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Trilayer micelles for combination delivery of rapamycin and siRNA targeting Y-box binding protein-1 (siYB-1)

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ABSTRACT

A three layer (trilayer) polymeric micelle system based on the self-association of the triblock polymer poly(ethylene glycol)-b-poly{N-[N-(2-aminoethyl)-2-aminoethyl] aspartamide}-b-poly(ε-caprolactone) (PEG-b-PAsp(DET)-b-PCL) has been synthesized and investigated for combination delivery of rapamycin (RAP) and siRNA targeting Y-box binding protein-1 (siYB-1). The trilayer micelle is composed of (a) a hydrophilic poly(ethylene glycol) (PEG) block constituting the outer layer to improve pharmacokinetics, (b) an intermediate compartment composed of the cationic poly{2-[(2-aminoethyl)amino] ethyl aspartamide} (PAsp(DET)) segment for interacting with siYB-1, and (c) an inner hydrophobic poly(ε-caprolactone) (PCL) compartment for encapsulation of RAP. A major advantage of this system is biocompatibility since PEG and PCL are both approved by the FDA, and PAsp(DET) is a non-toxic pH responsive cationic poly(amino acid)-based polymer. In this study, it has been shown that PCL can encapsulate RAP with high loading efficiencies, and PAsp(DET) can successfully interact with siRNA for efficient transfection/knockdown with negligible cytotoxicity. The enhanced therapeutic efficacy of RAP/siYB-1 micelles was demonstrated in cell cultures and in a PC3 xenograft nude mouse model of human prostate cancer. Herein, we demonstrate that trilayer micelles are a promising approach to improve the simultaneous delivery of combination siRNA/drug therapies.

1. Introduction

Rapamycin (RAP) (914 g/mol) was originally used as an antifungal agent [1], and later developed as an approved immunosuppressant and potential anticancer drug [2–5]. Preclinical data with RAP revealed inhibition of tumor growth in a number of cell lines, including lung [2], cervix [4], colon [6], prostate [2,7], and breast [8] cell carcinomas with a typical $IC_{50} < 50$ nM. The anticancer mechanism of the drug involves blocking vascular endothelial growth factor (VEGF) production, stimulating endothelial cells [9,10] and inducing cell cycle arrest at the transition from G1-S phase [11,12]. In addition, RAP is the most commonly used chemical to induce autophagy, a lysosome-based pathway that is responsible for the degradation and recycling of proteins and intracellular components for maintaining cellular homeostasis [13]. Unfortunately, autophagy protects some cancer cells by blocking the apoptotic pathway [14], and further suggests that RAP as a cancer therapeutic be administered in combination with other drugs. In addition, the systemic delivery of RAP constitutes a major challenge in the field of cancer therapy due to the drug's poor solubility in water (2.6 μg/ml) [15], low bioavailability, and dose-limiting toxicity [16]. To date, only one successful formulation of RAP into poly(ethylene glycol)-b-poly(ε-caprolactone) (PEG-b-PCL) micelles has been reported [17].

Small interfering RNA (siRNA) targeting Y-box binding protein-1 (siYB-1) has attracted great interest as a therapeutic agent because of its ability to efficiently knockdown (kd) genes associated with cancer cell proliferation and multiple drug resistance (MDR) [18]. YB-1 is an oncogenic transcription/translation factor and regulates DNA- and mRNA-dependent events in eukaryotic cells [19–21]. YB-1 is also a pronounced marker of tumors because of its overexpression in cancer cells [22–25]. Moreover, YB-1 translocation from the cytoplasm to the nucleus stimulates transcription of a number of genes encoding protective proteins, including those responsible for MDR [26–28]. As a result, the inhibition of YB-1 expression has been found to sensitize cancer cells to chemotherapeutic drugs such as doxorubicin (DOX) [29] or paclitaxel (PTX) [30]. Likewise, we have found that the combination of siYB-1 with RAP greatly enhanced sensitivity of human PC3 prostate cancer cells to RAP.

