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Elastin-based protein polymer nanoparticles carrying drug at both corona and core suppress tumor growth *in vivo*

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ABSTRACT

Numerous nanocarriers of small molecules depend on either non-specific physical encapsulation or direct covalent linkage. In contrast, this manuscript explores an alternative encapsulation strategy based on high-specificity avidity between a small molecule drug and its cognate protein target fused to the corona of protein polymer nanoparticles. With the new strategy, the drug associates tightly to the carrier and releases slowly, which may decrease toxicity and promote tumor accumulation via the enhanced permeability and retention effect. To test this hypothesis, the drug Rapamycin (Rapa) was selected for its potent anti-proliferative properties, which give it immunosuppressant and anti-tumor activity. Despite its potency, Rapa has low solubility, low oral bioavailability, and rapid systemic clearance, which make it an excellent candidate for nanoparticulate drug delivery. To explore this approach, genetically engineered diblock copolymers were constructed from elastin-like polypeptides (ELPs) that assemble small (<100 nm) nanoparticles. ELPs are protein polymers of the sequence $(Val\text{-}Pro\text{-}Gly\text{-}Xaa\text{-}Gly)_n$, where the identity of Xaa and n determine their assembly properties. Initially, a screening assay for model drug encapsulation in ELP nanoparticles was developed, which showed that Rose Bengal and Rapa have high non-specific encapsulation in the core of ELP nanoparticles with a sequence where Xaa = Ile or Phe. While excellent at entrapping these drugs, their release was relatively fast (2.2 h half-life) compared to their intended mean residence time in the human body. Having determined that Rapa can be non-specifically entrapped in the core of ELP nanoparticles, FK506 binding protein 12 (FKBP), which is the cognate protein target of Rapa, was genetically fused to the surface of these nanoparticles (FSI) to enhance their avidity towards Rapa. The fusion of FKBP to these nanoparticles slowed the terminal half-life of drug release to 57.8 h. To determine if this class of drug carriers has potential applications *in vivo*, FSI/Rapa was administered to mice carrying a human breast cancer model (MDA-MB-468). Compared to free drug, FSI encapsulation significantly decreased gross toxicity and enhanced the anti-cancer activity. In conclusion, protein polymer nanoparticles decorated with the cognate receptor of a high potency, low solubility drug (Rapa) efficiently improved drug loading capacity and its release. This approach has applications to the delivery of Rapa and its analogs; furthermore, this strategy has broader applications in the encapsulation, targeting, and release of other potent small molecules.

1. Introduction

Rapamycin (Rapa) is a cyclic and hydrophobic macrolide antibiotic which was discovered from a product of *Streptomyces hygroscopicus* in a sample of soil from Easter Island [1]. Because Rapa has great potency in suppressing immune response by inhibiting proliferation of lymphocytes, its clinical applications have shifted from anti-fungal to anti-transplant rejection formulations such as Rapamune [2]. Recently, Rapa's anti-proliferation properties have been explored, which have led to the clinical observation of anti-tumor efficacy in malignancy of the breast, prostate, and colon [3–6]. Rapa's anti-proliferation mechanism has also been revealed — inhibition of mTOR (mammalian target of rapamycin) pathway. When bound to its cognate receptor FKBP (K_d = 0.2 nM) [7], Rapa inhibits the mTOR pathway and then sequesters cancer cells in G_1 phase [8]. mTOR has essential functions in cell proliferation and growth. Screening studies confirmed that cancer cell lines having overexpression of S6K1 and expression of phosphorylated Akt *e.g.* MDA-MB-468 breast cancer cell are sensitive to Rapa treatment [9].

Although Rapa is extremely potent in cancer treatment, a number of drawbacks such as severe cytotoxicity, low bioavailability and rapid clearance limit wider usage of free Rapa. Recent studies have shown that Rapa and other macrolide mTOR inhibitors have serious

Abbreviations: Rapa, rapamycin; ELPs, elastin-like polypeptides; FKBP, FK506 binding protein 12; mTOR, mammalian target of rapamycin; ITC, inverse transition cycling; CMT, critical micelle temperature; CMC, critical micelle concentration; DLS, dynamic light scattering; TEM, transmission electron microscopy; cryo-TEM, cryogenic-transmission electron microscopy.

