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Synergistic antitumor efficacy of redox and pH dually responsive micelleplexes for co-delivery of camptothecin and genes

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ABSTRACT

Challenges remain to load and deliver two or multiple drugs of complementary effects for synergistic cancer therapies. In the current study, multiarmed amphiphilic copolymers of 4-arm poly(ethylene glycol) (PEG) and polyaspartate (PAsp) are created for conjugation of camptothecin (CPT) and condensation with tumor necrosis factor-α (TNF) plasmids. Diethylenetriamine (DET) is grafted on PAsp, and CPT is conjugated onto PAsp(DET) by disulfide linkages to form hydrophobic cores of micelles, followed by condensation with TNF plasmids to form micelleplexes. The *cis*-aconitic linkers are introduced between PEG and PAsp(DET) to remove PEG shells in response to acidic pH, resulting in destabilized micelleplexes and prompted endosomal escape into the cytosol. The micelleplex disintegration in response to reductive stimuli in the cytosol leads to an efficient CPT release and pDNA disassociation. The co-delivery of CPT with TNF plasmids enhances the gene transfection of micelleplexes at low N/P ratios, and shows synergetic cytotoxicities to tumor cells with 2.5 and 8 folds lower IC_{50}s compared with those after treatment with CPT or TNF alone, respectively. The micelleplex treatment on 4T1 tumor models dramatically extends the animal survival and suppresses the tumor growth with 2.3 and 3 folds lower in volume compared with CPT or TNF treatment alone, respectively. Histological and biochemical analyses display TNF expressions in tumor tissues after micelleplex treatment, resulting in significantly larger necrotic regions in tumors, higher cell apoptosis rates, and no obvious sign of tumor metastasis in lungs compared with other treatment. Therefore, the multifunctional micelleplexes based on multiarmed PEG-PAsp(DET) copolymers offer the targeted drug/gene delivery, dually responsive drug/gene release and synergistic antitumor efficacy, holding great promises for combination therapies.

Statement of Significance

Micelleplexes are constructed from multiarmed amphiphilic copolymers with conjugation of captothecin (CPT) and condensation of tumor necrosis factor-α (TNF) plasmid. The pH/redox stimuli realize co-delivery of CPT and pDNA in a sequential manner of folate-mediated endocytosis, endosomal escape induced by PEG cleavage, reduction-sensitive release of CPT in cytosol, and pDNA release from disintegrated polyplexes after CPT release. Compared with CPT or TNF treatment alone, the micelleplexes achieve 2.5 and 8 folds higher cytotoxicities to tumor cells, and suppress the tumor growth with 2.3 and 3 folds lower in volume, respectively. It demonstrates a feasible strategy to develop multifunctional micelleplexes with simultaneous drug conjugation and pDNA condensation, dually responsive drug/gene release and synergistic antitumor efficacy, holding great promise for combinational therapies.

1. Introduction

Cancer is recognized as a highly complex disorder caused by numerous genetic changes and continuous uncontrolled growths of abnormal cells *via* different mechanisms. Consequently, the cancer treatment relying on a single antitumor mechanism is far from satisfactory. Combination therapies through a simultaneous delivery of two or more drugs with different anticancer mechanisms, have entered clinical practice, demonstrated as a promising strategy to enhance the therapeutic efficacy [1]. Thus, to achieve a selective accumulation of chemotherapeutic agents in tumor tissues and relieve the systemic toxicity of standard cancer therapies, it is essential to construct highly efficient drug carriers to delivery

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more than one therapeutic agent to the action sites of tumor cells [2]. One of the strategies is the separate delivery of multiple drugs in different carriers, indicating advantages in the independent control of the release rate and timing of drug actions. However, it is difficult to achieve an efficient and harmonious biodistribution and cellular uptake of multiple drugs in different carriers [3]. The incorporation of two or multiple drugs into one carrier can optimize the pharmacokinetics and biodistribution of drugs, overcome the batch-to-batch variability, and achieve a ratiometric dose of drugs at the targeted sites [4]. This strategy has demonstrated much higher delivery efficiencies and more significant antitumor effects than the conventional cocktail therapy *via* a sequential delivery of drugs in separate vehicles [3].

In addition to the commonly used cancer chemotherapeutics, gene therapy using nucleic acid therapeutics, *e.g.* small interfering RNA and plasmid DNA, has been proposed to achieve combined effects with chemotherapies [5]. Unfortunately, it is particularly challenging to design nanocarriers capable of simultaneously co-loading anticancer drugs and nucleic acids owing to their different physicochemical features. To tackle this difficulty, various carriers have been explored for co-delivery of nucleic acids and chemotherapeutics based on polymeric micelles, liposomes, mesoporous silica nanoparticles and dendrimers [6]. All the synthetic carriers, which in most cases are cationic liposomes, peptides or polymers, have to form stable complexes with nucleic acids to prevent the enzymatic degradation by nucleases in blood and destabilization by electrostatic interactions with serum proteins [7]. Ediriwickrema et al. synthesized multilayered polymer nanoparticles, comprising of camptothecin (CPT)-loaded poly(lactic-co-glycolic acid) nanoparticles and surface coated polyethyleneimine (PEI) for pDNA complexation [8]. Shi et al. prepared triblock copolymers of PEG-poly(ε-caprolactone) (PCL)-g-PEI, which were self-assembled into micelles with physical entrapment of doxorubicin in the hydrophobic cores. By subsequently introducing pDNA to form complexes with PEI, the micelles improved the antitumor efficiency in the treatment of lung metastasis [9].