As such, multifunctional nanoparticles (NP) are highly desirable for the delivery of effective drug combinations and the only means to ensure that both drugs will simultaneously reach cancer cells. In drug delivery, chemical and/or physical optimizations are typically required of the vehicle to efficiently deliver any single one

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therapeutic agent. Obviously, co-delivery of two or more variety of drugs (e.g. siRNA/drug or drug/drug) requires compartmentalization in the design of the NP to satisfy the different chemical properties of each drug. To date, there have been few drug delivery systems reported in the literature for the co-delivery of both drug and gene. The need for drug compartmentalization was demonstrated with cationic core–shell NP encapsulating PTX in the core and condensing either plasmid DNA (pDNA) or siRNA on the cationic surface [31]. In another drug compartmentalization system, NP were formed by conjugating DOX to poly(lactic-co-glycolide) (PLGA) polymers, and these in turn were entrapped within liposomes made of PEG-*block*-distearoylphosphatidylethanolamine (PEG-b-DSPE), phosphatidylcholine and cholesterol for optimal loading of combretastatin within the outer lipid bilayer (combretastatin is an anti-angiogenesis drug) [32]. Still, another design utilized NP made from PEG-b-PLGA polymers decorated with nucleic acid ligands (aptamers) to encapsulate docetaxel in the core and intercalate DOX within the aptamers at the surface [33]. Although promising, some of these systems rely on complicated NP assemblies that are ultimately inherently too complex to scale up.

In the present study, we report on the synthesis and characterization of three layer micelles formed from the triblock polymer PEG-b-PAsp(DET)-b-PCL for delivery of RAP and siYB-1. Each drug is encapsulated within a different compartment of the micelle (Fig. 1), and the design successfully incorporates biocompatibility, simultaneous loading of RAP and siYB-1 at high loading capacities, and relies on self-association of polymer chains to form NP. More specifically, the trilayer micelle is composed of (a) a hydrophilic PEG block constituting the outer layer to improve pharmacokinetics, (b) an intermediate compartment composed of the cationic PAsp(DET) segment for interacting with siYB-1, and (c) an inner hydrophobic PCL compartment for encapsulation of RAP. In addition, we also report on the therapeutic efficacy of combination RAP/siYB-1 micelles in cultured cells and in a PC3 xenograft mouse model of human prostate cancer.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals and biologicals

L-aspartic acid β-benzyl ester, triphosgene, diethylenetriamine (DET), benzene, anhydrous dichloromethane (DCM), hexane and N,N-dimethylformamide (DMF) were purchased from Sigma–Aldrich (Milwaukee, WI). Methoxy-ω-amino PEG (PEG5k-NH_2, Mn = 5 KDa, Mw/Mn = 1.04) was purchased from Polymer Science. Dialysis tubing (MWCO = 3500) was obtained from SpectraPor. The monomer ε-caprolactone (Alfa Aesar) was distilled over CaH_2 and used fresh. The catalyst trimethylaluminum ($AlMe_3$) was purchased from TCI (Portland, OR). The anticancer drug RAP was purchased from LC systems. For gel retardation studies, plasmid DNA (pDNA) coding for luciferase (pGL4) contained 5320 base pairs (Promega). For drug release studies, total RNA (tRNA) was isolated from mouse placenta by using a Qiagen RNA purification kit. For kd studies, siRNA against luciferase (siLuc) was obtained from Promega (Madison, WI). For cytotoxicity studies, siYB-1 (sequence was previously reported [30]) was obtained from IDT (sense: 5′-UUUGCUG-GUAAUUGCGUGGdTdT-3′; antisense: 5′-CCACGCAAUUACCAGCAAAdTdT-3′).

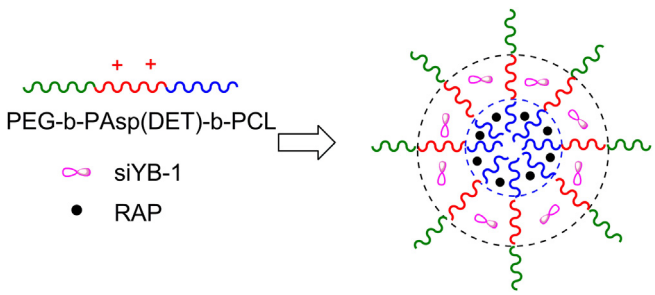

Fig. 1. The trilayer micelle is composed of (a) a hydrophilic PEG block constituting the outer layer to improve pharmacokinetics, (b) an intermediate compartment composed of the cationic PAsp(DET) segment for interacting with siYB-1, and (c) an inner hydrophobic PCL compartment for encapsulation of RAP.

2.1.2. Cell culture

Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), trypsin-EDTA (0.05% trypsin, 0.48 mM EDTA in HBSS) and penicillin-streptomycin were purchased from Cellgro (MediaTech, Herndon, VA). LNCap and PC3 are human prostate cancer cells obtained from ATCC and cultured according to ATCC protocols. LNCap cells were transduced with lentiviral vectors per standard protocols to generate a stable cell line expressing luciferase (LNCap-Luc).

2.1.3. Knockdown and cytotoxicity assays

For kd assays, the Luciferase Assay System was purchased from Promega (Madison, WI). Luminescence was measured with a single tube luminometer (Berthold Technologies). Protein content was obtained using the DC protein assay kit from BioRad (Hercules, CA), absorbance was measured with the Spectramax 190 microplate reader (Molecular Devices, Sunnyvale, CA), and results were fit to a known protein calibration curve. Cytotoxicity was assessed through the resazurin dye.

2.1.4. Animals

All aspects of the animal studies were performed in accordance with the guidelines defined by the Animal Research Committee of the University of Wisconsin. Nude male mice (7–8 week old) were obtained from Jackson Laboratory and divided into five groups (5 mice per group) for xenograft tumor studies. General anesthesia to animals was induced with 1.5% isoflurane/oxygen. Tumor volume (volume = $0.5 \times l \times w^2$) and animal body weights (bw) were monitored daily for 10 days.

2.1.5. Instruments

^{1}H NMR spectra were obtained with a Varian Unity-Inova 400 MHz NMR spectrometer (Palo Alto, CA), with temperature regulation at 80 °C or otherwise as indicated. Chemical shifts are reported in ppm, with respect to the deuterated solvent used. All polymers were characterized by ^{1}H NMR and gel permeation chromatography (GPC) (Shodex LF-804 GPC column) at every step of the synthesis.

2.1.6. Statistical analyses

Results were presented as mean ± SEM, $n = 3$–6. To compare between data sets, Graphpad Prism 5 Software was used to perform one way analysis of variance (ANOVA). A $p < 0.01$ and $p < 0.001$ were considered significant and denoted by * or ** respectively to indicate statistical differences.

2.2. Methods

2.2.1. Synthesis of poly(ethylene glycol)-block-poly(β-benzyl L-aspartate) or PEG-b-PBLA

The monomer β-benzyl-L-aspartate N-carboxy-anhydride (BLA-NCA) was prepared using the Fuchs-Farthing method, through the cyclization of β-benzyl-L-aspartic acid with triphosgene, followed with purification by crystallization in dry THF/hexane. Next, diblock polymer PEG-b-PBLA was prepared by the ring-opening polymerization of BLA-NCA initiated by the terminal $-NH_2$ of PEG5k-NH_2 (MW 5 kDa) as previously reported [34]. Typically, BLA-NCA (349 mg, 1.4 mmol) was dissolved in 1 ml DMF and 5 ml DCM, and added into PEG5k-NH_2 (200 mg, 0.04 mmol) dissolved in 5 ml DCM. The reaction was stirred at 35 °C under argon for 24 h. The mixture was then precipitated in diethyl ether three times, and freeze-dried with benzene to yield a solid white powder. The degree of polymerization (DP) and size distribution of the polymer were checked by ^{1}H NMR and GPC.

2.2.2. Synthesis of the intermediate triblock poly(ethylene glycol)-block-poly(β-benzyl l-aspartate)-block-poly(ε-caprolactone) or PEG-b-PBLA-b-PCL

PEG-b-PBLA was used as a macro-initiator for the ring-opening polymerization of ε-caprolactone. Generally, freshly distilled ε-caprolactone (166 mg, 1.45 mmol) was added to PEG-b-PBLA (200 mg, 0.016 mmol) dissolved in 10 ml DCM, followed by addition of 6 equivalent $AlMe_3$ (0.096 mmol). The reaction was allowed to proceed for 24 h at 40 °C. The organic solvent and $AlMe_3$ were then removed by rotovap under vacuum. The residues were re-dissolved in DCM, precipitated in diethyl ether, and freeze dried with benzene. The DP for the PCL block was determined by integrating the corresponding proton peak intensities of PCL (-OC$\underline{H}_2$(CH_2)$_4$CO–, $\delta = 4.0$ ppm) to the methylene protons of the PEG chain (–OC$\underline{H}_2$C$\underline{H}_2$–, $\delta = 3.5$ ppm) in DMSO-d_6 at 80 °C.

2.2.3. Synthesis of the final triblock polymer poly(ethylene glycol)-block-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide}-block-poly(ε-caprolactone) or PEG-b-PAsp(DET)-b-PCL

DET side chains were substituted into the PBLA segments of PEG-b-PBLA-b-PCL via an aminolysis reaction. Briefly, DET (148.56 mg, 1.44 mmol) was added to PEG-b-PBLA-b-PCL (180 mg, 0.008 mmol) dissolved in 10 ml DMSO, and the reaction

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