in aqueous solutions (approximately 40 mg mL^{-1}) [22]. Considering its unique solubility, poly(D-L-lactide-co-glycolide) (PLGA) [22,23] and polymethylmethacrylate [24] nanoparticles were chosen to load carboplatin, but the drug LC was quite low, less than 0.35%

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and 0.86%, respectively. Therefore, achieving efficient carboplatin loading and delivery via nanoparticles is presently a big challenge.

To solve these tough problems, here we designed a novel amphiphilic amino-functionalized polyphosphazene, poly[(methoxy-poly(ethylene glycol)) (*N*-[bis(2-amino ethyl) ethyl amino] amino phenyl amide) phosphazene] (poly[(mPEG)(AEPA)phosphazene], PEAP), for carboplatin delivery. The introduction of AEPA groups into PEAP was intended to facilitate drug loading by means of the considerable hydrogen interaction between the primary amines of AEPA and the ester groups of carboplatin. Furthermore, we previously validated that amphiphilic graft polyphosphazenes with relatively high hydrophobic side groups tended to form self-assembled vesicles [25]. Therefore, by regulating the graft ratio of mPEG to AEPA, PEAP was supposed to become vesicle-like nanoparticles, which would further favor loading of hydrophilic carboplatin. We first synthesized PEAP-1 and PEAP-2 with different primary amine contents and investigated the effect of the chemical structure of PEAP on carboplatin loading capacity using non-amino-containing poly[(methoxy-poly(ethylene glycol)) (ethyl-p-aminobenzoate) phosphazene] (poly[(mPEG)(EAB) phosphazene], PEEP) as a control [25,26]. Then, we conducted a series of experiments *in vitro* and *in vivo* based on the PEAP-2 polymersomes. The results certified that encapsulation by PEAP-2 could not only efficiently enhance the cellular uptake of carboplatin by tumor cells, significantly prolong its half-life *in vivo*, and ultimately improve drug efficacy *in vivo* but also ease its most serious myelotoxicity by raising three routine blood indexes.

2. Materials and methods

2.1. Reagents, cells and animals

Hexachlorocyclotriphosphazene (Acros Organics, Morris Plains, USA) was used after purifying by sublimation at 90 °C. PEG_{2000} (Fluka, St. Louis, USA) was used by azeotropically distilled with dry toluene. Ethyl-p-aminobenzoate (EAB) (Acros Organics, Morris Plains, USA) and tris (2-aminoethyl) amine (TAA) (Aladdin, Shanghai, China) were used directly without further purification. Carboplatin was obtained from Zhejiang Haizheng Pharmaceutical Co., Ltd. (Hangzhou, China).

Murine colon adenocarcinoma cells CT-26 were supplied by the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences (Shanghai, China). Cells were incubated in RPMI-1640 medium (Gibco BRL, Grand Island, USA.) supplemented with 10% fetal bovine serum (Sijiqing Biologic, Hangzhou, China), streptomycin at 100 μg/mL, and penicillin at 100 units/mL. The cells were maintained in a humidified 5% CO_2 atmosphere at 37 °C, and were used in logarithmic growth phase for all cell experiments.

Five-to-six-week-old female Balb/c mice (SLAC Laboratory Animal Co. Ltd, Shanghai, China) were maintained in a sterile laboratory environment. All the animal experiments were done under the international legal and institutional guidelines and approved by the Institutional Animal Care and Use Committee of Zhejiang University.

