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# An amphipathic alpha-helical peptide from apolipoprotein A1 stabilizes protein polymer vesicles

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ABSTRACT

L4F, an alpha helical peptide inspired by the lipid-binding domain of the ApoA1 protein, has potential applications in the reduction of inflammation involved with cardiovascular disease as well as an antioxidant effect that inhibits liver fibrosis. In addition to its biological activity, amphipathic peptides such as L4F are likely candidates to direct the molecular assembly of peptide nanostructures. Here we describe the stabilization of the amphipathic L4F peptide through fusion to a high molecular weight protein polymer. Comprised of multiple pentameric repeats, elastin-like polypeptides (ELPs) are biodegradable protein polymers inspired from the human gene for tropoelastin. Dynamic light scattering confirmed that the fusion peptide forms nanoparticles with a hydrodynamic radius of approximately 50 nm, which is unexpectedly above that observed for the free ELP (~5.1 nm). To further investigate their morphology, conventional and cryogenic transmission electron microscopy were used to reveal that they are unilamellar vesicles. On average, these vesicles are 49 nm in radius with lamellae 8 nm in thickness. To evaluate their therapeutic potential, the L4F nanoparticles were incubated with hepatic stellate cells. Stellate cell activation leads to hepatic fibrosis; furthermore, their activation is suppressed by anti-oxidant activity of ApoA1 mimetic peptides. Consistent with this observation, L4F nanoparticles were found to suppress hepatic stellate cell activation *in vitro*. To evaluate the *in vivo* potential for these nanostructures, their plasma pharmacokinetics were evaluated in rats. Despite the assembly of nanostructures, both free L4F and L4F nanoparticles exhibited similar half-lives of approximately 1 h in plasma. This is the first study reporting the stabilization of peptide-based vesicles using ApoA1 mimetic peptides fused to a protein polymer; furthermore, this platform for peptide-vesicle assembly may have utility in the design of biodegradable nanostructures.



## 1. Introduction

The prevalence of secondary diseases associated with obesity, such as non-alcoholic fatty liver disease (NAFLD), is expected to rise with the high prevalence of obesity in Western nations [1]. NAFLD, characterized by the deposition fat and the presence of inflammation, can progress to hepatic fibrosis and cirrhosis [2]. A candidate for the prevention of progression of NAFLD is the apolipoprotein A-1 mimetic peptide D4F, which prevents hepatic fibrosis in murine models through an anti-oxidative effect [3]. D4F, a peptide comprised of D-amino acids, and its natural enantiomer L4F, made up of L-amino acids, have been extensively studied in animal models related to lipid oxidation and inflammatory diseases. Based on the 18A peptide [4], a synthetic 18 amino acid peptide designed to mimic amphipathic helices found in apolipoproteins and originally intended to displace apolipoproteins from HDL [5,6], the 4F peptides are amphipathic and create type A α-helices due to their 4 hydrophobic phenylalanine residues. The 4F peptides bind oxidized lipids with an affinity 4–6 orders of magnitude higher than ApoA-1 [7]. 4F's anti-oxidant properties are attributed to their affinity for pro-inflammatory oxidized lipids. L4F has been demonstrated to decrease cytokines including IL-6, TNF-α, and IL-1β in obese mice [8,9] as well as inhibit activation of the transcription factor NF-κB in chronic kidney disease rat models [10].

While D4F is anti-fibrotic *in vivo* [3], we chose to develop the L-amino acid – L4F – peptide for three reasons. First, unlike D4F, the L4F peptide can be directly engineered onto an recombinant ELP protein polymer to modulate its self-assembly properties, which may impact its biodistribution and efficacy. Secondly, chronic use of D-amino acids results in high tissue accumulation due to impaired breakdown, which

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is not an obstacle using biodegradable enatiomers [4]. Lastly, when administered subcutaneously, the L form of the 4F molecule was just as effective at treating atherosclerosis as the D form [11,12]. It is therefore likely that D4F's anti-fibrotic mechanism is conserved in its L4F enantiomer.

To capitalize on ability of the L4F to form an amphipathic secondary structure, we here explore the addition of high molecular weight elastin-like polypeptide (ELPs) to modulate its assembly properties. ELPs are derived from the human gene for tropoelastin and are repetitive polypeptide chains of the amino acid sequence (Val–Pro–Gly–*Xaa*–Gly)$_n$, where *Xaa* can be substituted with virtually any amino acid [13]. ELPs phase separate above a transition temperature, $T_t$, which can be tuned by selection of *Xaa* and *n*. ELPs are emerging as a platform to display and manipulate the assembly of protein polymer nanoparticles, partly because they are biodegradable, biocompatible, and non-immunogenic [14,15]. Since they are genetically encoded, they lend themselves to direct fusion with biologically functional proteins. In addition, the fusion of ELPs to low molecular weight peptides has been shown to both increase the half-life of the peptide as well as decrease systemic clearance [16,17].

In this study, we report the surprising formation of L4F ELP fusion proteins into nanoparticles 50 nm in radius at 37 °C, significantly below the transition temperature of the parent ELPs. We also demonstrate that L4F nanoparticles inhibit the activation of hepatic stellate cells *in vitro*; furthermore, we show that the pharmacokinetics of these nanoparticles are not compromised in comparison to the free peptide. We expect these data to act as a springboard for the development of a biologically active nanostructures assembled by ApoA-1 mimetic peptides.

## 2. Materials and methods

### 2.1. Construction of L4F ELP fusions

A DNA sequence encoding for the peptide L4F followed by a thrombin cleavage site and ELP insertion site (IDT Technologies, Coralville, IA) was cloned into a pET25b+ vector (Clontech, Mountain View, CA). A forward primer (TATGGATTGGTTCAAAGCGTTTTATGATAAAGTGGCG GAAAAATTCAAAGAAGCGTTCGGTCTGGTTCCGCGTGGTTCTGGTTACTG ATCTCCTCG) and a reverse primer (GATCCGAGGAGATCAGTAACCAGA ACCACGCGGAACCAGACCGAACGCTTCTTTGAATTTTTCCGCCACTTTATCA TAAAACGCTTTGAACCAATCCA) were annealed and ligated into a pET25b+ vector digested with NdeI/ BamHI to generate a 2 base pair overhang created by digestion at an amino terminal BseRI cut site. Various ELP genes [18] were ligated downstream of the L4F encoding sequence using BseRI/BamHI cut sites in both L4F and ELP plasmids to digest and 1 μL T4 DNA ligase (Invitrogen, Carlsbad, CA) to ligate, resulting in N-L4F-thrombin-ELP-C (Table 1). The resulting fusion protein constructs were expressed in BLR *E. coli* and purified using ELP-mediated phase separation [19]. Purity was determined by running 20 μg of polymer on a 4–20% SDS-PAGE gel stained with copper chloride.

### 2.2. Optical characterization of the ELP phase diagram

The phase behaviors of ELPs were characterized as a function of molecular weight and concentration by measuring the solution turbidity at 350 nm of protein polymer as a function of temperature. Protein polymers (300 μL) in phosphate buffered saline (PBS, 0.2 g/L KCl, 0.2 g/L $KH_2PO_4$, 8 g/L NaCl, 1.15 g/L $Na_2HPO_4$), with concentrations ranging from 5 to 100 μM, were observed in a Beckman *Tm* microcell at a constant ramp rate of 1 °C min$^{-1}$ and measurements were captured 3× min$^{-1}$ by a UV visible spectrophotometer (DU800 Spectrophotometer, Beckman Coulter, CA). The maximum first derivative of the curve was defined as the transition temperature, $T_t$.

### 2.3. Dynamic light scattering

A Dynapro plate reader (Wyatt Technology Inc., Santa Barbara, CA) was used for all hydrodynamic radius and polydispersity measurements. Polymers were prepared at 25 μM concentrations in PBS, filtered through 0.2 μm cellulose acetate filters, and centrifuged at 4 °C, 1200 rpm to remove air bubbles. Mineral oil was added to the top of the sample to prevent evaporation. Polymers were observed over a range of temperatures from 4 to 60 °C in 1 °C increments.

### 2.4. Conventional and cryogenic transition electron microscopy

TEM and cryo-TEM images were captured on an FEI Tecnai 12 TWIN Transmission Electron Microscope, operating at 100 kV for TEM and 80 kV for cryo-TEM. TEM samples (25 μM) were pipetted onto a carbon-coated copper grid (Electron Microscopy Sciences, Hatfield, PA). Filter paper was used to wick away excess solution. Ten microliters of 2 wt.% aqueous uranyl acetate was used to stain samples. For cryo-TEM, 25 μM samples were pipetted onto lacey carbon coated TEM grids (LC325-Cu, Electron Microscopy Sciences) pre-treated with plasma air to make the lacey carbon film hydrophilic. Samples were vitrified by plunging them into a liquid ethane reservoir precooled with liquid nitrogen. Both TEM and cryo-TEM images were acquired with a 16 bit 2k × 2k FEI Eagle bottom mount camera (Hillsboro, OR). Images were processed with ImageJ (NIH, Bethesda, MD). Nanoparticle size was averaged from three areas of view with more than 50 particles per image for TEM and 20 particles for cryo-TEM. Bilayer thickness was averaged across three points along the circumference of the vesicle. Numbers are presented as averages ± 95% confidence interval.

### 2.5. α-smooth muscle actin assay

Primary mouse hepatic stellate cells (HSCs) were isolated by collagenase/pronase digestion and Stractan density gradient

**Table 1**
Recombinant protein polymers examined during this study.

| Label | Encoded amino acid sequence* | MW [kDa] | $T_t$ [°C]** | Property at 37 °C | Slope [°C/log$_{10}$(μM)]*** | *Y*-intercept [°C] |
|---|---|---|---|---|---|---|
| A192 | G(VPG**A**G)$_{192}$Y | 73.7 | 61.9 | Soluble | −9.2 [−9.9 to −8.6] | 74.9 [74.0–75.8] |
| L4F-A192 | MDWFKAFYDKVAEKFKEAFGLVPRGS G(VPG**A**G)$_{192}$Y | 76.6 | 45.8 | Nanoparticle | −2.6 [−3.6 to −1.7] | 49.6 [48.–0.9] |
| V2A192 | G(VPG**V**G VPG**A**G VPG**A**G)$_{64}$Y | 75.4 | 47.2 | Soluble | −5.8 [−6.7 to −4.9] | 55.9 [54.–7.1] |
| L4F-V2A192 | MDWFKAFYDKVAEKFKEAFGLVPRGS G(VPG**V**G VPG**A**G VPG**A**G)$_{64}$Y | 79.0 | 34.3 | Coacervate | −1.3 [−1.6 to −1.0] | 36.2 [35.–6.6] |

* Genes encoding for L4F-A192, V2A192, and L4F-V2A192 were constructed similarly to A192.
** The observed transition temperature at 25 μM in PBS.
*** The slope, *b*, and *Y*-intercept, *m*, were derived from the log-linear regression analysis for transition temperature vs concentration fit to the equation $T_t = m \log_{10}[C_{ELP}] + b$. Mean [95% CI].