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Constructing aptamer anchored nanovesicles for enhanced tumor penetration and cellular uptake of water soluble chemotherapeutics

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ABSTRACT

Polymersomes represent a promising pharmaceutical vehicle for the delivery of hydrophilic therapeutic agents. However, modification of polymersomes with molecules that confer targeting functions remains challenging because of the strict requirements regarding the weight fractions of the hydrophilic and hydrophobic block polymers. In this study, based on the compatibility between cholesterol and polymeric carriers, polymersomes self-assembled by amphiphilic graft polyphosphazenes were endowed with a targeting function by incorporating the cholesterol-linked aptamer through a simple dialysis method. The aqueous interior of the polymersomes was employed to encapsulate water-soluble doxorubicin hydrochloride. *In vivo* experiments in tumor-bearing mice showed that the aptamer-anchored vesicle targeted accumulation at the tumor site, favorable penetration through tumor tissue, and incremental endocytosis into tumor cells. Correspondingly, the aptamer-anchored vesicle decreased systemic toxicity and effectively suppressed the growth of subcutaneous MCF-7 xenografts. These findings suggested that vesicles modified with targeted groups via hydrophobic supermolecular interactions could provide a platform for selective delivery of hydrophilic drug.

Statement of Significance

Polymersomes have represented a promising type of pharmaceutical vehicles due to their predominant physical properties. However, it is still a challenge to endow polymersomes with active target function because of strict requirements of the weight fractions of hydrophilic polymer block to hydrophobic one. In this research, by taking advantage of the supermolecular interactions between amphiphilic graft polyphosphazene and cholesterol which was linked to aptamer AS1411, we prepared a targeted functional polymersome (PEP-DOX·HCl-Ap) through a simple method with high loading of water soluble anti-cancer drug doxorubicin hydrochloride. The *in vivo* experiments in MCF-7 tumor-bearing mice demonstrated several advantages of PEP-DOX·HCl-Ap vesicle such as prolonged circulation time in blood, targeted accumulation at tumor site, permeation through the tumor tissue and incremental endocytosis by tumor cells, which consequently resulted in the significantly improved anti-cancer efficacy. Moreover, this novel polymersome designed in this study has built a research platform to achieve targeted delivery of hydrophilic chemotherapeutics for cancer therapy.

1. Introduction

Polymersomes are nanometer-sized vesicles self-assembled from synthetic amphiphilic copolymers. In comparison with micelles, polymersomes can act as carriers for both hydrophilic and hydrophobic molecules because of their unique architecture with an aqueous interior and thick lamellar membranes. In addition, polymersomes have been regarded as an improved alternative to liposomes because of their advantageous physical properties, including greater mechanical strength, better colloidal stability, and longer circulation time [1–4].

Endowing drug-carrying nanoparticles with targeting functions improves treatment efficacy and reduces the occurrence of side

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effects in cancer chemotherapy [5–7]. Several types of micelles and liposomes with active targeting groups on surfaces have been reported [8–11]. However, endowing polymersomes with active targeting functions remains challenging, because the self-assembly behavior of polymersomes requires a particular ratio of hydrophilic to hydrophobic block fractions. Once ligands are conjugated to the hydrophilic chain of the polymersomes, and prior to aggregate formation, the amphiphile ratio of the polymers inevitably changes, leading to a change in the structural morphology of the assemblies (e.g. from vesicles to micelles) [12]. To prevent this type of interference with self-assembly, several approaches to attach ligands onto the polymersome surface after vesicle formation have been reported; for example, biotin-streptavidin binding [13–17] or azide-alkyne click chemistry [18,19]. Although such attachment approaches have been proven feasible, there are obstacles to their therapeutic application, including human intolerance to streptavidin and toxic effects owing to the possible presence of residue from the copper used to catalyze the alkyne-azide click reaction [20]. Moreover, the targeting efficiency of these ligand-polymersome systems has not been clearly verified *in vivo*. So, In this study we expected to construct a targeted polymersome through a simple method with the aim of avoiding any influence on its self-assembly behavior.

To achieve this goal, amphiphilic graft poly[methoxy-poly (ethylene glycol)/ethyl-*p*-aminobenzoate phosphazene]s (PEPs) with the ability to self-assemble into polymersomes in water were synthesized. Generally, polymersome self-assembled from poly (ethylene glycol)-polycaprolactone or poly(ethylene glycol)-polylactic acid [21,22] has a relative low payload for water-soluble doxorubicin hydrochloride (DOX·HCl). However, the PEP polymersomes loading DOX·HCl could achieve high drug loading content (LC) and encapsulation efficiency (EE) during the self-assemble process in water, which is an outstanding advantage for drug delivery and cancer treatment [23].