2.2. Synthesis and characterization of polymers

Firstly, PEEP was prepared by successively grafting amino-terminal PEG_{2000} (PEG_{2000}-NH_2) and EAB onto poly(dichlorophosphazene) backbone through nucleophilic substitutions as illustrated in Fig. 1. Specifically, PEG_{2000}-NH_2 (synthesized by PEG via a two-step reaction. 1.60 g, 0.80 mmol) solution in dry toluene (30 mL) containing equivalent mole freshly distilled triethylamine (TEA) was dripped slowly into poly(dichlorophosphazene) (0.50 g, 8.62 mmol P—Cl bonds) in dry toluene (30 mL) and reacted overnight by magnetically stirring at 30 °C. Then an excess of EAB (1.40 g, 8.47 mmol) together with equivalent mole dry TEA in dry toluene (30 mL) was added dropwise into the reaction mixture with further 24 h stirring at 50 °C. The product, abbreviated as PEEP ($[NP(PEG)(EAB)]_n$), was collected and purified by precipitation in ether twice and dialyzing (MWCO 8.0-14.0 kDa, Spectrum Laboratories, Laguna Hills, USA) against pure water for 2 days. Secondly, PEAP-1 and PEAP-2 were prepared by changing partial or all EAB groups to AEPA via amidation reaction of PEEP with different amount of TAA. For PEAP-1, PEEP (0.50 g, containing 0.72 mmol EAB) solution in *N*,*N*-dimethylformamide (DMF, 10 mL) was added slowly into TAA (0.06 g, 0.43 mmol, which was 60% mole of EAB) in DMF (5 mL) and reacted overnight by stirring at 70 °C. For PEAP-2, the synthesis procedure was the same as that of PEAP-1 except the use of an excess of TAA (0.30 g, 2.16 mmol). The products abbreviated as PEAP-1 ($[NP(PEG)(EAB)(AEPA)]_n$), and PEAP-2 ($[NP(PEG)(AEPA)]_n$), were purified and collected by dialyzing (MWCO 8.0–14.0 kDa) against pure water and freeze-drying.

The chemical composition of the PEEP, PEAP-1 and PEAP-2 were validated separately by infrared spectroscopy using a FTIR spectrometer (VECTOR 22, Bruker, Germany) and ^1H NMR spectra using a NMR spectrometer (Avance III 500, Bruker, Germany) with $CDCl_3$. The molecular weights of the polymers were determined by gel permeation chromatograph (GPC, PL-GPC 220, Polymer Laboratories Ltd., Epsom, UK) dissolved in DMF with narrow-disperse polystyrene as the calibration criterion.

2.3. Preparation and characterization of blank and carboplatin-loaded nanoparticles

The nanoparticles of PEEP, PEAP-1, and PEAP-2 were prepared by a simple dialysis method. In detail, for blank nanoparticles, PEEP, PEAP-1 or PEAP-2 was dissolved at 20 mg/mL concentration in DMF, and added dropwisely into purified water at 1:1 volume ratio. Then, the solution was transferred into a dialysis bag (MWCO 8.0–14.0 kDa) and dialyzed against distilled water for 6 h with frequent water change. As DMF was gradually removed, the blank nanoparticles were formed. And the nanoparticle powders were collected after freeze drying. The same way was applied for the carboplatin-loaded nanoparticles except that polymers (20 mg/mL) and carboplatin (0.4 mg/mL) were dissolved in DMF separately and mixed afterwards. And the carboplain-loaded nanoparticles were abbreviated as PEEP-C, PEAP-1-C, PEAP-2-C, respectively.

The morphologies of nanoparticles were examined using a transmission electron microscopy (TEM, JEM-1230, JEOL Ltd., Akishima, Japan). Each sample was obtained by dipping a formvar-coated copper grid into the nanoparticle (5 mg/mL), blotting with filter paper, and drying in air. In order to further confirm the morphologies, liposoluble cell membrane dye DiI (Beyotime, Shanghai, China) and water-soluble FITC-dextran (Sigma Aldrich, St. Louis, USA) were loaded into PEEP, PEAP-1, PEAP-2 using dialysis method at a ratio of 1:20 separately. The samples were transferred to slides with 50% glycerin. Red fluorescence of DiI and green fluorescence of FITC were observed using a super-resolution structured illumination microscopy (SIM; Nikon, Japan). The hydrodynamic diameters of the nanoparticles dissolved in purified water at 2 mg/mL were measured by dynamic light scattering (DLS, Malvern Nano-ZS 90, Malvern Instrument, Malvern, UK). The drug payloads were analyzed by HPLC (LC-20AT, Shimadzu, Kyoto, Japan) using a 5 μm ODS C18 column (250 × 4.6 mm) after the carboplatin-loaded nanoparticles were destroyed using acetonitrile. The mobile phase consisted of 5% acetonitrile and 95% deionized water with a flow rate of 0.5 mL/min. Carboplatin was quantified by UV detection at a wavelength of 229 nm at a retention time of 4.8 min. The drug