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A genetically engineered thermally responsive sustained release curcumin depot to treat neuroinflammation

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ABSTRACT

Radiculopathy, a painful neuroinflammation that can accompany intervertebral disc herniation, is associated with locally increased levels of the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα). Systemic administration of TNF antagonists for radiculopathy in the clinic has shown mixed results, and there is growing interest in the local delivery of anti-inflammatory drugs to treat this pathology as well as similar inflammatory events of peripheral nerve injury. Curcumin, a known antagonist of TNFα in multiple cell types and tissues, was chemically modified and conjugated to a thermally responsive elastin-like polypeptide (ELP) to create an injectable depot for sustained, local delivery of curcumin to treat neuroinflammation. ELPs are biopolymers capable of thermally-triggered *in situ* depot formation that have been successfully employed as drug carriers and biomaterials in several applications. ELP–curcumin conjugates were shown to display high drug loading, rapidly release curcumin *in vitro via* degradable carbamate bonds, and retain *in vitro* bioactivity against TNFα-induced cytotoxicity and monocyte activation with IC_{50} only two-fold higher than curcumin. When injected proximal to the sciatic nerve in mice *via* intramuscular (i.m.) injection, ELP–curcumin conjugates underwent a thermally triggered soluble–insoluble phase transition, leading to *in situ* formation of a depot that released curcumin over 4 days post-injection and decreased plasma AUC 7-fold.

1. Introduction

Intervertebral disc (IVD) herniation, or "ruptured disc," is a common cause of low back pain that can cause significant back pain, radiating leg pain (a.k.a. sciatica or radiculopathy), neurological impairments, prolonged patient disability, and lost wages [1–3]. Painful symptoms from IVD herniation are believed to result from compression of the nerve root by extruded or prolapsed tissue, an inflammatory response to soluble factors within the IVD, or a combination of these two pathophysiologies [4–7]. At the biological level, radiculopathy and peripheral nerve injury are both characterized by neuroinflammation, immune system activation, and neuronal dysfunction [4,8,9]. Following injury, resident macrophages called microglia in the spinal cord and dorsal root ganglion (DRG) are activated and release inflammatory cytokines, chemokines, and neurotransmitters that contribute to pain sensitization [10–12]. These biochemical signals attract peripheral immune cells, which infiltrate the injured nerve site, DRG, or ruptured disc and contribute to chronic inflammation and pain over time [9,13,14].

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine implicated as a key mediator of immune activation and neuroinflammation in radiculopathy and peripheral nerve injury. TNFα is secreted by multiple cell types in response to these injuries, both at the injury site and upstream in the DRG [15,16], and these responses are, in part, regulated by nuclear factor kappa B (NF-κB) activation [17]. TNFα is expressed at higher levels in degenerated and herniated IVDs [18–23], and application of exogenous TNFα to lumbar DRG in the rat [24,25], much like animal models of compression-induced nerve root injury, can produce changes in nerve conduction velocity, microglial activation, and inflammatory mediator levels [24,26].

The central role of TNFα in animal models of neuroinflammation has led to clinical interest in the delivery of TNFα-blocking antibodies and TNFα antagonists to treat IVD herniation-associated pain. However, the high costs and poor clinical results associated with systemic delivery of antibodies or soluble receptors to antagonize TNFα have motivated interest in local drug delivery approaches [27–30], as well as alternative small-molecule compounds such as minocycline [31], neurotransmitter receptor antagonists [32,33], and resveratrol [34].

One such compound that has generated significant interest is curcumin, a natural product derived from the rhizome (turmeric) of

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the herb *Curcuma longa* with known anti-carcinogenic, anti-bacterial, and anti-inflammatory activities [35]. At micromolar concentrations, curcumin suppresses TNF-induced or IL-1-induced activation of NF-κB and downregulates cell adhesion molecules and pro-inflammatory cytokines in multiple cell lines [36–43]. Recently, curcumin has been shown to be a potent modulator of the microglial transcriptome with an ability to alter the activation, migration, and pro-inflammatory phenotype of microglia [44], cells which have been shown to be key initiators of neuroinflammatory pathology in models of radiculopathy and nerve injury [9,10]. In addition to promoting a neuroprotective phenotype in microglia, curcumin demonstrates neuroprotective activity against IL-1β in rat DRGs at micromolar levels [45] and ameliorates neuropathic pain sensitivities in a mouse model of peripheral nerve injury [46]. Clinically, curcumin suffers from exceptionally low bioavailability due to low solubility and poor absorption into systemic circulation [47]. Many investigators have sought to make curcumin more soluble in aqueous solutions by developing structural derivatives [48–52], or incorporating insoluble curcumin particles into soluble nanoparticles [53]. To prolong systemic circulation of curcumin, investigators have entrapped curcumin in micro- or nano-sized polymeric particles including poly(N-isopropylacrylamide) (poly(NIPAAM)) (*i.e.* "nanocurcumin" [54,55]), liposomes [56], micellar di-block copolymers [57–59], PLGA microspheres [60,61], phosphatidylcholine-based phytosomes (Meriva®, Indena S.p.A., Milan, Italy) [62], and self-assembling peptide hydrogels [63]. Curcumin has also been chemically conjugated to drug carriers like poly(ethylene glycol) (PEG) [64], poly(amidoamine) (PAMAM) dendrimers [65], and incorporated into the polymer backbone of a hydrogel system *via* degradable carbonate bonds [66].

