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The humanin peptide mediates ELP nanoassembly and protects human retinal pigment epithelial cells from oxidative stress

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Abstract

Humanin (HN) is a hydrophobic 24-amino acid peptide derived from mitochondrial DNA that modulates cellular responses to oxidative stress and protects human retinal pigment epithelium (RPE) cells from apoptosis. To solubilize HN, this report describes two genetically-encoded fusions between HN and elastin-like polypeptides (ELP). ELPs provide steric stabilization and/or thermo-responsive phase separation. Fusions were designed to either remain soluble or phase separate at the physiological temperature of the retina. Interestingly, the soluble fusion assembles stable colloids with a hydrodynamic radius of 39.1 nm at 37 °C. As intended, the thermo-responsive fusion forms large coacervates (> 1,000 nm) at 37 °C. Both fusions bind human RPE cells and protect against oxidative stress-induction of apoptosis (TUNEL, caspase-3 activation). Their activity is mediated through STAT3; furthermore, STAT3 inhibition eliminates their protection. These findings suggest that HN polypeptides may facilitate cellular delivery of biodegradable nanoparticles with potential protection against age-related diseases, including macular degeneration.

Graphical Abstract

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Humanin (HN), a 24-mer peptide of mitochondrial origin, elicits multiple cellular functions and is a potential therapeutic. When recombinantly fused to an elastin-like polypeptide (ELP), HN peptide mediates assembly of spherical nanostructures with lower critical solution behavior. These nanoparticles maintain their ability to recognize and protect human retinal pigment epithelial (RPE) cells against oxidative stress-induced apoptosis through STAT3 mediated pathway, suggesting their potential applications in ocular diseases.

Keywords

protein-polymer; mitochondria-derived peptide; dynamic light scattering; lower critical solution temperature; nanoparticle tracking analysis

1. Background

Mitochondria are key players in aging and in the pathogenesis of age-related diseases.[1–4] Recent studies on the human mitochondrial genome revealed the existence of a family of peptides encoded in open reading frames.[5] Among these a 24-amino acid peptide named humanin (HN) became the first mitochondrial-derived peptide to be discovered. It plays diverse roles across many biological processes, including apoptosis, substrate metabolism, inflammation, and cell stress response.[6–8] Based on these observations, HN has become an attractive therapeutic candidate. Our team recently showed that HN and its receptors are expressed in human retinal pigment epithelium (RPE) cells.[9] RPE cells are the melanin-pigmented cells at the posterior segment of the eye that actively protect the retinal neuro-receptors from photo-oxidative stress. Exogenous treatment with free HN protects RPE cells from oxidative stress-induced cell death, an important causative factor in wet and dry age-related macular degeneration (AMD). Unlike wet AMD, which is responsive to anti-angiogenic therapies, dry AMD has no approved therapy. This suggests that HN and related peptides have therapeutic potential for dry AMD. Despite potential applications across various diseases, a major *in vivo* barrier to HN therapies relates to the rapid clearance and poor stability of small peptides. Due to the unique anatomical and physiological barriers of the eye, the most effective way to deliver HN peptides to the retina is via intravitreal injection. Given the low retention time of small peptides, frequent dosing is often required to

maintain therapeutic effects in the vitreous or at the site of action, which may lead to undesired side effects and poor patient compliance.

To circumvent this limitation, we here propose a new delivery system by recombinantly fusing the HN peptide onto a high molecular weight protein-polymer to increase its apparent aqueous solubility and restrict its mass transport away from the site of administration. Composed of repeated motifs often inspired by naturally observed polypeptides, protein-polymers offer potential advantages of being biocompatible, biodegradable, and genetically encodable.[10] Among protein-polymers are the elastin-like polypeptides (ELPs), which are derived from the human tropoelastin protein.[11–15] The canonical ELP described by Dan Urry consists of a pentameric repeat of (Val-Pro-Gly- X_{aa} -Gly) $_n$, where X_{aa} can be any amino acid and n determines the molecular weight.[16] A primary feature of ELPs involves their ability to phase separate above a transition temperature (T_t) that can be tuned through the selection of guest residue X_{aa} and the number of repeats n . Taking advantage of this property, HN peptide has been engineered onto two ELPs of similar molecular weight with distinct solubility (soluble vs. insoluble) at physiological temperature (Table 1). The ELP V96 forms viscous coacervates with a T_t below 37 °C and thus was chosen as a backbone for depot formation, whereas S96 serves as a soluble carrier. Compared to unmodified ELPs, the addition of HN retains temperature-dependent phase separation yet promotes peptide-mediated nanoparticle assembly even below the T_t . This report describes how these HN-ELP fusions actively recognize and confer protection upon RPE cells against oxidative stress-induced apoptosis by inhibiting caspase-3 activation.