Aptamers are single-stranded RNA or DNA ligands that can specifically bind to various molecular targets with high affinity. Compared with other classes of ligands (peptides and small molecules), aptamers are easily modified via chemical synthesis *in vitro* and have less possibility to participate in systemic circulation for the negatively charged. In addition, they display high binding affinity to targets with dissociation constant (Kd) values in the nanomolar range [24] and show better tissue penetration [25]. Owing to these unique characteristics, aptamers have been used to modify nanoparticles for cancer therapy with some exciting results in recent studies [26–28]. AS1411 is a 26-mer DNA aptamer that has been confirmed to selectively bind to nucleolin [29], which is over-expressed on the membranes of human breast cancer MCF-7 cells but is absent in normal cells. This kind of particularly high affinity between aptamer AS1411 and nucleolin could be utilized for improving the endocytosis of nanoparticles into MCF-7 cells [30,31].

In this study, aptamer AS1411 was covalently linked to cholesterol, which served as a hydrophobic anchor to be firmly inserted into the hydrophobic EAB bilayer of PEP vesicles via supermolecular interactions. In this way, an aptamer-anchored DOX·HCl-loaded hybrid polymersome (PEP-DOX·HCl-Ap) was constructed. It is worth noting that the decoration of active targeting groups onto the polymersome surface can be accomplished during the self-assembly process of vesicle formation and drug loading in water. Also, it is a novel idea that take advantage of supermolecular interaction to modify the polymersome with targeted function. Based on these advantages of polymersomes and aptamers, we hypothesized that the PEP-DOX·HCl-Ap would accumulate at the tumor site, precisely recognized and be taken up by tumors cells, and consequently achieve satisfactory anti-cancer efficacy (Fig. 1). To validate this hypothesis, we performed the following experiments, including comprehensive *in vivo* studies.

2. Matrials and methods

2.1. Materials

Hexachlorocyclotriphosphazene (Acros Organics) was purified by sublimation at 80–90 °C. Methoxy-poly(ethylene glycol) (mPEG) (MW 2000, Acros Organics) was azeotropically distilled with toluene before use and amino-terminal mPEG2000 (mPEG2k-NH_2) was prepared in advance. Cholesterol-aptamer AS1411 (C-Ap, Ap sequence: 5′-GGT GGT GGT GGT TGT GGT GGT GGT GGT TTT TTT TTT TT-Cholesterol-3′) and Cholesterol-aptamer(mut) (C-Ap(mut), Ap sequence: 5′-TTC CTC CTC CTC CTT CTC CTC CTC CTC TTT TTT TTT TT-Cholesterol-3′) were synthesized by Sangon (Shanghai, China). All other reagents were commercially available.

Human breast tumor MCF-7 cells were kindly provided by the Cancer Research Center, Second Affiliated Hospital of Zhejiang University, China and were routinely cultured in RPMI-1640 medium containing 10% (v/v) fetal bovine serum and 100 IU/mL penicillin, 100 IU/mL streptomycin at 37 °C, 5% CO_2, and 95% humidity.

Five-to-six-week-old female BALB/c nude mice (20 ± 2 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Zhejiang University. The MCF-7 xenograft model was established by implanting with fragments of MCF-7 cancer subcutaneously in the right flank. Tumor volume was measured as width^2 × length × 0.5. When tumor volume reached about 100 mm^3, the mice were randomly divided into several groups for the pharmacokinetic study, *in vivo* biodistribution studies and *in vivo* tumor inhibition study.

2.2. Synthesis and characterization of poly[methoxy-poly(ethylene glycol)/ethyl-p-aminobenzoate phosphazene]s (PEPs)

For synthesis of PEPs, sequential substitution reactions were carried out between PEG2k-NH_2/EAB and chloride atoms on a poly(dichlorophosphazene) backbone according to our previous work [23]. The chemical compositions and formation of the PEPs were confirmed by ^{1}H NMR spectrometer (AC-80, Bruker Biospin., Germany) and Fourier transform infrared (FTIR) spectroscopy (FT/IR-4100, JASCO, Japan). The detailed information was shown in Supplementary data.

2.3. Preparation of aptamer anchored drug-loaded PEP vesicle

10 mg PEP copolymer was dissolved in DMF which was premixed with 1.5 mg DOX·HCl, followed by drop-wise addition of the same volume of C-Ap(mut) (0.8 OD) (a scrambled aptamer AS1411 conjugated with cholesterol) or C-Ap (0.8 OD) (an aptamer AS1411 conjugated with cholesterol) solution. Then, the mixed solution was dialyzed against distilled water. After DMF was gradually removed via dialysis, the PEP-DOX·HCl-Ap(mut) (a negative control) or PEP-DOX·HCl-Ap were formed. The obtained PEP-DOX·HCl-Ap(mut) or PEP-DOX·HCl-Ap solution was then filtered by 0.22 μm filter.

2.4. Confirmation of aptamer anchored PEP vesicle

Differential scanning calorimetry (DSC) was employed to evaluate the compatibility of PEP and cholesterol which was connected with aptamers. The thermograms of PEP, cholesterol and PEP-Chol mixture were acquired using a DSC-Q100 model (TA Instruments, UK). The PEP-Chol mixture (1:1) was fabricated by rorary-film evaporation method. The samples were sealed in aluminum pans