LENS EPITHELium DERIVED GROWTH FACTOR (1-326): A NEW PROTEIN DRUG FOR RETINAL DISEASES

By

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A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

Pharmaceutical Sciences

2013
This thesis for the Doctor of Philosophy degree by

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**Lens Epithelium Derived Growth Factor (1-326): A New Protein Drug for Retinal Diseases**

Thesis directed by Professor Uday B. Kompella

**ABSTRACT**

Although over 2.2 million people in the United States suffer from dry age related macular degeneration and retinitis pigmentosa, there are no FDA approved therapeutic agents. Lens epithelium derived growth factor (LEDGF), a transcription factor that promotes cell survival, although effective in reducing retinal degeneration, has a binding domain for the HIV integrase. To overcome this limitation, which promotes HIV infection, the purpose of this study was to design a fragment of LEDGF as a novel therapy for treating retinal degenerative diseases. Further, we hypothesized that the novel fragment of LEDGF is useful in treating protein aggregation disorders such as retinitis pigmentosa and that nanoassemblies of this protein can offer enhanced stability, delivery, and activity, while prolonging drug delivery.

We designed and synthesized LEDGF$_{1-326}$ as a new protein to avoid HIV integrase interactions of LEDGF, assessed its ability to reduce P23H rhodopsin protein aggregation induced stress, prepared stable formulations including nanoassemblies and assessed their cellular entry and in vivo delivery and efficacy. Additionally, LEDGF$_{1-326}$ nanoparticles in porous microparticles (NPinPMP) were engineered and characterized for morphology, drug release, and in vivo delivery.
LEDGF\textsubscript{1-326} expression decreased P23H rhodopsin aggregates and aggregation mediated retinal cell damage. LEDGF\textsubscript{1-326} was produced at about 20 mg/L of culture and purified with ~ 95 \% monomer content. A single intravitreal injection of LEDGF\textsubscript{1-326} protein in RCS rats significantly reduced the functional and morphological loss of photoreceptors for at least eight weeks. A liquid formulation of LEDGF\textsubscript{1-326} with stabilizing excipients improved protein stability and maintained protein activity in an ELISA assay for 60 days at 25 °C. LEDGF\textsubscript{1-326} nanoassemblies (~ 28 nm) increased cell uptake of LEDGF\textsubscript{1-326} to 2.5-fold and \textit{in vivo} mean residence time in Sprague Dawley rat choroid-retina by 21-fold from 0.27 to 5.7 days. Nanoassemblies prolonged LEDGF\textsubscript{1-326} efficacy and reduced retinal degeneration in RCS rats. NPinPMP sustained \textit{in vitro} LEDGF\textsubscript{1-326} release for 3 months and \textit{in vivo} delivery for at least 2 months.

LEDGF\textsubscript{1-326} is a new protein drug for treating retinal degenerative diseases. Nanoassemblies and NPinPMP are novel delivery systems for enhanced and sustained LEDGF\textsubscript{1-326} delivery to the back of the eye.

The form and content of this abstract are approved. I recommend its publication.

Approved: Uday B. Kompella
I dedicate this work to my son Vikramaditya for his naive and innocent love, sacrifice, support, and encouragement.
ACKNOWLEDGMENTS

First and foremost, I would like to express my sincere gratitude to my advisor Prof. Uday B. Kompella for providing me the opportunity and boundless support to perform my PhD study and research. I am deeply grateful to him for his patience, motivation, enthusiasm, and confidence towards me throughout my PhD. Because of his valuable scientific insights and meticulous comments, I was able to scientifically advance and accomplish a major milestone in my career. Under his guidance, I not only gained immense scientific knowledge but also honed my professional skills including communication, leadership, and decision-making. I could not have imagined having a better advisor and mentor for my Ph.D study. Without his encouragement, this dissertation would not have materialized.

Besides my advisor, I am deeply grateful to each of my dissertation committee members: Dr. Latoya Jones Braun (Chair), Dr. J Mark Petrash, Dr. Dhinakar Kompala, and Dr. Krishna Mallela for their encouragement, valuable recommendations, astute comments, and hard questions. I feel privileged to have them as my scientific guides. I would like to offer my special thanks and gratitude to Dr. N Karl Maluf who was my committee Chair during the initial years, for his constructive comments and warm encouragement.