To maximize the benefits of combination therapies, it is of urgent demand to construct nanocarriers endowed with an efficient cell-selective uptake and a stimuli-triggered release of both nucleic acids and chemotherapeutics at the targeting sites [10]. Various "intelligent" carriers in response to internal or external stimuli such as pH, redox, temperature and light have been actively pursued. Among them, pH and redox stimuli have gained the most attention, typically based on the cleavage of acid-liable bonds and the reduction of disulfide bonds by intracellular glutathione (GSH), respectively [11]. The pH-stimulated drug loading and release rely on acidic environments in cancerous tissues (pH 6.5–7.2), endosomes (pH 5.0–6.5) and lysosomes (pH 4.5–5.0) as compared to physiological pH of 7.4 in blood and normal tissues [12]. Guan et al. developed a co-delivery system by electrostatic binding of PEI-poly(L-lysine)-poly(L-glutamic acid) with *cis*-aconityl doxorubicin and pDNA, inducing an accelerated release of doxorubicin with the decreasing pH due to the acid-sensitive *cis*-aconityl linkage [13]. GSH is the most abundant reducing agent at a concentration range of 0.5–10 mM in the cytosol, while its concentration in blood and extracellular milieu is 100–1000 times lower [14]. Nam et al. synthesized paclitaxel-conjugated polymeric micelles, consisting of paclitaxel-conjugated PEG and arginine-grafted bioreducible poly(disulfide amine) for co-delivery of genes and drugs. The complexes showed a higher cytotoxicity in the reductive environment, an efficient cellular uptake and a higher anticancer potency than paclitaxel alone [15]. To date, only a few co-delivery nanocarriers in response to dual or multiple stimuli have been constructed to further improve drug performances. Chen et al. prepared ternary block copolymers consisting of PEG, pH-sensitive poly(2-(diisopropyl amino)ethyl methacrylate) and reduction-sensitive poly(N-(2,2′-dithiobis(ethylamine)) aspartamide), which were assembled into micelles by encapsulation of doxorubicin in the pH-sensitive cores and siRNA in the reduction-sensitive layers. The dual stimuli-responsiveness allowed microenvironment-specific releases of DOX and siRNA, which dramatically enhanced cell apoptosis and inhibited tumor growths [16]. In most cases, nonbiodegradable carriers and physical entrapment of anticancer drugs were employed in the co-delivery system.

Herein, biodegradable micelleplexes with pH and redox responsiveness were constructed from multiarmed amphiphilic copolymers with conjugation of captothecin (CPT) and condensation with tumor necrosis factor-α (TNF) plasmids. CPT exhibits high antitumor activities against a wide spectrum of human malignancies, but suffers from extremely low water solubility and pH-dependent structure disintegration [17]. TNF-α is identified as one of the apoptosis inducing agents to combat tumor cells, involving direct killing of tumor cells and promotion of tumor angiogenesis [18]. Compared with micelles with drug loading by physical entrapment, the micelles from CPT-conjugated polymers are supposed to achieve prolonged blood circulations, higher accumulations in tumor tissues, lower toxicities to normal tissues, and improved antitumor efficacies [19]. As illustrated in Scheme 1, the multiarmed amphiphilic carriers were constructed from 4-arm PEG, where folate moieties were grafted on 2 arms of PEG to enhance the specific endocytosis by cancer cells and the other 2 arms of PEG were conjugated with polyaspartate (PAsp) by pH sensitive linkers. Diethylenetriamine (DET) was further conjugated on PAsp, followed by coupling CPT onto a portion of PAsp(DET) *via* disulfide linkages to form hydrophobic cores of the micelles. In addition, the other portion of cationic PAsp(DET) side chains with a pKa value of 9.5 could form complexes with pDNA as the interlayer of micelleplexes to enhance the transfection efficiency and alleviate the enzyme degradation of pDNA.

The combined pH/redox stimuli were expected to achieve an on-demand delivery of CPT and pDNA in a sequential manner of folate-mediated endocytosis, endosomal escape induced by PEG cleavage and PAsp(DET) exposure, reduction-sensitive release of CPT in the cytosol, and pDNA release from disintegrated polyplexes after CPT release. Micelleplexes were supposed to remain the structural integrity in blood and tumor extracellular matrix. After uptake into endosome featured with considerably low pH, the cleavage of PEG shells from the micelleplexes resulted in an exposure of cationic PAsp(DET) and eventually facilitated the endosomal escape into the cytosol *via* the proton sponge effect. Subsequently, CPT was released from the polyplexes in response to enriched GSH in the cytosol, and the removal of hydrophobic CPT led to the polyplex disintegration and release pDNA [20]. More specifically, the micelleplex stability and pH/redox responsive release were investigated under different pH values and GSH concentrations. The cellular uptake, gene transfection efficiency, and synergistic antiproliferation efficacy were determined on breast carcinoma 4T1 cells. *In vivo* antitumor efficacy and the inhibition of tumor cell metastasis were evaluated on mice bearing 4T1 tumors.

2. Materials and method

2.1. Materials

The plasmid TNF, encoding fusion proteins of TNF-α/enhanced green fluorescent protein (eGFP) was received from Biowit Technologies Ltd. (Shenzhen, China). Propidium iodide, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dialysis membranes were obtained from Sigma–Aldrich (St. Louis, MO).