2. Methods

2.1 ELP gene design and construction

The pET25b(+) vectors containing genes for ELPs (V96/S96) were constructed by recursive directional ligation as described previously.[17] A DNA sequence encoding humanin peptide was optimized using the best *E.coli* codons in EditSeq (DNASTar, Madison, WI). The following oligonucleotides were synthesized (Integrated DNA Technologies, Coralville, IA) and cloned into the pET25b(+) vector via the BseRI restriction site: 5'-GATGGCTCCGCGTGGTTTCTCTTGCCCTGCTGCTGCTGACCTCTGAAATCGACCTGCCGGTTAAACGTCGTGCTGG -3' (forward) and 5'-AGCACGACGT TTAACCGGCAGGTCGATTTTCAGAGGTCAGCAGCAGCAGGCAAGAGAAACCACGC GGAGCCATCCC -3' (reverse). Success cloning of the fusion proteins was verified by DNA sequencing (Retrogen, San Diego, CA).

2.2 ELP expression and purification

Plain ELPs and the HN-ELPs were expressed in ClearColi BL21(DE3) electrocompetent cells (60810, Lucigen) following manufacturer's protocol and purified using the inverse transition cycling (ITC). To determine the concentration, purified protein in PBS was mixed with the same volume of 6M Guanidine hydrochloride and assayed for absorbance using Nanodrop (Thermo Fisher Scientific, Waltham, MA). The final concentration is calculated using Beer Lambert's law:

$$C_{ELP} = \frac{(A_{280} - A_{350})}{\epsilon \times l} \quad \text{Eq. 1}$$

where C_{ELP} is the molar concentration, A_{280} and A_{350} are absorbance at 280 and 350 nm respectively, l is the light path length in centimeters, and ϵ is an estimated molar extinction coefficient at 280 nm ($1,285 \text{ M}^{-1}\text{cm}^{-1}$ for V96 or S96; $1,410 \text{ M}^{-1}\text{cm}^{-1}$ for HN-V96 or HN-S96). Protein purity and identity were further determined by SDS-PAGE gels (Lonza, Morristown, NJ) stained with GelCode Blue Safe Protein Stain (Thermo Fisher Scientific, Waltham, MA). Samples were probed by Western blot using an anti-humanin antibody.⁹ Protein molecular weight was confirmed by MALDI-TOF analysis through Proteomics and Metabolomics Facility at Colorado State University. The endotoxicity burden was also verified to be less than 6 EU/mL using the HEK-Blue hTLR4 cell line from InvivoGen (San Diego, CA)

2.3 Characterization of ELP phase behavior

The phase diagrams for purified ELPs were characterized by measuring the optical density as a function of temperature and concentration. ELPs in PBS with concentrations ranging from 5 to 100 μM were loaded in thermal mount microcells and measured for absorbance at 350 nm using a DU800 UV visible spectrometer (Beckman Coulter) while the temperature was ramped from 10 $^{\circ}\text{C}$ to 80 $^{\circ}\text{C}$ at a constant rate of 1 $^{\circ}\text{C}/\text{min}$. The maximum first derivative of the optical density vs. temperature curve was defined as the transition temperature T_t . The data points were then fit to the following equation:

$$T_t = b - m \text{Log}_{10}[C_{ELP}] \quad \text{Eq. 2}$$

where C_{ELP} is the ELP concentration, m is the slope and b is the intercept transition temperature at 1 μM .

2.4 Light scattering analysis of ELP assembly

Two light scattering analyses were performed to determine the assembly properties of ELPs. The hydrodynamic radius (R_h) was monitored using dynamic light scattering (DLS).[18, 19] Briefly, 25 μM ELPs in PBS were filtered through 0.2 μm Supor membrane filters (Pall laboratory, Westborough, MA) and measured using a DynaPro plate reader (Wyatt Technology Inc., Santa Barbara, CA) over a range of temperatures from 10 to 40 $^{\circ}\text{C}$ in 1 $^{\circ}\text{C}$ increments. The results were analyzed using a Rayleigh sphere model and fitted into a regularization algorithm using the instrument's software.