I would like to offer special thanks to Dr. Robert Scheinman for his mentorship and deep rooted scientific discussions, which helped me in grasping and become proficient in the complex field of molecular biology. He continually and convincingly conveyed an immense spirit of adventure in research and scholarship and excitement in teaching.
I am in debt to Dr. Toshimichi Shinohara and Dr. Dhirendra Pratap Singh, University of Nebraska Medical Center for their guidance and for their help in providing lens epithelium derived growth factor (LEDGF) plasmid. My special thanks to Dr. Ron Kopito, Standford University for providing P23H rhodopsin plasmid. I would like to thank Dr. Raul Velez-Montoya and Dr. Jeffrey L Olson for their guidance in performing electroretinography experiments. I would also like to thank Dr. Joseph Brzezinski for his guidance in immunofluorescence studies and Dr. David Siegel for assisting me with confocal microscopy and western blot analyses. I also like to thank my collaborators from within the University of Colorado and other institutes: Dr. David Ross, Dr. Jeffrey L Olson, Dr. Eva N Grayck, Dr. Sangley P. Srinivas, Dr. Neena Haider, Dr. Radha Ayyagari, Dr. Venkataraman Mahalingam, Dr. Mark R. Prausnitz, Dr. Henry F. Edelhauser, Dr. Ram Kannan, Dr. Kirill Ostanin, and Dr. Shalesh Kaushal for giving me an opportunity to expand my research expertise beyond the dissertation project. I would like to express my sincere appreciation to all those who provided me the possibility to complete this dissertation.

I would also like to express my gratitude to University of Colorado Anschutz Medical Campus, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Nebraska Medical Center, and National Institute of Health for the financial support and core facilities.

I thank all my past and present members of my laboratory in University of Colorado Denver: Arun K Upadhyay, Jiban Jyoti Panda, Sarath Yandrapu, Ruchit Trivedi, Sunil Vooturi, Shelly Durazo, Rashmi Pacha Ravi, Namdev Shelke, Gajanan Yadav, Vidhya Rao, Jiban Anand Mishra, Ashish Thakur, Sneha Sundaram, Swita Singh,
and Puneet Tyagi for the stimulating discussions, and for all the fun we have had during my PhD. I would like to express my deepest appreciation to the past members of my laboratory Dr. Surya Ayalasomayajula, Dr. Nagesh Bandi, Dr. Aniruddha Amrite, and Dr. Jithan Aukunuru for their valuable recommendation, support, and guidance in my quest for future career opportunities.

I would like to offer my special thanks to my friends: Dr. Sundeep Sethia, Dr. Tushar Mukherjee, Dr. Saumitra Bagchi, and Abhijit Malvi, who have been my source of constant support and encouragement during my difficult times.

Last but not the least; I would like to thank my family: my parents Padam Agarwal and Renu Agarwal for their faith and confidence in me, my sister Seema Mittal and my brothers Kamalesh Agarwal and Rakesh Agarwal for their love and encouragement. My deepest appreciation goes to Rahul Baid who has always been my friend and source of encouragement. I owe a very important debt to my son Vikramaditya Baid for his sacrifice and letting me be away from him to pursue my research career. I owe my deepest gratitude to him for his innocent love, support, and smiles.
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CHAPTER I

STATEMENT OF PROBLEM

The objective of this project was to develop a novel therapeutic protein, for the treatment of retinal diseases such as dry age related macular degeneration and retinitis pigmentosa, and to develop novel sustained release formulations of this protein.

Retinal degeneration, the leading cause of blindness in United States, is a group of heterogeneous, chronic, and progressive diseases including dry age related macular degeneration (dry AMD) and retinitis pigmentosa (RP) (Congdon et al., 2004). Although belonging to genotypically diverse disorders, dry AMD and RP ultimately lead to vision loss or blindness due to photoreceptor cell death.