In this study, curcumin was chemically modified to include a degradable carbamate linkage and a reactive primary amine, so that it could be coupled to a thermally responsive drug carrier, an elastin-like polypeptide (ELP), for local, sustained release of bioactive curcumin to treat neuroinflammation. ELPs are thermally responsive biopolymers composed of a Val-Pro-Gly-Xaa-Gly pentapeptide repeat unit that is found to recur in tropoelastin, where Xaa can be any amino acid [67,68]. ELPs undergo an inverse phase transition at a specified transition temperature (T_t), above which the ELP transitions from a soluble chain to an insoluble, viscous coacervate [69]. The T_t of a given ELP is primarily a function of amino acid composition, solution concentration, and molecular weight, but also depends on the solution pH, ionic strength, polarity of the solvent, and the presence of any fused proteins or conjugated molecules. ELPs have been employed as drug carriers and biomaterials in a variety of applications owing to its facile recombinant synthesis, biocompatibility, biodegradability, and non-immunogenic nature [70,71]. In prior work, ELPs engineered to form depots at body temperature (T_t < 37 °C) were observed to reside in the perineural space of rats 7 times longer and reduce systemic exposure 14-fold compared to non-depot forming ELP [72]. ELPs have also been useful in forming intratumoral depots for local delivery of radionuclides [73,74], as well as subcutaneous depots for systemic delivery of glucagon-like peptide-1 for treatment of diabetes [75]. For these reasons, we designed a biodegradable ELP–curcumin conjugate that would rapidly form a depot upon physiological administration and slowly release bioactive curcumin within the perineural space to treat neuroinflammation. This paper reports on the synthesis, physicochemical characterization, *in vitro* bioactivity, and *in vivo* pharmacokinetics and clearance rates of the conjugate.

2. Materials and methods

2.1. Materials and reagents

All reagents were purchased from Sigma Aldrich (St. Louis, MO), unless otherwise noted. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), sulfo-N-hydroxysulfosuccinimide acetate (sulfo-NHS-acetate), and SnakeSkin® Pleated Dialysis Tubing (MWCO 7000 Da) were purchased from Pierce Biotechnology (Rockford, IL). Curcumin (95% purity) was purchased from Alfa Aesar (Ward Hill, MA), and fetal bovine serum (FBS) and phosphate-buffered saline (1× PBS) were purchased from Invitrogen (Gibco®, Carlsbad, CA).

2.2. Chemical synthesis of monofunctional curcumin carbamate (MCC)

See supplementary section S.1.

2.3. UV–vis characterization of curcumin and MCC

The absorbance of curcumin and MCC standards was measured *via* UV–vis spectrophotometry from 200 to 800 nm (CARY Bio 300 UV-vis, Agilent Technologies, Santa Clara, CA). The molar extinction coefficient of MCC at λ_{max} = 416 nm (ε_{416}) was calculated for MCC samples diluted from 100% acetonitrile (CH_3CN) stocks into a cold UV–vis buffer (50% CH_3CN:50% DI H_2O, pH = 7.4). ELP was acetylated (AcELP) in a process described below, and AcELP was added to each MCC standard in a fixed molar ratio of 1 mol AcELP to 5 mol MCC to mimic ELP–MCC conjugates, as the presence of proteins such as albumin [76] have been shown to affect curcumin absorbance. Absorbance of MCC with AcELP at 416 nm (OD_{416}) was obtained and plotted against the known concentration of MCC, and ε_{416} was determined from the linear regression of OD_{416} and concentration.

2.4. Conjugate synthesis and purification

Glutamate-rich ELPs with the amino acid sequence MSKGPG $[VPGXG]_{L = 60,80,160}$ WPC with X = V/I/E [1:3:1] (MW = 26.3, 34.9, and 68.8 kDa), where L is the number of total pentapeptide repeats, were synthesized, expressed, and purified as previously described [77,78]. Hydrophilic glutamate residues were periodically and precisely placed along the polymer backbone to provide carboxylates convenient for drug attachment; and we hypothesized that unreacted carboxylates remaining after conjugation would compensate for the increase in hydrophobicity following conjugation of curcumin (distribution coefficient at pH = 7.4, $\log D_{pH = 7.4}$ = 2.9).

2.4.1. Acetylation of ELP terminal amines

To prevent intra- and inter-ELP chain crosslinking, the two terminal amine groups present on purified ELP (the amino terminus and the ε-amine of lysine) were blocked by reacting ELP in 1X PBS (pH = 8.3) at a concentration of 1 mM with sulfo-NHS-acetate (Pierce) dissolved in dimethylformamide (DMF) at a molar ratio of 25 to 1 acetates to amines for 1 h at room temperature. The reaction solution was dialyzed against de-ionized H_2O (DI H_2O, pH = 7.1–7.4) for 48 h with at least 2 buffer changes, and the dialysate was frozen and lyophilized.

Blocking efficiency was confirmed with a 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay [79]. Dry AcELP and unmodified ELP were weighed and dissolved in a known volume of 0.1 M sodium bicarbonate ($NaHCO_3$, pH = 8.3). AcELP was diluted to 50 μM and a standard curve of unmodified ELP ranging from 10 to 100 μM was prepared. TNBS acid stock was diluted to 0.01% acid in the same buffer and 50 μl was added to 50 μl of sample or standard in a 96-well plate. The plate was incubated at 60 °C (15 min.), allowed to cool at room temperature (5 min.), and absorbance was recorded at 340 nm (background correction at 595 nm) (Enspire Multimode Plate Reader, Perkin Elmer, Waltham, MA). Successful blocking was defined as a reduction in 340 nm absorbance by ≥90% of unmodified ELP. This process resulted in a net increase in molecular weight of 43 Da per acetyl group, or 86 Da per ELP.

2.4.2. Conjugate synthesis

ELP–MCC ($AcELP_{L = 60,80,160}$) conjugates were synthesized *via* carbodiimide coupling chemistry and termed MCC60, MCC80, or