To further estimate the molecular weight and coordination number of the ELP assemblies, soluble HN-S96 nanoparticles were analyzed using size exclusion chromatography with multi-angle light scattering (SEC-MALS). Briefly, 100 μL of 25 μM ELPs was injected onto a Shodex PROTEIN KW-803 column (Showa Denko America, New York, NY) equilibrated with PBS at a flow rate of 5 mL/min. Elution from the column was analyzed by the following in-line detectors: i) UV absorbance at 210 nm (SYS-LC-1200, Agilent Technologies, Santa Clara, CA); ii) multi-angle light scattering (DAWN HELEOS, Wyatt Technology, Santa Barbara, CA); and iii) differential refraction (Optilab rEX, Wyatt

Technology, Santa Barbara, CA). Data analysis and molecular weight calculations were performed using ASTRA 6 software (Wyatt Technology, Santa Barbara, CA). To evaluate stability of HN-S96 nanoparticles, filtered samples were incubated at 4 °C and 37 °C for four days and analyzed for their hydrodynamic diameter and zeta potential in 1 % phosphate buffered saline diluted into water using a ZetaView particle tracking analyzer (PMX-120, Particle Metrix GmbH, Germany).

2.5 Transmission electron microscopy (TEM) imaging

The TEM imaging was carried out on a JEM-2100 Transmission Electron Microscope (JEOL USA, Peabody, MA) at 80 kV.[20] Samples were prepared by mixing a 25 µM HN-S96 solution with same volume of 2% uranyl acetate and then deposited on a gold grid. Excess solution was removed by filter paper. Grids were dried at room temperature for 30 minutes prior to imaging.

2.6 Retinal pigment epithelial (RPE) cell culture

All experiments and procedures were conducted in compliance with the tenets of the Declaration of Helsinki and Association for Research in Vision and Ophthalmology (ARVO) guidelines. RPE cells were isolated from human fetal eyes obtained from Advanced Bioscience Resources Inc. (Alameda, CA) and maintained in DMEM supplemented with 10% FBS as previously described.[9] Cell cultures from passages 2 to 4 were used for cell protection, uptake and STAT3 inhibition assays.

2.7 Protection of RPE cells from oxidative stress

The anti-apoptotic activity of HN-ELPs was evaluated using TUNEL staining and immunofluorescent detection of cleaved caspase-3 in RPE cells challenged with tert-Butyl Hydroperoxide (tBH).[9] Briefly, confluent RPE cells grown on 4-well chamber slides were co-treated with 10 µM of ELPs (S96, V96, HN-S96, HN-V96) plus 150 µM tBH or 150 µM tBH alone for 24 h in serum-free medium. For TUNEL staining, cell death was accessed using an *In Situ* Cell Detection Kit (Roche Applied Science, Indianapolis, IN) and nuclei were stained with DAPI. The TUNEL-positive cells were then counted and data were presented as percent of total cells undergoing cell death. Activated caspase-3 was detected using a rabbit antibody against cleaved caspase-3 (#9661, Cell Signaling Technology, Danvers, MA) followed by a fluorescein-conjugated anti-rabbit secondary antibody. Images were obtained using a Keyence fluorescence digital microscope (Keyence, Itasca, IL).

2.8 Cellular uptake assay

Confluent RPE cells were serum starved overnight and treated with 10 µM ELPs (S96, V96, HN-S96, HN-V96) in DMEM for indicated time periods at 37 °C. To label mitochondria, MitoTracker Red CMXRos (500 nM, Thermo Fisher Scientific, Waltham, MA) was added to the medium 15 minutes before cessation of treatment. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Primary antibody staining was done using the mouse-derived AK1 monoclonal anti-ELP antibody[21] (1:300 dilution) followed by an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:200 dilution, #A11001, Thermo Fisher Scientific, Waltham, MA). Nuclei were stained with

DAPI, and images were captured on a LSM800 confocal laser-scanning microscope (Carl Zeiss Microscopy, Thornwood, NY) with a Plan-Apochromat 63x oil objective.

2.9 STAT3 inhibition study

The effects of HN-ELPs on STAT3 pathway activation and inhibition by STAT3 inhibitor VII were studied using immunoblotting, immunofluorescence and TUNEL. Confluent RPE cells were serum starved overnight and treated with 10 μ M ELPs (S96, V96, HN-S96, HN-V96) in DMEM for 4 hours. For STAT3 inhibition experiments, 90% confluent RPE cells were pre-treated for 2 h with 0.5 μ M STAT3 inhibitor VII (Calbiochem, San Diego, CA, USA) followed by co-incubation with 10 μ M HN-ELPs, 150 μ M tBH, and 0.5 μ M inhibitor. More details are included in the supplementary data file.

2.10 Statistics

Data presented are representative curves or mean $\pm$ SD. All experiments were replicated at least three times. Statistical analysis was performed using either a non-paired t-test or one-way ANOVA followed by Tukey's *post hoc* test. A p value less than 0.05 was considered statistically significant.

3. Results

3.1 Construction and purification of ELPs fused to the HN peptide

Two ELPs were evaluated in this study as potential carriers for the HN peptide (Table 1, Figure 1a,b). Both V96 and S96 have 96 pentameric repeat units and share similar molecular weight; however, they vary in the hydrophobicity of their guest residue *X*. Based on these differences, it was expected that these two fusions result in different T_t . [17] Purification of all four ELPs from bacterial lysate was conducted using a chromatography-free protocol of inverse transition cycling, yielding ~40 mg proteins per liter culture. Purified materials were further characterized using SDS-PAGE stained with Coomassie blue and Western blot probed by an HN antibody (Figure 1b, c). Each of the fusion proteins appears as a major band around 40 kDa, which corresponds to the predicted and observed molecular weight as confirmed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) (Table 1).

3.2 HN peptide shifts the phase diagram differently for V96 and S96

To characterize the phase behavior of ELP fusions, optical density was measured as a function of temperature over a range of concentrations from 5 to 100 μ M (Figure 2). Soluble ELPs have low optical density below their T_t . When heated above T_t , ELPs coacervate into droplets that scatter light and sharply increase solution turbidity (Figure 2a–e). As observed for other ELPs, [17] concentration-temperature phase diagrams for all four constructs follow a log-linear relationship (Figure 2c, f, Table 1). V96, which contains the relatively hydrophobic guest residue valine, phase separates above 30 °C. The addition of the humanin peptide decreases the transition temperature to ~22 °C. In contrast, S96 phase separates between 50 °C and 63 °C but HN fusion increases the transition temperature to above 70 °C. Although the fusion proteins behave differently in terms of shifting the phase diagram, HN-

S96 and HN-V96 remain good candidates as soluble and insoluble formulations during in *vitro* characterization at physiological temperatures.

3.3 HN peptide mediates the assembly of ELP-stabilized nanoparticles

While optical density is useful in determining phase separation temperature, dynamic light scattering (DLS) is necessary to confirm the size of ELPs before and after transition. In Figure 3, both V96 and S96 remained monomeric and showed a hydrodynamic radius (R_h) of 5 nm when below their transition temperatures, whereas the addition of HN significantly increased their radius to ~40 nm. Further investigation of the size distribution using regularization analysis also supported the differences in radius as well as polydispersity between HN fusion and plain ELPs (Figure 3c, d). Both HN-V96 and HN-S96 assembled a significant population of nanoparticles (46.1 ± 0.5 nm at 15 °C, 39.1 ± 0.3 nm at 37 °C, respectively) with relatively high polydispersity at 15 °C (23.8%) and 37 °C (20.9%), respectively. In contrast, V96 and S96 controls only showed a monodisperse population with a hydrodynamic radius equivalent to monomers (4.9 ± 0.1 at 15 °C, 5.0 ± 0.1 at 37 °C, nm respectively). Since V96 and S96 themselves do not assemble structures until above their T_t , the increase in hydrodynamic radius upon fusion suggests that the HN peptide alone mediates the multimeric assembly. This is consistent with the observation that the hydrophobic HN peptide aggregates in solution.[22]

The morphology of these nanostructures was investigated using transmission electron microscopy (TEM) (Figure 4a, b). Since HN-V96 and HN-S96 showed similar size distributions below T_t and because only HN-S96 remained soluble for sample preparation at room temperature, HN-S96 alone was characterized to learn more about their shape and aspect ratio. Negative contrast images of HN-S96 confirmed their polydispersity and revealed the presence of spherical structures. Image analysis revealed they have an average radius of 25.2 ± 8.2 nm, which is slightly smaller compared to the measurements by DLS (Figure 3). The reduction in particle size could be an artifact of TEM sample preparation. Furthermore, the nanostructures of HN-S96 was also studied independently using multi-angle light scattering (Figure 4c). In addition to confirming assembly of particles with a radius of gyration (R_g) of 52.6 nm, the absolute molecular weight measured as 10,000 kDa suggests that the multimers are made up of an average of ~244 monomers. For HN-S96 at room temperature, the R_g/R_h ratio is 1.3, which is consistent with an extended, hydrated polymeric nanostructure. Formulation stability was further examined by studying the distribution of HN-S96 nanoparticles incubated at either 4 or 37 °C for 4 days. HN-V96 was not assessed as coacervates into large droplets at 37 °C (Figure 3B). As assessed by nanoparticle tracking analysis (NTA), the particle diameter and zeta potential appeared to be nearly unaffected by the incubation temperature (Table 2) and were consistent with hydrodynamic radii of particles observed by dynamic light scattering (Figure 3). Taken together, these results consistently suggest that HN peptide promotes the assembly of the fused ELPs into relatively stable spherical nanoparticles.

3.4 Exogenous HN-ELPs specifically bind and activate RPE cells

Having established that the HN-ELP fusions assemble into multimeric nanoparticles, it was unclear whether the peptide would remain accessible to cellular targets. To study this, RPE

cells were used as an *in vitro* model. Our team demonstrated that RPE cells express receptors for HN, which play an important role in the activation of STAT3 pathway.[9] Thus, the recognition of RPE cells by HN-ELPs was compared with that of unmodified ELPs as detected using an anti-ELP antibody. As shown in Figure 5a, both HN-S96 and HN-V96 bound efficiently to the RPE cells within 4 h of incubation, and this was even more evident after 24 h. In contrast, no positive signal was observed for S96 or V96 over a time period of 24 h. Interestingly, HN-V96 exhibited much stronger cell-associated fluorescence compared to HN-S96, which is consistent with its formation of large coacervates at 37 °C on the cell surface. It should be noted that the HN-ELPs associated with RPE cells did not colocalize with mitochondria, whereas our previous study observed a significant colocalization of free HN peptide with mitochondria.[9] The differences in cellular localization could be reasoned by the distinct sizes between HN-ELP fusion and the free HN peptide, which may result in different internalization trafficking. Despite this impact on cellular uptake, both HN-ELP fusions still activate STAT3, as indicated by a phosphorylation signal (Figure 5b). Similar to untreated controls, ELPs without the HN peptide failed to activate phosphorylation of STAT3.

3.5 Exogenous HN-ELPs protect RPE cells from oxidative-stress induced apoptosis

The evidence that both HN-ELPs successfully recognized and activated RPE cells, prompted us to explore the anti-apoptotic properties of these fusions using RPE cells under oxidative stress. RPE cells are particularly sensitive to oxidative stress and oxidative stress-induced RPE apoptosis is known to be a major factor in the etiology of AMD.[23, 24] Therefore, confluent RPE cultures were challenged with 150 μ M tBH and co-treated with varying concentrations of HN-S96 or HN-V96 for 24 h (Figures 6, Supplementary Figure S1). Our data revealed a dose-dependent cellular protection for both HN-S96 and HN-V96; at doses 5 and 10 μ M of fusion proteins significantly protected RPE cells against oxidative stress induced cell death ($p < 0.0001$ vs tBH treated group). However, cell death decreased significantly from 13% in 5 μ M HN-S96 group to 7% in 10 μ M HN-S96 group ($p = 0.0034$). Similar trend was also observed with HN-V96 groups where 50% more cell survival was obtained with 10 μ M HN-V96 over 5 μ M HN-V96. However, control ELPs (V96 or S96) failed to show protective activities (Figures 7a, Supplementary Figure S2a). In addition, oxidative stress induced activation of caspase-3 was reduced in HN-ELPs compared to ELPs alone (Figures 7b, Supplementary Figure S2b). Collectively, these results demonstrate that despite the involvement of the HN peptide in the assembly of peptide vesicles, they retain their intrinsic ability to bind biological targets associated with their anti-apoptotic properties and protect RPE cells from oxidative-stress induced cell death.

3.6 Cellular protection by HN-ELPs in RPE cells is mediated through STAT3 activation.

HN exerts its protective function through multiple pathways including both intra- and extra-cellular mechanisms of action.[25] Our team has demonstrated that in RPE cells HN activates both mitochondrial function as well as the STAT3 pathway.[9] However, in this study, we did not observe any evidence of mitochondrial translocation of HN-ELP nanoparticles (Figure 5a). Instead, HN-ELPs remained on the cell surface and induced the phosphorylation of STAT3 in RPE cells up to 24 h (Figure 5b). We therefore hypothesized that STAT3 plays a major role in the HN-ELP mediated protection. To access the

involvement of STAT3 in HN-ELP mediated cellular protection, RPE were pretreated for 2 h with a specific inhibitor of STAT3 (0.5 μ M), followed by co-incubation with 150 μ M tBH and 10 μ M HN-ELPs (HN-S96, HN-V96). Cell death was studied by TUNEL staining and activated caspase-3 staining. Treatment of RPE cells with tBH showed ~20% cell death (Figure 8a), which was significantly blocked by the HN-ELP fusions ($p < 0.0001$). Remarkably, the inhibition of STAT3 completely eliminated protection against TUNEL-positive cells under oxidative stress, suggesting the active involvement of STAT3 pathway in cellular protection. Consistent with STAT3 activation, caspase-3 activation was also evident in the presence of tBH treatment (Figure 8b), while co-treatment with HN-ELPs suppressed caspase-3 activation. Thus, the STAT3 pathway appears to be a major pathway involved in HN-ELP mediated cellular protection as its blockage significantly augments RPE cell death as evidenced by increased cell death and caspase-3 activation, indicating an essential role for STAT3 in HN-mediated cellular protection.

4. Discussion

Age-related macular degeneration (AMD) is the leading cause of severe and irreversible vision loss among aging populations worldwide. Thought to be a primary cause of AMD, oxidative stress is associated with RPE cell loss and photoreceptor degeneration.[26] Recent discovery of HN has created a new category of biologically active peptides with protective effects against oxidative stress.[5] As more research elucidates its physiological role, HN has emerged as a potential therapeutic for treating human diseases.[6–8]

Studies in multiple disease models reveal that administration of HN prevents polyglutamine toxicity,[27] stroke,[28] myocardial ischemia-reperfusion injury[29] and Alzheimer's disease-related stress.[30–33] More recently, Sreekumar et al. reported that HN is efficient in protecting human RPE cells from oxidative stress-induced apoptosis, suggesting it has potential as a cytoprotective agent against AMD.[9] Unfortunately, the main drawback of small, hydrophobic peptides like HN is their poor solubility, stability, and clearance *in vivo*. [34] Multiple and frequent dosing of HN is necessary to achieve substantial effects in other disease models; furthermore, in a clinical setting of intravitreal administration it will be necessary to reduce injection frequency to on the order of once per month.[35]

To overcome this formulation challenge, in this study the HN was genetically engineered with high molecular weight ELP protein polymers that have the potential to modulate its solubility, local retention, and cellular bioactivity. Two ELPs were evaluated, including a temperature sensitive V96 which forms coacervates at 37 °C and a soluble S96 that remains a stable colloid at physiological temperatures. Similar to their parent ELPs, both HN-S96 and HN-V96 exhibit temperature-dependent phase separation (Figure 2). Although the addition of HN shifts the phase diagram differently for V96 and S96, their proposed properties (soluble *vs.* insoluble) at body temperature remain as desired. Surprisingly, HN, a 24-amino acid peptide, mediates the self-assembly of ELP nanoparticles, which are dominant even below the ELP T_t . DLS measurements indicate that both HN-ELP fusions form nanoparticles that are stable up to their T_t (Figure 3). The apparent size and shape of these multimeric species was further confirmed by TEM and multi-angle light scattering (Figure 4).

These observations also raised the question of whether the nanoparticle assembly influences the biological activity of HN. Thus, the cytoprotective function of HN-V96 and HN-S96 was assessed using an *in vitro* primary cell model. Human RPE cells undergo apoptosis with mild oxidative stress when tBH reaches a toxic dose. Similar to the previous studies of HN, treatment of HN-V96 or HN-S96 shows a dose-dependent cell protection by inhibiting caspase-3 activation (Figures 6, 7, Supplementary Figures S1, S2). [9] It should be noted that the minimum effective dose of HN-V96 is 5 μ M, which is slightly higher than what has been reported before for HN (10 μ g/mL, equivalent to 3.7 μ M). This indicates that the involvement of HN peptide in the particle assembly may affect its interaction with biological targets responsible for the anti-apoptotic activity.

Humanin is known to exert neuroprotective functions through different pathways by binding to either extracellular receptors or intracellular targets.[36, 37] Sreekumar et al. demonstrated that HN protection of RPE cells from oxidative stress is mediated via dual mechanisms: 1) enhancing mitochondria function through an intracellular pathway; 2) activating receptors for HN through an extracellular pathway.[9] Given that HN-ELP fusions assemble into multimeric nanoparticles, we hypothesized that they may work differently from the free peptide. The evidence of cell surface accumulation supported that these fusion proteins act mainly through extracellular binding partners (Figure 5). Furthermore, pharmacological inhibition of downstream STAT3 completely blocked the cytoprotective effects (Figure 8). While the *ex vivo* results are promising, a deeper understanding of the mechanisms of intracellular trafficking and signaling for HN-polypeptides will be necessary if they are to be further evaluated *in vivo* for clinical translation.

In the past few decades, considerable advances have been made in the development of ocular delivery systems.[38–40] The main challenge of designing a therapeutic system for the eye is to achieve an optimal concentration of a drug at the active site for the appropriate duration to provide ocular delivery systems with high therapeutic efficacy. To this end, many systems have been developed for controlled and sustained release of the therapeutic agents, including gels, microparticles, nanoparticles liposomes as well as solid implants or polymeric films. [41–46] In this study, the fact that HN-S96 and HN-V96 assemble distinct structures at physiological temperatures represents two different platform designs, stable colloid nanoparticles *vs.* coacervate microparticles, using one class of protein-polymers. These different designs suggest the potential utility and flexibility of ELPs as vehicles for hydrophobic, biologically active peptides.

In conclusion, this manuscript reports that fusions between ELP protein-polymers and the cytoprotective HN peptide can assemble stable nanostructures; furthermore, HN-polypeptides can be designed either to remain stable or undergo bulk phase separation at physiological temperatures. Surprisingly, both fusion proteins maintain their ability to bind and protect RPE cells from oxidative stress via STAT3 activation. Both fusions are protective to a similar degree using *ex vivo* studies of human RPE cells, suggesting their potential ophthalmological applications. Future characterizations of their respective ocular pharmacokinetics and pharmacodynamics will clarify their relative clinical opportunities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Elastin-like polypeptide fusions with the HN peptide.

a) Schematic showing the construction of plain and fusion ELPs. HN peptide was expressed at the N-terminus of ELPs. b) SDS-PAGE stained with Coomassie blue was used to determine the purity and molecular weight of purified protein polymers. c) Western blot probed with an anti-humanin polyclonal antibody was used to further confirm the identity of the HN polypeptides.

Figure 2. HN polypeptides can phase separate either above or below physiological temperature. ELPs with similar molecular weight, but different hydrophobicity (V96 and S96) were fused to HN and characterized for optical density as a function of temperature and concentration. **a)** Hydrophilic S96 phase separates above physiological temperature ($\sim 60^{\circ}\text{C}$). **b)** Fusion of HN to S96 increased its transition temperature to $\sim 80^{\circ}\text{C}$. **c)** Concentration-temperature phase diagrams for S96 and HN-S96 followed a log-linear relationship. Lines indicate the fit of T_t to Eq.3 (Table 1) with dashed-line 95% prediction bands. **d)** Hydrophobic V96 phase separates below physiological temperature ($\sim 30^{\circ}\text{C}$). **e)** Addition of HN to V96 decreased the phase transition temperature to $\sim 22^{\circ}\text{C}$. **f)** Concentration-temperature phase diagrams for V96 and HN-V96 followed a log-linear relationship. Lines indicate the fit of T_t to Eq.3 (Table 1) with dashed-line 95% prediction bands.

Figure 3. HN polypeptides assemble nanoparticles below ELP-mediated phase transition temperature.

a,b) The hydrodynamic radius (R_h) of ELPs were measured as a function of temperature at a concentration of 25 μM in PBS. Cumulative fit suggests **a)** HN-S96 and **b)** HN-V96 have a larger radius than their parent ELP S96 and V96, even below transition temperature. **c,d)** Regularization analysis indicates both **c)** HN-S96 (37 $^{\circ}\text{C}$) and **d)** HN-V96 (15 $^{\circ}\text{C}$) assemble a population of multimeric nanoparticles when below their phase transition temperatures.

Figure 4. HN-S96 assembles nanoparticles below their transition temperature.

a) TEM imaging was used to characterize the morphology of assembled HN-S96 multimers. Representative images of negatively-stained samples revealed the evidence of spherical nanoparticles with an average radius of 25.2 ± 8.2 nm (mean $\pm$ SD, $n=53$). Scale bar: 50 nm.

b) The distribution of radius for HN-S96 is quantified from the TEM images. **c)** SEC-MALS analysis on HN-S96 particles shows a molecular weight of 1.0×10^4 ($\pm 1.1\%$) kDa and a radius gyration (R_g) of 52.6 ($\pm 1.4\%$) nm.

Figure 5. HN polypeptides mediate cell-binding and STAT3 activation in human RPE cells.

a) Representative images showing RPE recognition by HN fusions. Human RPE cells were incubated with 10 μ M ELPs (S96, HN-S96, V96, HN-V96) for 4 and 24 hours at 37 ° C. The presence of ELP was detected using a monoclonal anti-ELP antibody (Green) and mitochondria was labeled using Mitotracker (Red). Blue: DAPI. Scale bar: 10 μ m. **b)** Representative Western blot showing exogenous HN-ELPs (10 μ M, 4 hr) induced STAT3 phosphorylation, whereas unmodified ELPs had similar levels of activation to the untreated control.