While the molecular mechanism underlying dry AMD is not yet fully understood, the pathogenesis is characterized by accumulation of lipofuscin (fat-containing toxic “drusen”) in the retinal pigmented epithelium followed by choroidal ischemia and ultimately, oxidative damage and inflammation (Libby and Gould, 2010; Lim et al., 2012). This leads to central vision loss followed by complete blindness (Fine et al., 2000; Klein et al., 2004; Kuehn, 2005). Approximately 20% of the aging (> 70 yrs old) population currently suffers from AMD (Lim et al., 2012) and by 2050 it is predicted to reach approximately 17.8 million in the United States (Rein et al., 2009). Dry AMD accounts for 90% of the cases of AMD (Stuen, 2003). RP, on the other hand, is a genetically inherited disease (Ohguro et al., 2002) caused by more than 50 different gene mutations (Dryja et al., 1990b). The most common mutations are in the rhodopsin gene
(Berson et al., 1991), including P23H (Proline 23 → Histidine) mutation of rhodopsin, which leads to an aggregation prone protein (Mendes and Cheetham, 2008). Approximately, 1 in every 4,000 individuals are affected by RP, causing more than 1.75 million people, worldwide, to suffer from this disease (Hartong et al., 2006). Although dry AMD and RP are prevailing in a widespread population, there was no US FDA approved therapeutic agents until 2013. Current treatment options being evaluated are highly invasive and include recently approved (February 13, 2013) Argus® II retinal prosthesis system (Second Sight Medical Products, Inc. San Fernando Valley, CA).

Protein aggregation has been linked to many degenerative diseases including RP (Milam et al., 1998; Saliba et al., 2002; Stojanovic and Hwa, 2002), AMD (Klenotic et al., 2004), Alzheimer’s, Parkinson’s, and amyloid associated diseases (Tran and Miller, 1999; Saliba et al., 2002). The P23H mutation in the rhodopsin gene has been implicated in 10% of the cases of autosomal dominant RP (Noorwez et al., 2009). Mutations in the rhodopsin gene, including P23H mutation, lead to protein misfolding and aggregation (Sung et al., 1993; Mendes et al., 2005). Aggregation of P23H rhodopsin prevents translocation of P23H/wild type (WT) rhodopsin protein to the plasma membrane, an innate location for its functional activity, and results in accumulation within the photoreceptor cells. Apart from being toxic by itself, mutant P23H rhodopsin exerts a dominant negative effect on the biosynthesis, translocation, and degradation of WT rhodopsin (Colley et al., 1995; Kurada and O'Tousa, 1995; Saliba et al., 2002; Mendes et al., 2005).

The primary preventive measure, to slow the progression of RP, remains daily ingestion of up to 15,000 IU of Vitamin A palmitate (Berson et al., 1993a; Berson et al.,
Protein growth factors including brain-derived neurotropic factor (BDNF) (Faktorovich et al., 1992; LaVail et al., 1998; Delyfer et al., 2004; Leveillard et al., 2004a; Leveillard et al., 2004b; Andrieu-Soler et al., 2005; Delyfer et al., 2005) are under investigation as anti-apoptotic factors. Ciliary derived neurotrophic factor (CNTF) is undergoing phase III clinical trial for RP treatment (Sieving et al., 2006). Due to the large genetic heterogeneity of RP, gene therapies targeting a specific gene seem to be daunting. Since protein aggregation is one of the major forms of pathophysiology in RP, targeting protein aggregation seems to be quite feasible. Numerous substances including small molecules such as calnexin (Noorwez et al., 2009), pharmacological chaperones such as 11-cis-retinal (Mendes et al., 2005; Mendes and Cheetham, 2008), and anti-oxidants such as curcumin (Khajavi et al., 2005; Vasireddy et al., 2011) have been indicated to reduce P23H aggregation. Although the cause and molecular mechanism of P23H rhodopsin aggregation is well known (Sung et al., 1993; Kaushal and Khorana, 1994; Lem et al., 1999; Mendes et al., 2005), due to the multifactorial nature of RP, and complex pathophysiology, there is a dearth of potentially effective therapeutics that can prevent/reduce P23H aggregation and disease progression of RP.