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lung toxicity by causing interstitial pneumonitis [10]. Free Rapa has poor bioavailability because of its high hydrophobicity and low water solubility (ca. 2.6 μg/mL) [11]. As a result, organic solvents such as DMSO, polyethylene glycol (PEG) 400 and ethanol are presently used to deliver free Rapa [12]. However, most of these organic solvents are cytotoxic to the liver and kidney, and they may also cause hemolysis and acute hypersensitivity reactions [13,14]. It has also been determined that free Rapa has high tendency to partition into the erythrocytes which makes it more difficult to reach intratumoral targets [15]. Therefore, a well-designed Rapa formulation is currently in demand to overcome the limitations of this potent drug.

Derived from human tropoelastin, elastin-like polypeptides (ELPs) are repetitive protein polymers with the sequence of $(Val\text{-}Pro\text{-}Gly\text{-}Xaa\text{-}Gly)_n$, where Xaa is the guest residue and n is the length of the repetitive units [16]. ELPs undergo an inverse phase transition, which can be used to promote temperature-dependent self-assembly [17]. Below a tunable transition temperature (T_t), these ELPs are highly soluble. Above T_t they coacervate into a secondary aqueous phase, akin to a lower critical solution temperature. This phase separation can be used to purify ELPs and their fusion proteins by a process named inverse transition cycling (ITC). Here we explore two ELP diblock copolymers with a hydrophobic to hydrophilic length of n = 1:1 that form stable nanoparticles *e.g.* $G(Val\text{-}Pro\text{-}Gly\text{-}Ile\text{-}Gly)_{48}$ $(Val\text{-}Pro\text{-}Gly\text{-}Ser\text{-}Gly)_{48}Y$ and $G(Val\text{-}Pro\text{-}Gly\text{-}Phe\text{-}Gly)_{24}(Val\text{-}Pro\text{-}Gly\text{-}Ser\text{-}Gly)_{24}Y$, which are named I48S48 and F24S24 respectively. These diblock copolymers form nanoparticles that are potentially excellent drug carriers because: i) they are genetically engineered, which enables precise modification and fusion to proteins; ii) they can be biosynthesized efficiently in *E. coli*; iii) they form monodisperse nanoparticles that do not require electrostatic stabilization; iv) they are biodegradable into non-cytotoxic amino acid components. For example, the ELP nanoparticle I48S48 is effectively biodegraded in murine hepatocytes by elastase and collagenase endopeptidases without any obvious cytotoxicity [18]. With these advantages, we raised the hypothesis that drug (Rapa) encapsulation and release from ELP nanoparticles can be modulated by high-specificity avidity binding to the cognate protein receptor for the drug (FKBP) decorated at the nanoparticle surface. In such a formulation the drug can be encapsulated into the nanoparticle core via hydrophobic interactions, similar to other micelle delivery systems; however, it would also have specific drug binding capacity at its corona. The FKBP-bound Rapa was expected to remain tightly associated with decorated nanoparticles, which may promote tumor accumulation via the enhanced permeability and retention effect. To discover the capability of drug encapsulation using ELP nanoparticles, four different fluorescent small molecules were first screened for efficient encapsulation into the hydrophobic core of the I48S48 nanoparticle. Next, encapsulation and release experiments of the model drug Rose Bengal and the clinically-approved drug Rapa were performed using ELP micelles I48S48 and F24S24. Rapa has been previously demonstrated to bind competitively to an FKBP domain on an FKBP-ELP fusion protein. [19] To further enhance drug-specific encapsulation and improve drug release, the FKBP domain was genetically fused onto the corona of the nanoparticles formed from SI. This optimized construct FSI was then examined for Rapa encapsulation and release. Finally, cell proliferation assays and *in vivo* tumor regression studies were performed using FSI with encapsulated Rapa (FSI Rapa) and free Rapa in solvent (DMSO) to evaluate their relative toxicity and anti-tumor efficacy. These studies reveal an exciting new strategy for drug delivery and targeted encapsulation using genetically engineered nanoparticles.