Figure 6. Exogenous HN-S96 protects RPE cells from oxidative stress-induced cell death in a dose-dependent manner.

Human RPE cells were challenged with 150 μ M tBH and treated with varying doses of HN-S96 (0.5, 5, 10 μ M) for 24 hr. Cell death was assessed using TUNEL stain (Red). a) tBH challenge resulted in cell death and HN-S96 at doses of 5 and 10 μ M offered protection to RPE cells. Blue: DAPI, Scale bar: 50 μ m. b) Quantification of the TUNEL-positive cells. n=10. **p=0.0034, **** p<0.0001.

Figure 7. Exogenous Hn-S96, but not S96 protects RPE cells from oxidative stress-induced cell death.

Human RPE cells were challenged with 150 μ M tBH and treated with or without ELPs for 24 hr. a) Cell death was assessed using TUNEL stain. Representative confocal images showed that TUNEL-positive cells (Red) were reduced when cells were co-treated with 10 μ M HN-S96. Moreover, the negative ELP control S96 failed to protect cells from apoptosis.

b) The activation of caspase-3 (green) was assessed using indirect immunofluorescence. Representative confocal images showed that tBH-induced caspase-3 activation was inhibited in cells treated with 10 μ M HN-V96. The control S96 did not reduce caspase-3 activation. Blue: DAPI. Scale bar: 20 μ m.

Figure 8. STAT3 inhibition blocked the anti-apoptotic function of HN-ELPS.

Human RPE cells were pre-incubated with 0.5 μ M STAT3 inhibitor followed by co-incubation with different ELPs along with 150 μ M tBH for 24 hr. Control cells represent no treatment with either tBH or inhibitor (INH). **a)** TUNEL staining shows marked cytoprotection by HN-ELPS. Inhibition of STAT3 significantly ($****$ $p < 0.0001$) depressed the anti-apoptotic efficiency of HN-ELPs under tBH. Red. TUNEL-positive cells. $n=10$. **b)** The activation of caspase-3 (Green) as assessed using indirect immunofluorescence. While HN-ELPs treatment decreased the activation of caspase-3, STAT3 inhibition significantly ($****$ $p < 0.0001$) restored caspase-3 staining under challenge by tBH. Blue: DAPI. Scale bar: 50 μ m. $n=9$.

Table 1.

Recombinant protein-polymers evaluated in this study.

Label	Amino acid sequence	T_t^* [°C]	Property at 37 °C	Expected MW [†] [kD]	Observed MW [‡] [kD]	Intercept [§] , b [°C]	Slope [§] , m [°C] [log ₁₀ (μM)] ⁻¹
S96	G(VPGSG) ₉₆ Y	57.7	Soluble	38.39	38.35	62.8 [61.6 to 64.0]	3.9 [3.0 to 4.73]
HN-S96	GMAPRGFSCLLLTSEIDLVPKRRA G(VPGSG) ₉₆ Y	81.1	Nanoparticle	41.12	41.09	87.6 [81.9 to 93.4]	6.1 [2.1 to 10.1]
V96	G(VPGVG) ₉₆ Y	30.8	Coacervate	39.55	39.53	35.7 [35.2 to 36.2]	3.5 [3.1 to 3.8]
HN-V96	GMAPRGFSCLLLTSEIDLVPKRRA G(VPGVG) ₉₆ Y	22.4	Coacervate	42.28	42.31	23.6 [23.5 to 23.8]	0.9 [0.8 to 1.0]

* The observed transition temperature at 25 μM in PBS.

† Estimated from open reading frame excluding start codon.

‡ Measured by MALDI-TOF.

§ The Intercept b and slope m , were derived from the log-linear regression analysis for transition temperature vs. concentration (Figure 2) fit to Eq. 2. Data represent the mean [95% confidence interval].

Table 2.

Stability of diameter and surface charge for HN-S96 nanoparticles as assessed by Nanoparticle Tracking Analysis

Sample	Incubation temperature [*] [°C]	Particle diameter [†] (nm)	Zeta potential [‡] (mV)
HN-S96	4.0	132.6 [104.6 to 160.6]	−6.6 [−7.1 to −6.1]
HN-S96	37.0	132.6 [128.4 to 136.8]	−2.8 [−5.5 to −0.1]

^{*} Incubation temperature over 4 days.

[†] Average hydrodynamic diameter measured at 25 °C. Data represent the mean [95% confidence interval].

[‡] Average Zeta potential at 25 °C. Data represent the mean [95% confidence interval].