Lens epithelium derived growth factor (LEDGF) was discovered in 1999 and was identified as a transcription factor with anti-apoptotic activity (Singh et al., 1999; Singh et al., 2000). Singh and colleagues demonstrated that LEDGF reduced oxidative and thermal stress in various cells including lens epithelium, keratinocytes, and fibroblasts. Soon after its discovery, it was found that LEDGF upregulates transcription and expression of heat shock and stress associated genes (Singh et al., 2001; Kubo et al.,
The combined anti-apoptotic activity and ability to upregulate heat shock and stress related proteins makes LEDGF an attractive therapeutic protein for the treatment of retinal degenerative and other neurodegenerative diseases. In fact, LEDGF delayed retinal degeneration in light damaged rats and a retinal degenerative rat model (Royal College of Surgeons model) (Machida et al., 2001). The transcriptional activity of LEDGF is hypothesized to be the molecular mechanism of its action in the treatment of retinal degeneration.

Although LEDGF is a promising therapeutic, several reports found that full length LEDGF is associated with HIV integrase (Cherepanov et al., 2003), implicating that it can promote HIV infection; hence, it is currently a target for treatment of HIV associated diseases (Maertens et al., 2003; Vets et al., 2012). Thus, there is a need to delink HIV activity of LEDGF from its beneficial effects in degenerative diseases. Further, to date, there are no reports that demonstrate the effect of LEDGF on protein aggregation and therefore, the first objective of this study was to identify a critical fragment of LEDGF that has potential to reduce protein aggregation and investigate its efficacy in reducing protein aggregation.

With the advancement of biotechnology, protein therapeutics have dominated the spotlight in drug discovery and development for the treatment of multifarious disorders including malignant lymphomas (Spiekermann and Hiddemann, 2005), cancers (Scott et al., 2005), colitis (Feagan et al., 2005), cardiac disorders (Hershberger et al., 2005), diabetes (Xuan et al., 2005) as well as wet AMD (Heier et al., 2006b; Spaide et al., 2006; Emerson and Lauer, 2007). Numerous therapeutic proteins have been launched into the market, contributing to approximately 35 % of 37 new active substances (2001) as
published by PHARMA 2010. Data reported for 2008 indicated that the global biotech
drug sales growth rate was 12.5 % as compared to 6.4 % for the traditional small
molecule drugs for that year. As of 2008, biotechnology drugs accounted for one fifth of
all blockbuster drugs on the market (Malik, 2008). Proteins are interesting alternatives to
gene therapies as they are biologically active molecules and have been naturally designed
to exert their effect under normal physiological conditions. Further, proteins are
specifically suited for intravitreal dosing as they are retained for prolonged periods in the
vitreous compared to small molecule drugs, thus having an extended duration of
pharmacological action in the retina (Durairaj et al., 2009). Bevacizumab, a 150 kDa
protein, was reported to have a vitreal half-life of 4.32 days in rabbits and greater than 10
μg/ml of bevacizumab was maintained in the vitreous humor for 30 days after a single
intravitreal injection of 1.25 mg (Bakri et al., 2007b).

Proteins, although exciting therapeutics, are severely challenged due to inherent
instability, hurdles in biosynthesis and purification in large quantities for any formulation
development (Frokjaer and Otzen, 2005). However, several pharmaceutical companies
have overcome these challenges and have been very successful in creating protein drug
products. Recent approval and market entry of several protein therapeutics including
ranibizumab (Lucentis®, Genentech, CA), bevacizumab (Avastin®, Genentech, CA), and
VEGF Trap-Eye (Eylea®, Regeneron Pharmaceuticals, NY) have revolutionized the
treatment of posterior eye diseases. However, these therapeutics are effective in treatment
of choroidal neovascularization (CNV)/wet-AMD and not dry AMD. As mentioned
earlier, the majority of patients with AMD actually suffer from dry AMD, thus the quest
to find a therapy to treat dry AMD remains unaccomplished. The success of protein-
based therapeutics for CNV has sparked interest amongst several investigators to
discover, design, and develop new protein therapeutics with other targets for the
treatment of dry AMD (Damico et al., 2012). In our previous study, LEDGF$_{1-326}$ gene was
effective in protecting retinal cells from mutant P23H rhodopsin mediated cellular
damage, and reducing protein aggregation. Thus, LEDGF$_{1-326}$ has the potential to reduce
retinal degeneration. Therefore, the second objective of this study was to biosynthesize,
purify LEDGF$_{1-326}$ protein, characterize its biophysical parameters, and test its efficacy
in reducing retinal degeneration.

Proteins have a unique 3-D structure which makes them highly specific to their
target and at the same time this unique conformation poses a daunting challenge in the
development of protein formulation. The 3-D conformation of a protein is highly
susceptible to its surrounding environment such as pH, temperature, ionic composition,
and protein concentration (Wang, 1999). Unlike traditional small molecules which might
have chemical instability, proteins are susceptible to chemical as well as physical
instability. Chemical instabilities such as deamidation, disulphide scrambling,
isomerization, and hydrolysis lead to decomposition and fragmentation of proteins while
physical instability such as unfolding and denaturation lead to aggregation. Loss of
protein structural conformation is associated in most cases with loss of potency and may
also lead to immunogenicity (Cleland et al., 1993; Wang, 1999; Parkins and Lashmar,
2000). Proteins encounter various stresses such as temperature, air/water interface, and
agitation during their synthesis or purification stages as well as during storage and
transportation. Although precautions are taken to generally avoid harsh conditions such
as storing proteins at 4 °C or even as low as -70 °C, stabilization of formulations becomes
an unavoidable task to successfully employ a protein therapeutic. There is a plethora of pharmaceutical additives and formulation combinations available as a guide for a formulation scientist; however the major practical challenge is to quickly recognize a strategic combination of additives to formulate an acceptable stable protein preparation. Among the most commonly used pharmaceutical additives are buffering agents such as citrate, and tris, salts such as sodium chloride, non-reducing sugars including sucrose, and trehalose, surfactants such as polysorbate 20, and antioxidants to name a few (Kamerzell et al., 2011). Therefore, the third objective of this study was to increase the stability of LEDGF\textsubscript{1-326} in an aqueous formulation.

Protein intravitreal injection is a common practice in the clinic since it delivers drug in close proximity to the retina and evades dynamic barriers (blinking, tear film, tear turnover, induced lachrymation) and other membrane barriers (sclera, conjunctiva, RPE, etc.). Due to these dynamic and membrane barriers, topical and periocular routes of administration are inefficient in delivering drugs to the back of the eye (Gaudana et al., 2010; Thrimawithana et al., 2011). However, lifelong frequent intravitreal injections not only lead to retinal detachment, retinal hemorrhage, endophthalmitis and increased intraocular pressure but also cause patient incompliance, and infections (Peyman et al., 2009; Wu and Chen, 2009). Thus a system capable of delivering drug over an extended period of time in the eye is much warranted.

We hypothesized that complexation of proteins with metal ions can form various nanostructures that can control protein release and or persistence. Metal ions are known to be an integral part of many metalloproteins specifically transcription factors, which not only stabilize the protein structure, but are also essential for their biological function
(Zhou et al., 2005; Andreini et al., 2006). An example of a metal ion protein complex that sustains the release of a protein under a day is insulin protein hexamer formulation with zinc. To date the interaction of zinc with proteins has been limited to the formation of oligomers of proteins (Gaskin and Kress, 1977) and protein precipitation for purification (Yang et al., 2000). There are no reports on long-term drug release or formation of controlled nanoassemblies with zinc. Further, formation of stable self-assembly of proteins in the presence of zinc and their use as a drug delivery device to extend the persistence of a protein in eye has never been explored. **Therefore, the fourth objective of this study was to develop a novel nanoassembly of LEDGF1-326, using metal ion that can persist for a prolonged period in ocular tissues.**