2. Materials and methods

2.1. Materials and reagents

Rose Bengal, copper chloride, phosphate buffered saline (PBS) tablets, polyethylenimine (PEI), Congo Red, Thioflavin and Erythrosin were purchased from Sigma-Aldrich (St. Louis, MO). Rapamycin was ordered from LC Laboratories (Woburn, MA). TB dry growth medium was obtained from MO BIO Laboratories, Inc. (Carlsbad, CA). pET25b(+) vector and BLR (DE3) *E. coli* cell were ordered from Novagen Inc. (Madison, WI). MDA-MB-468 and MDA-MB-231 cells were purchased from the American Type Tissue Culture Collection. MDA-MB-468 cells were cultured at 37 °C humidified in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM)/F12 medium with 10% fetal bovine serum. MDA-MB-231 cells were cultured at 37 °C humidified in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum.

2.2. Biosynthesis of ELPs

To generate the ELPs evaluated during this study, synthetic genes encoding for both ELP mono and diblock copolymers were constructed (Table 1). BLR (DE3) *E. coli* cells were transformed with recombinant pET25b(+) vectors containing ELP genes [19,20]. The *E. coli* cells were incubated using 50 mL of TB dry growth medium with 100 μg/mL ampicillin overnight at a 37 °C orbital shaker. The culture was then centrifuged at 4000 rpm for 10 min and the pellet was re-suspended with 5 mL fresh TB dry growth medium. 500 μL of the re-suspended culture media was inoculated in 1 L TB dry growth medium with 100 μg/mL ampicillin and then incubated for 24 h at a 37 °C orbital shaker. The *E. coli* cells were harvested by being centrifuged at 4000 rpm for 15 min and then re-suspended in PBS and lysed by ultrasonication. The lysate was centrifuged at 12,000 rpm for 15 min to remove insoluble cell debris, and nucleic acids were precipitated by PEI (0.5% w/v final concentration) and removed by 12,000 rpm 15 min centrifugation. Inverse transition cycling (ITC), which has been described previously, was used to further purify the cell lysate containing ELPs [19,20]. In brief, ELP was heated to 37 °C and

Table 1
ELP protein polymers evaluated in this article.

ELP nomenclature	Amino acid sequence[a]	T_t (°C)[b]	Calculated ELP MW (Da)[c]	Observed ELP MW (Da)[d]
I48	$G(VPGIG)_{48}Y$	22.0	20,566.9	20,309.9
S192	$G(VPGSG)_{192}Y$	56.5	80,000.8	79,779.5
I48S48	$G(VPGIG)_{48}(VPGSG)_{48}Y$	27.0	39,643.6	39,435.5
F24S24	$G(VPGFG)_{24}(VPGSG)_{24}Y$	n.a.	20,757.3	20,493.9
SI	$G(VPGSG)_{48}(VPGIG)_{48}Y$	27.0	39,643.6	39,445.0
FSI[e]	$FKBP\text{-}G(VPGSG)_{48}(VPGIG)_{48}Y$	24.5	51,445.2	51,446.8

[a] ELP gene sequences confirmed by DNA sequencing from N and C termini and diagnostic digestions.
[b] Transition temperature (*Tt*) (25 μM, pH 7.4) for I48, S192; Critical micelle temperature (CMT) for I48S48, SI, and FSI; The CMT of F24S24 was not applicable (n.a.) as nanoparticles form below 4 °C (Fig. 1d).
[c] Estimated from open reading frame excluding methionine start codon.
[d] Results from matrix assisted laser desorption ion time of flight (MALDI-ToF) mass spectrometry.
[e] FKBP amino acid sequence: "MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE".