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Synthesis and characterization of a thermally-responsive tumor necrosis factor antagonist

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ABSTRACT

Numerous antagonists of tumor necrosis factor alpha (TNFα) have been developed to attenuate inflammation and accompanying pain in many disease processes. Soluble TNF receptor type II (sTNFRII) is one such antagonist that sequesters TNFα away from target receptors and attenuates its activity. Systemic delivery of soluble TNF receptors or other antagonists may have deleterious side effects associated with immune suppression, so that strategies for locally targeted drug delivery are of interest. Elastin-like polypeptides (ELPs) are biopolymers capable of *in situ* drug depot formation through thermally-driven supramolecular complexes at physiological temperatures. A recombinant fusion protein between ELP and sTNFRII was designed and evaluated for retention of bivalent functionality. Thermal sensitivity was observed by formation of supramolecular submicron-sized particles at 32 °C, with gradual resolubilization from the depot observed at physiological temperatures. *In vitro* refolding of the sTNFRII domain was required and the purified product exhibited an equilibrium dissociation constant for interacting with TNFα that was seven-fold higher than free sTNFRII. Furthermore, anti-TNF activity was observed in inhibiting TNFα-mediated cytotoxicity in the murine L929 fibrosarcoma assay. Potential advantages of this ELP-sTNFRII fusion protein as an anti-TNFa drug depot include facility of injection, *in situ* depot formation, low endotoxin content, and functionality against TNFα.

1. Introduction

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine that has been implicated as a key mediator of inflammation in multiple systemic and local disease processes including rheumatoid arthritis [1–3], inflammatory bowel disease [4,5], sepsis [6–8], and radiculopathy [9–11]. TNFα acts through two transmembrane receptors, TNFRI and TNFRII, that dissociate the cytotoxic and pro-inflammatory effects in target cells [12]. Soluble TNF receptors (sTNFRI and sTNFRII) are the extracellular domains of these membrane receptors that are liberated from the transmembrane domain by proteolytic cleavage. Upon removal, these truncated receptors become soluble proteins and retain the appropriate tertiary structure to act as decoy receptors sequestering TNFα away from the cell surface receptors [13,14]. Cleavage products of the Type I and Type II TNF receptors (sTNFRI and sTNFRII respectively) have 27.5% sequence identity [15] and have TNFα equilibrium dissociation constants of 1.2 nM and 0.35 nM [16].

Both TNFα-binding antibodies (infliximab and adalimumab) and molecules based on sTNFRI (pegsuncercept) or sTNFRII (etanercept) are used to sequester the cytokine away from target cell surface receptors in inflammatory disease [13,17,18]. These therapeutic high molecular weight conjugates shift clearance from renal to hepatic providing for longevity in the systemic circulation [13,19]. Such agents are delivered subcutaneously or intravenously causing systemic toxicity in patients due to immunosuppression [18]. While systemic diseases such as rheumatoid arthritis, Crohn's disease, and psoriasis demand parenteral treatment, local inflammation in monoarticular osteoarthritis or disc-herniation radiculopathy exhibit local upregulation of TNFα-activity and may benefit from local treatment [20]. A delivery system that sustains sTNFRII activity in a targeted compartment may increase therapeutic efficacy and minimize serum exposure and associated side effects. Soluble TNF receptors are complex proteins with tertiary structure of extensive disulfide bridging [21] requiring expression in *E. coli* to include *in vitro* oxidation–reduction refolding [22]. Purification of such agents, alone or conjugated to a carrier, has involved affinity purification with a TNFα-functionalized column [14,17,23].

Thermally-responsive elastin-like polypeptides (ELPs) have been evaluated as drug carriers to diarthrodial joints [24], dorsal root ganglia [25], and solid tumors [26]. ELPs consist of pentapeptide

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repeats of a Val-Pro-Gly-Xaa-Gly sequence with structural homology to mammalian elastin (Xaa is a guest residue other than proline) [27]. Aqueous solutions of these polymers exhibit inverse phase transition behavior: ELPs are soluble monomers below a characteristic transition temperature (T_t), but upon heating the solution above their T_t, they undergo a reversible hydrophobic association into micron-sized, supramolecular complexes. This property can be exploited to form drug depots *in situ* by delivering ELPs in solution at room temperature that spontaneously associate upon delivery to a local cavity at body temperature. Indeed, prior studies have demonstrated that an ELP designed to undergo thermal phase transition upon intra-articular injection exhibited a 25-fold increase in its intra-articular half-life compared to a soluble, non-transitioning ELP [24]. In other work on local delivery, a depot-forming ELP delivered to the dorsal root ganglion exhibited a seven-fold increase in local half-life compared to a soluble ELP of comparable molecular weight [25]. Furthermore, both studies revealed that systemic exposure to the depot-forming ELP was substantially decreased by the phase-transitioning property of the polymer. It remains unclear if this longevity will provide for greater or sustained activity of conjugated therapeutics delivered locally in a disease model. Local targeting after systemic delivery has also been accomplished by intravenous delivery of soluble ELPs with a T_t of 40 °C followed by application of local hyperthermia to trigger the phase transition and permit ELP phase separation and accumulation within a tumor [28,29]. These results support that the inverse phase transition behavior of an ELP conjugated to a drug may provide a facile means of generating an *in situ* forming depot, with slow release increasing drug longevity in the targeted compartment and reducing serum exposure to the attached therapeutic. Following intravenous administration, ELPs are cleared with a terminal half-life of 8.4 h [30]. The reported biodistribution studies relate only to the ELP carrier, and it remains uncertain if therapeutic fusion proteins would exhibit those desired benefits of sustained release and attenuated serum exposure to potent immunosuppressive agents. Drugs conjugated with ELPs gain properties of thermally-induced phase transition and also maintain *in vitro* bioactivity. This has been shown for chemically-conjugated chemotherapeutics such as doxorubicin [26], recombinant oligopeptide fusions with cell penetrating peptides [31] and a *c-myc* oncogene inhibitor [32], and recombinant protein fusions with interleukin-1 receptor antagonist [33] and other proteins [34,35]. Surfaces coated with an ELP fused to the RGD or fibronectin CS5 cell binding sequence also retain an ability to support *in vitro* endothelial cell adhesion and spreading [36]. Other applications of ELP, including entrapment of small molecules such as dexamethasone [37], have also been investigated and are elsewhere reviewed [38].

The primary objective of this study was to create a fusion protein between an ELP and sTNFRII that would retain the ELP inverse phase transition behavior and sTNFRII domain bioactivity. This study is the first step towards realizing the long-term objective exploring the feasibility of attenuating local inflammation from TNFα hyperactivity in joint, nerve, and intervertebral disc spaces via local delivery and sustained release of the immunomodulator therapeutic. An ELP-sTNFRII gene was designed and the fusion protein was expressed in Escherichia coli. Results are presented for the phase transition and resolubilization behavior of the fusion protein, as well as binding affinity of ELP-sTNFRII to immobilized TNFα and *in vitro* anti-TNF bioactivity. The results indicate that ELP-sTNFRII retains functionality of both domains, establishing the potential of this therapeutic as an injectable local immunomodulatory protein.

2. Materials and methods

2.1. Fusion protein synthesis

The gene encoding human sTNFRII was inserted into a pUC57 cloning vector (GenScript, Piscataway, NJ) with the coding sequence flanked by unique XbaI and HindIII restriction sites, with the inclusion of an *SfiI* restriction site at the 3′ end. This plasmid was linearized with *SfiI* and treated with *Calf Intestinal Phosphatase* (CIP, New England Biolabs, Ipswich, MA). A cassette for the ELP gene encoding $(VPGVG)_{60}$ was removed from a pUC19 cloning vector (generously provided by Dr. Chilkoti, Duke University) by double digestion using *PflmI* and *BglI*, followed by electrophoretic separation and agarose gel extraction. The ELP cassette was then ligated into the linearized pUC57 vector. The fusion gene cassette was removed by double digestion using *XbaI* and *HindIII*, followed by electrophoretic separation and agarose gel extraction. Separately, a pET25b(+) expression vector was modified by double digestion with *XbaI* and *HindIII* and ligation with annealed custom-designed non-phosphorylated oligonucleotides (Integrated DNA Technologies, Coralville, IA) to move the *XbaI* site and downstream to the Shine–Delgarno variant ribosomal-binding site. This modified pET25b(+) vector was double digested with *XbaI* and *HindIII*, treated with *CIP*, agarose gel purified, and then ligated with the fusion gene cassette to yield the target fusion gene (ELP-sTNFRII) in an expression vector. The BL21trxB(DE3) expression strain of *E. coli* was transformed with the ligation mixture using heat shock poration.

One liter of TB-Dry (MoBio, Carlsbad, CA) media with 100 μg/mL of ampicillin (Sigma-Aldrich, St. Louis, MO) was inoculated with the expression strain and grown using a hyperexpression protocol [39]. Cells were harvested by centrifugation (3200 ×*g*, 15 min) and resuspended in 35 mL PBS. They were lysed by sonication at 4 °C and centrifuged (11,500 ×*g*, 15 min, 4 °C) to eliminate cell debris. Nucleic acids were precipitated using polyethyleneimine (0.5% w/v) and removed by centrifugation (11,500 ×*g*, 15 min, 4 °C). Fusion protein was purified by four rounds of inverse transition cycling (ITC) as described previously [40] under reducing conditions with 0.15% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) for samples to be refolded, or non-reducing conditions for samples to be directly evaluated. SDS-PAGE was performed at 80 mV with ReadyGel™ 4–20% gradient gels (Bio-Rad, Hercules, CA) and stained with SimplyBlue™ (Invitrogen, Carlsbad, CA) to confirm fusion protein purity. Concentrations were determined spectrophotometrically using calculated extinction coefficients [41].

2.2. Thermal characterization

The phase-transitioning behavior of the ELP-sTNFRII protein (MW=44.3 kDa) was characterized in order to confirm retention of thermal transitioning behavior. Dynamic light scattering (DLS) at a 90° scattering angle (DynaPro LSR with Peltier temperature control; Wyatt Technology Corp., Santa Barbara, CA) was used to evaluate the temperature-dependence of the hydrodynamic radius of the soluble ELP fusion protein and its supramolecular complexes for a 25 μM solution in PBS filtered through a 20 nm Whatman Anodisc filter. This low protein concentration on the order of 0.1% w/v helps control the solution viscosity to allow accurate estimates of particle size from the Stokes–Einstein equation, while also helping protect against the complicating feature of multiple scattering events. A broader range of fusion protein concentrations (500 nM to 100 μM) in both PBS and cell culture media was evaluated for the inverse phase transition using turbidity analysis, optical density at 350 nm (OD_{350}), measured over the 15–60 °C range (Cary 300 UV–VIS spectrophotometer with multicell thermoelectric temperature controller; Varian, Walnut Creek, CA) with the transition temperature defined as the temperature at which the OD_{350} is increased by 5% of the maximal observed change.

2.3. Fusion protein resolubilization

Fusion protein resolubilization was tested to evaluate the kinetics of monomer resolubilization from the multiparticle depots at