A thermo-responsive protein treatment for dry eyes

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ABSTRACT

Millions of Americans suffer from dry eye disease, and there are few effective therapies capable of treating these patients. A decade ago, an abundant protein component of human tears was discovered and named lacritin (Lacrict). Lacrt has prosecretory activity in the lacrimal gland and mitogenic activity at the corneal epithelium. Similar to other proteins placed on the ocular surface, the durability of its effect is limited by rapid tear turnover. Motivated by the rationale that a thermo-responsive coacervate containing Lacrt would have better retention upon administration, we have constructed and tested the activity of a thermo-responsive Lacrt fused to an elastin-like polypeptide (ELP). Inspired from the human tropoelastin protein, ELP protein polymers reversibly phase separate into viscous coacervates above a tunable transition temperature. This fusion construct exhibited the prosecretory function of native Lacrt as illustrated by its ability to stimulate β-hexosaminidase secretion from primary rabbit lacrimal gland acinar cells. It also increased tear secretion from non-obese diabetic (NOD) mice, a model of autoimmune dacyroadenitis, when administered via intra-lacrimal injection. Lacrt ELP fusion proteins undergo temperature-mediated assembly to form a depot inside the lacrimal gland. We propose that these Lacrt ELP fusion proteins represent a potential therapy for dry eye disease and the strategy of ELP-mediated phase separation may have applicability to other diseases of the ocular surface.

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1. Introduction

The lacrimal–corneal axis plays a critical role in maintaining ocular surface health. While the avascular cornea serves as both a protective barrier and the main refractive element of the visual system, the lacrimal gland (LG) is the major organ secreting key proteins and electrolytes into the tear film that bathes the cornea and, through nutrient and antimicrobial proteins, sustains its function [1,2]. Dry eye disease (DED) is a multifactorial disease of the ocular surface causing visual disturbance and tear film instability [3] and can be due to either aqueous tear insufficiency originating with defects in aqueous tear production by the LG [4] or evaporative dry eye associated with meibomian gland insufficiency [5,6]. Accordingly to reports, severe DED affects approximately 5 million Americans above age 50 and its global prevalence ranges from 5% to 35% of the population [3]. Traditional approaches to treat DED include topical administration of artificial tears or the conservation of secreted tears using tear plugs [7] and eye-shields [8]. Since many cases of DED are associated with inflammation [9,10], some treatments for DED have been proposed that inhibit inflammation of the LG [11]. None of these methods are satisfactory in replacing the lost regulatory functions provided by the many components found in normal tears. To better sustain the health and homeostasis of the ocular surface there remains a need for efficient, sustained and targeted DED therapy. In humans, the inferior palpebral lobe of the LG is accessible for injection beneath the eyelid; furthermore, if coupled with a sustained release strategy this route of administration might have clinical relevance, similar to intra-vitreal injection or subconjunctival injection.

The discovery of the glycosylated human tear protein, lacritin (Lacrict), provided critical insight into the potential use of regulatory tear proteins to treat DED [12,13]. Lacrt was found in a systematic oligonucleotide screen of a human LG cDNA library and exhibited LG specificity [14]. Subsequent studies have proven its efficacy in stimulating perforase secretion in cultured rat [14], and both lactoferrin and lipocalin secretion in cultured monkey lacrimal acinar cells [15]. Lacrt also promotes constitutive tear secretion by New Zealand white rabbits and Aire KO mice via topical treatment [16,17], proliferation of transformed human corneal epithelial cells [18,19], and restored health of...
transformed human corneal epithelial cells, primary human corneal epithelial cells [19] and primary monkey lacrimal acinar cells [15] that had been stressed with the inflammatory cytokines interferon-γ and tumor necrosis factor. Interestingly, Lacrt displays growth factor-like behavior; however, its specificity for target cells of the ocular surface system results from a unique ‘off–on’ switch controlled by heparan sulfate glycansation of the cell surface protein, syndecan-1 [20], which both exposes and generates a Lacrt binding site [21] as a prerequisite for mitogenic signaling. Confirmed by 2-D electrophoresis, mass spectrometry and surface-enhanced laser desorption/ionization studies, Lacrt [22] is down regulated in blepharitis (chronic inflammation of the eyelid) vs. normal tears [23], and most aqueous deficient dry eye [24]. Whether down regulation of Lacrt provokes disease is a key unresolved question, but its prosesory and corneal mitogenic activity suggest that it might have activity as a protein therapeutic for ocular surface diseases.

Great strides have been made to improve the bioavailability and simplify the administration of existing drugs, which include depot formulations that deliver short peptides such as leuprolide and bioadhesive polymers used in buccal drug-delivery systems [25]. Recently, stimuli-responsive polypeptides have emerged as an attractive controlled release strategy. One such type of biomaterial is the elastin-like polypeptides (ELPs) [26]. Biologically inspired from human tropoelastin, ELPs are composed of a pentapeptide repeat (VPGXG)n, where the ‘guest residue’ X can be any amino acid and n determines molecular weight. One unique property of ELPs is their inverse temperature phase transition behavior. ELPs are soluble in aqueous solutions below their transition temperature (Tc) and self-assemble into various-sized particles above Tc [27]. Tc can be precisely modulated by adjusting the number of pentapeptide repeats, n, and the hydrophobicity of the guest residue, X, which can determine whether the ELP remains a soluble macromolecular drug carrier [28], assembles a nanoparticle [29], or phase separates into micron-sized coacervates [30] at physiological temperature. With their distinctive thermo-responsive, elastic, and biocompatible properties, ELPs have impacted fields such as protein purification [31], stimuli responsive hydrogels [32], tissue engineering [33,34], and targeted cancer treatment [35,36]. Yet, the application of ELPs in ophthalmology has just started [37,38].

To explore the concept of a thermo-responsive reservoir drug as a potential novel treatment for DED [7], we generated a novel Lacrt-ELP fusion with Tc below physiological temperature. The construct exhibits thermo-responsiveness of the parent ELPs while retaining prosesory efficacy of native Lacrt, as demonstrated by its ability to stimulate dose-dependent β-hexosaminidase secretion from primary rabbit lacrimal gland acinar cells (LGACs). Moreover, the Lacrt-ELP fusion enhanced tear secretion from the non-obese diabetic (NOD) mouse model of autoimmune dacyrooadenitis when given via intra-lacrimal injection. This treatment formed a depot that lasted over 24 h inside the LG, which was confirmed by confocal laser scanning microscopy. Finally, we captured the intracellular trafficking and transcytosis of exogenous Lacrt in LGACs using time-lapse confocal fluorescence microscopy, which was prolonged by fusion to the ELP. These findings support the potential enhancement of Lacrt therapeutics via the linkage to a thermo-responsive ELP, which may have broader implications in the treatment of DED.

2. Material and methods

2.1. Animals

In vitro studies were conducted using LG from Female New Zealand White rabbits (2.2–2.5 kg) obtained from Irish Farms (Norco, CA). In vivo studies were conducted using LG isolated from 12-week-old male/female C57BL/6 (Jackson Labs, Bar Harbor/ME, USA) or in house bred non-obese diabetic (NOD) (Taconic Farms, Germantown/NY, USA) mice. All procedures performed were in accordance to the university approved IACUC protocol.

2.2. Instruments and reagents

Terrific broth dry powder growth medium was purchased from MO BIO Laboratories, Inc. (Carlsbad, CA). Isopropyl β-D-1-thiogalactopyranoside, OmniPur®. 99.0% min. was purchased from VWR (Visuala, CA). Amicon Ultra concentrators were purchased from Millipore (Billerica, MA). Thrombin CleanCleave™ Kit, carbobal (CCh) and insulin–transferrin–sodium selenite media supplement were purchased from Sigma-Aldrich (St. Louis, MO). 4–20% Tris–Glycine PAGE gel was purchased from LONZA (Allendale, NJ). Cell culture reagents were from Life-Technologies (Carlsbad, CA). Peter’s Complete Medium (PCM) consisted of 50% Ham’s F-12 plus 50% DME (low glucose) supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), glutamine (4 mM), hydrocortisone (5 nM), transferring (5 μg/ml), insulin (5 μg/ml), butryate (2 mM), linoileic acid (0.084 mg/l), carbobal (1 μM), laminin (5 mg/l) and insulin–transferrin–sodium selenite (ITS) media supplement (5 μg/ml).

2.3. Biosynthesis of Lacrt–ELP fusions

A sequence encoding human Lacrt without a secretion signal peptide was designed using the best Escherichia coli codons in EditSeq (DNASTar Lasergene, WI) [31]. A thrombin cleavage site was encoded between the Lacrt sequence and ELP tag via insertion at the BseBI site. A custom gene flanked by Ndel and BamHI restriction digestions sites at the 5’ and 3’ ends was purchased in the pIDTS-Kant-KAN vector from Integrated DNA Technologies (IDT) as follows:

5′-CATATGGAAGACCCCTTCTTCGACTCTACCGGTCGAGGACGGCTTCGACCCGTGTCGTGCTGCTGTTCAGGGTACCGCTAAAGTTACCTCTTCTCGTCAGGAACCTACCCCTGCTGACCCGGCTC

AGGAACGCTTCACTCITAAACCGAGAAGAAATCTCTGTTCTCCCGCTTG

AACCCGCCTCTCGCCGGAACACCCAACACCGTGTCGGAAAGAAGCTCT

CTGCTGCTGTTCAGGGTACCGCTAAAGTTACCTCTTCTCGTCAGGAAC

TGAACTCCCTGCTGAAATCTCTCTGCTGAAATCTCTCTGCTGCAAGAC

AGCCCTGCTGCAATAGCTGTTAAGGATGTCACCGGCTGTTGCTGCTGCTG

GTAAACTACCTGCAAGAACGGCTTCGTGACCCGGCTGCTGCTGCTG

AAAATTCTCTTGATGAAACCTGGCCGCTGTTGCTGCTGCTGCTG

CTGCTGACTGACCTCTCCGAGACCTG

1.5′

The gene encoding for V96 was synthesized by recombinant directional ligation in a modified pET25b (+) vector as previously reported [40,41]. The Lacrt-thrombin gene was subcloned into the pET25b (+) vector between the Ndel and BamHI sites. LV96 gene fusions were synthesized by ligation of a gene encoding for the ELP V96 via the BseBI restriction site, resulting in placement of the thrombin cleavage site between Lacrt and ELP. Correct cloning of the fusion protein gene was confirmed by DNA sequencing. The amino acid sequences of ELPs used in this study are described in Table 1.

2.4. Expression and purification of Lacrt ELP fusion protein

Plain ELP V96 and the Lacrt fusion LV96 were expressed in BLR (DE3) E. coli (Novagen Inc., Milwaukee, WI). Briefly, V96 was expressed for 24 h in an orbital shaker at 37 °C at 250 rpm. For LV96, 500 μl IPTG was added to the culture when the OD 600 nm reached 0.5, at which point the temperature was decreased to 25 °C for protein expression for 3 h. Cell cultures were harvested and re-suspended in phosphate buffer saline (PBS). Proteins were purified from clarified cell supernatant by inverse transition cycling [39] until ELP purity was determined to be approximately 99% by SDS-PAGE stained with CuCl2. Due to partial proteolysis of LV96 during biosynthesis, fusion proteins were further purified to homogeneity using a Superose 6 (GE Healthcare Bio-Sciences, Piscataway, NJ) size exclusion column at 4 °C. After equilibration with PBS (pH 7.4), 10 mg LV96 was loaded onto the column and washed out by isocratic flow of PBS at 0.5 ml/min. PI, representing LV96 (Supplementary Fig. S1), was collected and concentrated using an Amicon Ultra concentrator (10 kD). When desired, free Lacrt was...
released by thrombin cleavage of LV96 fusion protein. Briefly, 300 μl of
thrombin bead slurry (Sigma-Aldrich) was added to 200 mg of purified
LV96 and incubated at room temperature for 3 h. After pelleting the
thrombin beads at 250 rpm, the solution was warmed up to 37 °C and
centrifuged at 4000 rpm for 10 min to remove ELP coacervates. The super-
nant was then concentrated using an Amicon Ultra concentrator
with a 3 kD M.W. cut-off (MWCO). Protein concentrations were deter-
mined by UV−VIS spectroscopy at 280 nm (εELP = 1285 M−1 cm−1, εV96 = 6990 M−1 cm−1, εLacrt = 5500 M−1 cm−1). Protein molecular
weight was further confirmed by MALDI-TOF mass spectrometry
(AXIMA Assurance, Shimadzu).

2.5. Thermal characterization of Lacrt ELP fusion proteins

Self-assembly of purified V96 and LV96 fusion proteins was
characterized by optical density using a DU800 UV−VIS spectrophotom-
er equipped with the High Performance Transport and Peltier
Temperature-Controlled Cell Holder (Beckman Coulter, Brea, CA). Consis-
tent with previous reports [27,28,36], optical density was measured
at 350 nm as a function of temperature, a wavelength at which LV96
and V96 contribute little absorption. ELPS (5 to 100 μM) were observed
in PBS under a temperature gradient of 1 °C/min (10 to 45 °C). The cu-
vette provides minimal insulation between the sample and the cell
holder. At this slow temperature gradient, the sample and cell holder
are engineered to remain in close agreement to avoid over or under
heating. The inverse transition temperature (Tt) of each solution was
defined as the temperature at which the first derivative of the optical
density with respect to the temperature reached a maximum. The ELP
transition temperature has been observed as a function of concentration
as follows:

\[ T_t = b - m \log_{10}[C_{ELP}] \]  

(1)

where b is the intercept, m is the slope, and C_{ELP} is the ELP concentration. Eq. (1) was fit to data obtained for V96 and LV96 (Table 1).

2.6. Dynamic light scattering

To characterize the assembly process of LV96 coacervates, the hy-
drodynamic radius (Rs) was monitored as a function of temperature.
Samples were suspended (25 μM) in PBS and were filtered through
Whatman Anotop 10 syringe filters with a pore size of 0.02 μm (GE
Healthcare Bio-Sciences, Piscataway, NJ) at 4 °C. Light scattering data
were collected at regular temperature intervals (1 °C) as solutions
were heated from 5 to 60 °C using a DynaPro-LSR Plate Reader (Wyatt
Technology, Santa Barbara, CA). The results were then analyzed using
a Rayleigh sphere model.

2.7. Stability of Lacrt

To determine the cleavage half-life of Lacrt, the purified proteins
were incubated in PBS at 37 °C for 72 h followed by SDS-PAGE
analysis. Peptide sequence analysis was performed using MALDI-TOF
(AXIMA Assurance, Shimadzu). Cleavage products were assigned by
MALDI-TOF mass by comparison of measured with predicted mass to
charge ratios (m/z) with +1 charge ionization ([M + H]+). For Western
blotting of purified Lacrt, 50 μg purified protein was loaded onto 4–20% Tris–HCl polyacrylamide gels; with blocking buffer at room tempera-
ture for 1 h and blotted with rabbit anti-N-terminal or anti-C-terminal
(1:200) Lacrt antibody [42] overnight at 4 °C followed by blotting with
IRDye680 Donkey anti-rabbit IgG (H + L) (Rockland) (1:3000) at
room temperature for 1 h. Images were taken using the Odyssey infra-
red imaging system (Li-Cor, Lincoln, NE).

2.8. Cell isolation, culture and treatments

Isolation of primary cultured LGAC from female New Zealand white rabbits was performed in accordance with the Guiding Principles for
Use of Animals in Research. Specifically, LGACs were isolated from rab-
bit LGAC and cultured by the method of da Costa [43] in Peter’s Com-
plete Medium (PCM) medium for 2–3 days.

2.9. Secretion of \( \beta \)-hexosaminidase

Fresh PCM medium was added to wells containing LGAC and incuba-
tions were continued for additional 2 h. Baseline samples were then taken from each well, and the cells were stimulated with 100 μM carba-
chol (CCh), Lacrt, V96, or LV96 at various concentrations as indicated for
1 h. After stimulation, the cell supernatant was collected and \( \beta \)-hexosaminidase activity in each aliquot was measured using a 246 model substrate, methylumbelliferyl-N-acetyl-\( \beta \)-glucosaminide. As-
says of catalytic activity were performed in black-well plates, and re-
action product absorbance was determined with a plate reader at
460 nm (Tecan Genios Plus; Phenix Research Products, Candler, NC).
Signals were analyzed with the manufacturer’s software package
(Magellan v6.6; Phenix Research Products). Medium was then aspirated
from all wells and 500 μl 0.5 M NaOH was added into each well and in-
cubated at 4 °C for overnight to lyse the acini and solubilize all protein.
Total protein in each well was measured by the bicinchoninic acid assay
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10.1016/j.jconrel.2014.11.016
where $\Delta h_{\text{ex}_{\text{treatment}}}$ is the sample activity, $\beta_{\text{hex}_{\text{Ch}}}$ is the activity released in the absence of stimulation, and $\beta_{\text{hex}_{\text{Ch}+}}$ is the activity released upon stimulation with Ch.

2.10. Live cell imaging of actin remodeling

LifeAct-RFP adenovirus was generated as described previously [44].

2.11. Cellular uptake of Lacrt and Lacrt ELP fusion proteins

Lacrt, V96 and LV96 were conjugated with NHS-rhodamine (Thermo Fisher Scientific Inc, Rockford, IL) via covalent modification of the amino terminus. Conjugation was performed in 100 mM borate buffer (pH 8.0) for 2 h (LV96 and Lacrt) or overnight (V96) at 4 °C followed by desalting on a PD10 column (GE Healthcare Bio-Sciences, Piscataway, NJ) to remove free dye. Degree of labeling was estimated following the manufacturer’s instructions as follows:

$$C_{\text{rhodamine}} = \frac{A_{550}}{\epsilon_{\text{rhodamine}}}$$

(3)

$$C_{\text{protein}} = \frac{A_{280} - (A_{280} \cdot C_{\text{rhodamine}})}{\epsilon_{\text{protein}}}$$

(4)

where $\epsilon_{\text{rhodamine}} = 80,000 \, \text{M}^{-1} \cdot \text{cm}^{-1}$; $C_{\text{rhodamine}} = 0.34$. Cellular uptake was studied on 35 mm glass coverslip-bottomed dishes. Briefly, after washing with warm fresh medium, LCAGs were cultured in medium containing 10 μM of proteins conjugated with rhodamine. After incubation at 37 °C for different time points, the cells were rinsed three times with room temperature medium and images were acquired using confocal fluorescence microscopy. The washing step does not conserve coacervate particles, which are disrupted below 27 °C, but does enable the observation of their intracellular uptake and trafficking.

2.12. Intra-lacrimal administration, retention, and tear secretion in mice

For intra-lacrimal injection, mice were anesthetized with an i.p. injection of xylazine/ketamine (60–70 mg + 5 mg/kg), administered a subcutaneous injection of buprenorphine (0.02 mg/kg), and placed on a heating pad. After removing fur from the cheek and cleansing the area with alcohol, a small incision (5 mm) was made to visualize the LG. 5 μl of 50 μM carbamyl (CCH), 100 μM LV96, 100 μM V96 or 100 μM Lacrt was injected into the LG using a 33 gauge blunt needle. The mice were monitored on the heating pad until fully recovered from anesthesia. For quantification of tear secretion, a glass capillary (Microcaps Drummond disposable micropipettes 2 μl) was placed on the lower eyelid of the mice to collect tears (2 LG/each mouse, 30 min/each gland). To evaluate protein retention in the LG, each time point was repeated in three LGs and a representative image was shown. Three sections from each sample were imaged and quantified using ImageJ (n = 9). Data were then analyzed using two-way ANOVA followed by Tukey’s post-hoc test (GraphPad Prism).

3. Results

3.1. Construction and purification of a Lacrt ELP fusion protein

We designed the a gene encoding LV96 in the pET25b(+) vector resulting in the amino acid sequence shown in Table 1. LV96 forms viscous coacervates with T below physiological temperatures of 37 °C [40] and thus was chosen as the ELP backbone for potential depot formation. LV96 was also used to generate free Lacrt control protein utilizing selective cleavage of a thrombin cleavage site (Fig. 1A). In contrast to the previously reported intein system for Lacrt purification [14], LV96 fermentation yielded more than 40 mg/l using the inverse transition cycling purification approach followed by size exclusion chromatography at high purity (Fig. 1B). Interestingly, SDS-PAGE analysis of purified LV96 (Supplementary Fig. S1) suggested the spontaneous cleavage of ELP (V96) from the fusion construct, which yielded a combination of fusion protein and the ELP tag after purification. After optimization, a size exclusion chromatography was optimized to remove free ELP tags as a final purification step (Supplementary Fig. S1). The internal Lacrt control was liberated from the ELP tag via thrombin cleavage. Similar to previous reports [23], Lacrt ran higher on SDS-PAGE than the expected M.W. of 12 kDa (Fig. 1B); however, its expected mass was confirmed by mass spectrometry (Table 1).

Optical density was used to characterize the phase behavior for all three constructs (Table 1), which revealed that only LV96 and V96 phase separate at physiological temperatures (Fig. 1C). The phase separation for LV96 was similarly confirmed using confocal microscopy and also dynamic light scattering (Supplementary Fig. S2) The LV96 phase transition curve at 25 μM (Fig. 1C) was consistent with the phase transition behavior of the parent V96 with an ~5 °C decrease. Further characterization of the concentration–temperature phase diagrams shows that LV96 is less dependent on concentration compared to V96 as it forms a hexagonal phase at 50 μM (Fig. 1D).

Therefore, the cleavage half-life of disappearance for Lacrt is about one day (Fig. 1C).

2.13. Statistical analysis

All experiments were replicated at least three times. Values are expressed as the mean ± SD. For $\beta$-hexosaminidase secretion, data were analyzed using two-way ANOVA followed by the Bonferroni post-hoc analysis (GraphPad Prism). Within each experiment, each treatment was performed in triplicate and three independent experiments have been performed (n = 9). For LCAC uptake studies, treatments were performed in triplicate and three representative acini in each plate were compared (n = 9). Data were then analyzed using two-way ANOVA followed by Tukey’s multiple comparisons test (GraphPad Prism). For mouse tear secretion studies, each treatment was performed on three mice and three independent experiments were performed (n = 9). The results were analyzed using one-way ANOVA followed by Tukey’s post-hoc test (GraphPad Prism). To evaluate protein retention in the LG, each time point was repeated in three LGs and a representative image was shown. Three sections from each sample were imaged and quantified using ImageJ (n = 9). Data were then analyzed using two-way ANOVA followed by the Bonferroni post-hoc analysis (GraphPad Prism). A p value less than 0.05 was considered statistically significant.

3.2. Controls

Lactacystin (LC594, 10 μM), a proteosome inhibitor, and 10 μM PD98059, a specific MEK inhibitor, was added to the cells for 4 h before and during the stimulation. Western blotting with the anti-Lacrt antibodies (Supplementary Fig. S4) showed that LC594 down regulates Lacrt expression, while PD98059 had little effect. These results are consistent with previous reports on the role of the proteosome in Lacrt regulation.

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cocktail (Supplementary Fig. S3). Despite this apparent biodegradation, by optimizing the purification strategy and maintaining proteins on ice, both Lacrt and LV96 were available at high purity and yields necessary for further study (Fig. 1B).

3.2. LV96 stimulates β-hexosaminidase secretion from primary rabbit LGACs

The concentration-dependent prosecretory activity of recombinant human Lacrt was first reported using freshly isolated rat lacrimal acinar cells with peroxidase as the marker of secretory activity [14]. Signaling was effective in cells exposed to 10 to 20 μM Lacrt as a coating solution and at 0.8 to 13 nM when presented as soluble Lacrt. The latter was confirmed in assays of several cultured human cell lines over a broad dose range [18]. Yet, Lacrt partially purified from monkey tears is apparently optimal at 1 μM. Further in vivo studies indicated that 0.8 to 8 μM Lacrt topically administered either as a single dose or chronically over two weeks elevated basal tearing of healthy New Zealand White adult female rabbits [46]. Rabbit LGACs do not secrete peroxidase; therefore, we monitored β-hexosaminidase secretion, a robust secretory marker, from primary rabbit LGACs treated with a similar dose range for Lacrt or LV96 (Fig. 3). For all secretory studies, the small molecule carbachol (CCh) was used as a positive control. CCh acts on a broad spectrum of muscarinic and nicotinic acetylcholine GPCRs, including targets in the LG. This non-specificity makes CCh a poor therapeutic; however, as a positive control it can be used to assay maximal prosecretory capacity for the LG. With respect to no treatment or CCh, three treatment groups (Lacrt, LV96 and V96) were evaluated at four concentrations (0.1 to 20 μM) using a two-way ANOVA followed by Bonferroni post-hoc analysis (GraphPad Prism). Each treatment was performed in triplicate and three independent experiments were performed (n = 9). Compared to V96, the LV96 coacervate significantly stimulated secretion at a concentration of 10 μM (p < 0.01) and 20 μM (p < 0.001) while significant Lacrt-triggered stimulation was observed at 20 μM (p < 0.05) (Fig. 3B); the effects of either Lacrt or LV96 at 1 μM or 0.1 μM were not statistically significant. This data suggests that receptors exist on rabbit LGACs that respond to human Lacrt delivered by an ELP fusion.

In response to secretagogues, LGACs exocytose mature secretory vesicles containing tear proteins at their apical membranes for release into the acinar lumen, an event that involves F-actin remodeling around secretory vesicles at the luminal region [47]. Motivated to understand the cellular mechanisms of LGAC secretory activity in response to LV96, cellular morphology was tracked in live LGACs transduced with adenovirus Ad-LifeAct-RFP to observe changes in F-actin filament rearrangement at the apical and basolateral membranes during exocytosis (Fig. 3C). CCh acutely increased significant F-actin filament turnover and promoted transient actin coat assembly around apparent fusion intermediates in 15 min, as previously reported [47]. In contrast, LV96 exhibited a slower and sustained effect on F-actin remodeling, which triggered increased irregularity in the actin filaments around the lumen and formation of actin-coated structures beneath apical membranes of LGACs (white arrows) after 20 min. No significant remodeling of actin filaments was observed in the V96 control group. This data confirms that LV96, even when incubated above its phase transition temperature, induces F-actin remodeling in rabbit LGACs, which is consistent with their secretion of β-hexosaminidase (Fig. 3B).

3.3. Fusion with V96 influences cellular uptake of exogenous Lacrt into LGACs

Secreted by LGAC, transported via ducts and deposited onto rapidly renewing ocular surface epithelia, Lacrt is thought to be preferentially...
mitogenic or prosecretory for the cell types that it normally contacts during its glandular outward flow, such as the corneal, limbal and conjunctival epithelial cells, meibomian and lacrimal epithelium, retina, and retinal pigmented epithelium/choroid [48]. Yet, no previous studies have captured the real-time binding and transport of Lacrt in live cells. Herein, live-cell confocal microscopy was used to document the time-dependent uptake of exogenous Lacrt and LV96 in rabbit LGACs (Fig. 4). Binding of native Lacrt to the basolateral membrane of LGAC was observed from 10 min of exposure (Fig. 4A). Significant levels of fluorescent puncta were observed in the cytosol after 30 min. Interestingly, there was an increase in accumulation in the apical lumen (white *), which suggests possible transcytosis of Lacrt from the basolateral to the apical membranes in LGACs. Similarly, LV96 was observed in intracellular puncta within LGACs, while LV96 treated cells showed lower levels of basolateral staining than Lacrt (Fig. 4B). The unmodified ELP, V96, did not undergo significant internalization into these cells.

Table 2

Representative Lacritin cleavage sequences identified by MALDI-TOF.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Amino acid sequence*</th>
<th>Exp. M.W. [kDa]</th>
<th>Obs. M.W. [kDa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GEDASSDSTCDPQAEQGTSKPNIEISGPAEPASPPPETTATQETSAAAVQCTAKVTSSRQELN</td>
<td>12.84</td>
<td>12.96</td>
</tr>
<tr>
<td>2</td>
<td>GEDASSDSTCDPQAEQGTSKPNIEISGPAEPASPPPETTATQETSAAAVQCTAKVTSSRQELN</td>
<td>11.84/11.97</td>
<td>12.00</td>
</tr>
<tr>
<td>3</td>
<td>GEDASSDSTCDPQAEQGTSKPNIEISGPAEPASPPPETTATQETSAAAVQCTAKVTSSRQELN</td>
<td>11.25/11.38</td>
<td>11.35/11.47/11.50</td>
</tr>
<tr>
<td>4</td>
<td>PNEISGPAEPASPPPETTATQETSAAAVQCTAKVTSSRQELN</td>
<td>6.42</td>
<td>6.36</td>
</tr>
</tbody>
</table>

* Underlined sequence: Syndecan-1 binding site.
* Expected M.W. (kDa) for fragments was calculated by DNAStar Lasergene Editseq.
* Observed M.W. (kDa) was measured by MALDI-TOF.

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3.4. LV96 stimulates tear secretion from non-obese diabetic (NOD) mice

To explore Lacrt’s efficacy as a secretagogue in a murine model, Lacrt and LV96 were administered into non-obese diabetic (NOD) mice via intra-lacrimal injection and assessed for tear secretion (Fig. 5A). NOD mice have been established as a model for type-1 insulin-dependent diabetes mellitus [49] and one of the most utilized models for the study of DED symptomatic of Sjögren’s syndrome [50–52], which is characterized by reduced production of aqueous tears [53] and autoimmune infiltration of LGs and salivary tissues [54]. Paradoxically, although Sjögren’s syndrome is more prevalent in female patients, the 10–12 weeks of age female NOD mice maintain normal LG morphology [54–56] and increased expression of matrix metalloproteinases [56]. At 10–12 weeks of age, female NOD mice maintain normal LG morphology [54–56] and increased expression of matrix metalloproteinases [56].

Using 10–12 week old NOD mice, four treatments were compared in an acute tear secretion study: CCh, V96, LV96 and Lacrt. Within each experiment, treatments were performed on three mice, and this experiment was repeated three times (n = 9). In each mouse, both LGs were treated. The tears were collected for 30 min from each eye and pooled to obtain the volume secreted per mouse. Results were then analyzed using a one-way ANOVA followed by Tukey’s post-hoc test (GraphPad Prism). Immunohistochemistry revealed that intra-lacrimal injection of LV96 generates a local depot that is positive for human Lacrt immediately after injection (Fig. 5B). This depot was independent-ly observed following intra-lacrimal injection into healthy mice, which revealed significantly more staining for LV96 than for free Lacrt (Supplementary Fig. S4). In normal mice, there was a small increase in tear volume for LV96 compared to the control group, however, it was not significant (Supplementary Fig. S4). In contrast, for the NOD disease model LV96 and free Lacrt produced strong prosecretory activity in both LG, including severe lymphocytic infiltration (Fig. 5C), decreased production of lacrimal fluid, significant extracellular matrix degradation and increased expression of matrix metalloproteinases [55]. At 10–12 weeks of age female NOD mice maintain normal LG morphology (Fig. 5D); however, they do develop severe pathology in salivary tissues [54].

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Using 10–12 week old NOD mice, four treatments were compared in an acute tear secretion study: CCh, V96, LV96 and Lacrt. Within each experiment, treatments were performed on three mice, and this experiment was repeated three times (n = 9). In each mouse, both LGs were treated. The tears were collected for 30 min from each eye and pooled to obtain the volume secreted per mouse. Results were then analyzed using a one-way ANOVA followed by Tukey’s post-hoc test (GraphPad Prism). Immunohistochemistry revealed that intra-lacrimal injection of LV96 generates a local depot that is positive for human Lacrt immediately after injection (Fig. 5B). This depot was independently observed following intra-lacrimal injection into healthy mice, which revealed significantly more staining for LV96 than for free Lacrt (Supplementary Fig. S4). In normal mice, there was a small increase in tear volume for LV96 compared to the control group, however, it was not significant (Supplementary Fig. S4). In contrast, for the NOD disease model LV96 and free Lacrt produced strong prosecretory activity in both LG.
males and females (Fig. 5E, F). Compared to CCh stimulation (100%), LV96’s prosecretory effect was 40.9% in males and 50.0% in females, both significantly higher than V96 treatment (p < 0.01). Lacrt’s efficacy was 29.6% in males and 42.9% in females. This data confirms the surprising in vitro finding (Fig. 3) that the phase separation of LV96 does not inhibit Lacrt-specific activity. Due to the acute nature of this assay, it was not expected that LV96 would produce a greater tear volume than free Lacrt.

To differentiate the in vivo potential of LV96 and free Lacrt, it was necessary to follow the LG up to a day after intra-lacrimal injection. In humans, the inferior palpebral lobe of the lacrimal gland is accessible for injection beneath the eyelid, which makes it a clinically relevant location for sustained release formulations. Unfortunately, in a murine model injection of the lacrimal gland requires surgical exposure of the injection site. This invasive procedure near the eye makes it challenging to attribute differences in basal tear production to the formulations. Therefore, to obtain evidence supporting the sustained retention of LV96, as compared to free Lacrt, the LG biodistribution of rhodamine labeled LV96 and Lacrt were assessed as a function of time at 2, 4, and 24 h after intra-LG injection (Fig. 6). Typically, eight slices (thickness: 8 μm/slice) of each sample with an interval of 80 μm were imaged to reflect the protein distribution in the whole LG. Each treatment and time point has been repeated in three independent LGs. Fluorescence intensity of the entire view or within a defined area was quantified using ImageJ. As shown in Figs. 6A, 2 h after implantation LV96 remained in a deposit at the site of injection, which was more intense compared to Lacrt alone (Fig. 6A, B). This effect was quantified by image analysis (Fig. 6C). After 24 h LV96 coacervates remained obvious and showed little decrease in fluorescent intensity. In contrast, signal from free Lacrt was undetectable after 2 h (Fig. 6C). In addition, the depot maintains significant signal both at the center of the injection and also at a reference point taken 300 μm away (Fig. 6D, E). Between 4 and 24 h the intensity taken at the reference point changed minimally, which suggests the possibility that fluorescent LV96 is being released from the depot. In contrast, free Lacrt was not observed anywhere within the gland at 4 or 24 h.

4. Discussion

The eye is now a frequent target for development of new drugs, especially novel biological therapies [56] due to the increased numbers of...
patients with aging, ocular allergies and DED [57]. Local ocular delivery provides unique opportunities to enhance the therapeutic index of ophthalmic drugs by extending local residence time while minimizing off-target effects and dose frequency [58]. Over the past several decades, protein therapeutics have become highly successful because of their high target specificity, reduced interference with normal biological processes and minimal immune responses to human self-proteins [59]. The discovery of Lacrt offers a new therapeutic opportunity for DED and an alternative to conventional approaches [12]. Its basic structural features [12], prosecretory and mitogenic significance [14], as well as associated downstream signaling transduction mechanisms [18,20] have been gradually elucidated over the past decade.

As a proof of concept, this study characterizes a thermo-responsive Lacrt–ELP fusion protein for extended retention. The ELP V96 was fused to Lacrt to confer multiple functions: i) re-engineer Lacrt with the ability to form an intra-lacrimal depot at physiological temperatures; ii) to maintain Lacrt-mediated cell signaling. Together, these properties support the further development of Lacrt or other biologicals into sustained-release biopharmaceuticals for ophthalmology. The transition temperature (Fig. 1C, D) and thermo-responsive assembly of LV96 (Supplementary Fig. S2) supports the hypothesis that Lacrt fused to an ELP exhibits similar phase separation and self-assembly properties relative to the parent ELP. Significantly enhanced β-hexosaminidase secretion and actin remodeling from primary rabbit LGACs (Fig. 3) and increased tear secretion from both male and female NOD mice (Fig. 5) corroborated the prosecretory activity of LV96, even above its phase transition temperature. Despite having similar prosecretory activity, cellular internalization studies revealed a distinctly slower pattern of uptake for LV96 cocervates compared to free Lacrt (Fig. 4). Based on this assessment, the microbiodistribution of LV96 following intra-lacrimal administration was characterized via indirect immunofluorescence (Fig. 5B, Supplementary Fig. S4) and by covalent labeling (Fig. 6). These data definitively show that Lacrt fused to an ELP maintains significantly more fluorescence than free Lacrt at all times post-injection. In other disease models, it was recently shown that phase separation of ELPs in a tumor slowed the local half-life of clearance by more than an order of magnitude [60]. Similarly, extended control over blood glucose level was observed using a depot of a therapeutic ELP [61]. Thus, the ocular data presented here support the hypothesis that Lacrt fused to an ELP remains prosecretory both in vitro and in vivo; furthermore, its ability to form a local depot is consistent with previous literature in other disease models.

Interestingly, Lacrt demonstrated a susceptibility to protease degradation based on MALDI-TOF analysis (Fig. 2B) and time-dependent analysis of degradation by SDS-PAGE (Fig. 2C), which together suggest that native Lacrt has a cleavage half-life of about one day at 37 °C [94] (Fig. 2D). The biodegradation of Lacrt was consistent with the generation of peptides that were cleaved between lysine residues found in human Lacrt (Table 2). Trypsin-like serine proteases cleave peptide bonds next to lysine or arginine residues, with serine performing the nucleophilic attack and negatively charged aspartic acid controlling the specificity [62–64]. In silico analysis by the Protease Specificity Prediction Server suggested Lacrt’s serine protease sensitivity liberates the C-terminal amphipathic α-helix intact for downstream co-receptor binding to syndecan-1 (Table 2) [65]. Recent reports suggest that this proteolysis releases an α-helical carboxy-terminal peptide from Lacrt that displays bactericidal activity, which may represent an innate defensive immunity on the ocular surface [66]. The cleavage may be regulated by serine proteases, as specific protease inhibitors (chymostatin, leupeptin) or boiling were reported to inhibit proteolysis. This report confirms that this proteolytic activity can be inhibited by a standard cocktail of protease inhibitors (Supplemental Fig. S3). This proteolysis was also observed for LV96 during purification (Supplemental Fig. S1). To maintain a single band by SDS-PAGE for purified LV96 (Fig. 1B), same-dilute aliquots were frozen after purification and thawed on ice. Future studies are required to determine the source of this proteolysis, which may be attributed to either: i) trace proteases from bacterial fermentation; or ii) the possibility that Lacrt exhibits autolytic activation similar to trypsin [67]. In addition, the impact of glycosylation or other regulatory mechanisms to control the cleavage of native Lacrt remain to be determined.

Previously, Lacrt’s in vivo prosecretory activity has been reported using New Zealand white rabbits via topical administration [16]. Recently, Vijmansi et al. tested Aire-knockout mice and proved that a long-term topical Lacrt treatment promoted tear secretion, restored ocular surface integrity, and reduced CD4 + T cell infiltration of the LG [17]. Aire-knockout mice are an aqueous-deficient dry eye mouse model that is deficient in the autoimmune regulator (Aire) gene. This model was derived from the non-obese diabetic (NOD) and Balb/c mice backcross and is highly efficient in the autoimmune regulator (Aire) gene. This model was derived from the non-obese diabetic (NOD) and Balb/c mice backcross and is highly efficient in the autoimmune regulator (Aire) gene.
known to bind specifically to heparanase activated syndecan-1 [20]. Vijmani and coworkers hypothesized that via topical administration, Lacrt may play a functional role in maintaining corneal innervation during homeostasis, interrupting or mitigating the inflammatory cycle, and thus protecting the LG from focal infiltration of auto-antigen primed CD4+ T cells [17]. This report provides the first in vivo evidence of Lacrt’s prosecretory effect upon direct interaction with the LG. Further investigations regarding syndecan-1 and heparanase expression level in different mice LGs, Lacrt’s affinity towards receptors expressed in the LG, its neuronal stimulation mechanism and differences among various mice strains will be necessary to better understand the mechanism of Lacrt therapies.

Exhibiting prosecretory, mitogenic, cytoprotective and bactericidal functions, Lacrt may recruit different signaling pathways for each of these activities. For example, previous reports have explored intracellular ca\textsuperscript{2+} changes in response to Lacrt, which is readily evident in the corneal epithelium [39]. Being an important early messenger in signal transduction cascades, ca\textsuperscript{2+} can indicate different signals by changing its transient cytosolic oscillation frequency [68]. In the LG acinar cells, cholinergic agonists stimulate protein secretion by binding to receptors in the basolateral membrane of secretory cells and activating phospholipase C to break down phosphatidylinositol bisphosphate into 1,4,5-inositol triphosphate (1,4,5-IP\textsubscript{3}) and diacylglycerol (DAG). 1,4,5-IP\textsubscript{3} causes intracellular release of ca\textsuperscript{2+}, which works together with calmodulin and further activates specific protein kinases that may be involved in secretion [69]. Interestingly, of all the treatment groups tested herein, only carbachol (CCh) triggered cytoplasmic ca\textsuperscript{2+} concentration change in LGACs (Supplementary Fig. S5), while no significant signals were observed in EGF, Lacrt or LV96 treatment groups. Consistent with this observation, Sanghi and coworkers discussed a similar loss of ca\textsuperscript{2+} activation in Lacrt’s prosecretory activity pathway [14]. Although Lacrt and LV96 only exhibited 10–30\% of the control CCh response in the β-hexosaminidase secretion assay (Fig. 3B), these findings are consistent with the possibility that Lacrt may recruit alternative pathways for its prosecretory function. While CCh acts on a broad spectrum of muscarinic acetylcholine G-protein coupled receptors (GPCRs) as well as nicotinic acetylcholine receptors (ligand-gated ion channels), Lacrt’s signaling pathway may be more specific.

This specificity may be due to its involvement with deglycanated syndecan-1, which enables it to act on an as of yet unidentified GPCR [12]. The carboxy-terminal amphiphilic α-helix of Lacrt has been reported to associate with co-receptor syndecan-1, which thus regulates functional specificity [20] and maintains corneal epithelium homeostasis [19]. New data suggested that the same region undergoes proteolytic processing and demonstrates crucial bactericidal activity in tears [66]. Similar to our recent reports in SV40-transduced human corneal epithelial cells (HCE-Ts) [39], Lacrt triggers a cascade of mitogenic events involving G\textsubscript{o}i or G\textsubscript{o}–PKC\textsubscript{α}–PLC–Ca\textsuperscript{2+}–calcinurin–NFATC1.

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The concept of a ‘reservoir drug’ [70] is intended to tackle the problem of inefficient target delivery and rapid payload clearance. By modulating the precise location and residence time of the therapeutic agent, side effects (such as those produced by CCh) can be reduced and efficacy may be enhanced [71]. ELPs have shown promise as reservoir scaffolds [30,34], and now provide an emerging alternative to PLGA [72] or catechol-based gels [73]. Prior to this study, it was unclear if polymer modification and phase separation would sterically hinder Lacrt and thus impair its activity. Thus, we have now verified both in vitro and in vivo that elastin-like polymers are equivalent in potency to free Lacrt. Moreover, the phase separation of the V96 tag slows cellular uptake and promotes retention in the LG. Based on this report, and of those noted above for free Lacrt, we can propose a mechanism of action for an LV96 drug depot (Supplementary Fig. S6); however, additional studies will be required to confirm this model. Further research of Lacrt ELP fusions are required to explore the local and systemic pharmacokinetics as well as the molecular mechanisms of Lacrt release, receptor-mediated binding, and cellular signaling involved with tear secretion.

5. Conclusion

Achieving sustained delivery of therapeutic proteins is one of the major challenges of ophthalmology. In pursuing this goal, ELPs were fused with the model dry eye disease biopharmaceutical, Lacrt. Lacrt-ELP fusion proteins demonstrated thermo-responsive phase separation, similar to that exhibited by the parent ELPs. They also gained the prosecretory activity of human Lacrt. ELP modifi ed proteins displayed thermo-responsive phase separation, macokinetics as well as the molecular mechanisms of Lacrt release, and sustained basal tearing and is well tolerated, Invest. Ophthalmol. Vis. Sci. 52 (2011) 6265–6270.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2014.11.016.

References