In the past, biodegradable polymeric nano- and micro- particles have gained much attention as potential sustained delivery system. They can release drug molecule for weeks to months and have the advantage of being fine-tuned to tissue specific uptake (Singh et al., 2009). Singh et al. showed that RGD and transferrin functionalized nanoparticles accumulated selectively in the neovascular eye and inhibited the progression of neovascularization in laser induced CNV rat model. Although polymeric particles have been very successful in delivering small molecules and genes, protein encapsulation in these particles has its own challenges. Conventional methods of preparation of nano- and micro- particles expose proteins to harsh conditions such as organic solvent, sonication and high temperature leading to denaturation and loss of biological activity of encapsulated protein. Supercritical (SC) CO\textsubscript{2} has been used to formulate porous microparticles with reduced organic solvent content (Koushik and
Kompella, 2004) for the delivery of deslorelin (1.29 kDa) a peptide drug for one week. The organic solvent content in the porous particles was below the detection limit as compared to 4500 ppm in untreated particles made by the conventional emulsion solvent method. Further SC CO$_2$ differentially altered and expanded the polymeric particles depending upon the amorphous or crystallin nature of the polymer involved. Thus the fifth and last objective of this study was to develop a unique nanoparticle in porous microparticle (NPinPMP) system encapsulating LEDGF$_{1-326}$ using supercritical CO$_2$ technology.

**Specific Aims**

**Specific Aim 1**

*To determine the efficacy of LEDGF$_{1-326}$ gene to reduce protein aggregation and cell death in retinal pigment epithelial cells*

**Hypothesis**

LEDGF$_{1-326}$ reduces P23H aggregation and the associated cytotoxicity in retinal cells. The tasks for this specific aim are as follows:

**Task 1.** To establish an in vitro cell culture model expressing aggregation prone P23H rhodopsin protein

**Task 2.** To determine whether mutant P23H rhodopsin aggregates are toxic to ARPE-19 cells

**Task 3.** To determine whether LEDGF$_{1-326}$ improves ARPE-19 cell viability

**Task 4.** To determine whether LEDGF$_{1-326}$ reduces mutant P23H rhodopsin aggregates
Specific Aim 2

To biosynthesize, purify and characterize LEDGF\textsubscript{1-326} protein and determine its efficacy in reducing retinal degeneration in Royal College of Surgeons (RCS) rats

**Hypothesis**

LEDGF\textsubscript{1-326} protein improves photoreceptor survival and thus is effective in reducing retinal degeneration in RCS rats. The tasks for this specific aim are as follows:

**Task 1.** To clone and establish an *Escherichia coli* bacterial system expressing LEDGF\textsubscript{1-326}.

**Task 2.** To develop a large scale purification method for LEDGF\textsubscript{1-326} protein.

**Task 3.** To establish physical and biophysical properties of LEDGF\textsubscript{1-326}.

**Task 4.** To determine LEDGF\textsubscript{1-326} efficacy to reduce retinal degeneration in RCS rats.

Specific Aim 3

To reduce LEDGF\textsubscript{1-326} aggregation, and retain its immunoreactivity during storage at 25 °C in aqueous formulation

**Hypothesis**

Pharmaceutical additives will reduce the physical and chemical instability of LEDGF\textsubscript{1-326} in aqueous formulation. The tasks for this specific aim are as follows:

**Task 1.** To identify an optimum pH for a LEDGF\textsubscript{1-326} aqueous formulation.

**Task 2.** To optimize formulation additives having the potential to decrease LEDGF\textsubscript{1-326} physical and chemical degradation.

**Task 3.** To develop an ELISA method for determining LEDGF\textsubscript{1-326} activity in the formulation
Specific Aim 4

To engineer metal ion induced LEDGF$_{1-326}$ nanoassemblies to increase cellular uptake and in vivo persistence and activity.

Hypothesis

LEDGF$_{1-326}$ forms nano-sized self-assembles in the presence of Zn(II), and these assemblies increase LEDGF$_{1-326}$ physical and chemical stability, cellular uptake and in vivo persistence and activity. The tasks for this specific aim are as follows:

Task 1. To prepare and characterize the LEDGF$_{1-326}$ nanoassemblies.

Task 2. To determine LEDGF$_{1-326}$ nanoassembly uptake in retinal cells.

Task 3. To study persistence, delivery, and pharmacokinetics of LEDGF$_{1-326}$ nanoassemblies in various ocular tissues following intravitreal injection.

Task 4. To determine the efficacy of LEDGF$_{1-326}$ nanoassemblies to reduce retinal degeneration in RCS rats.

Specific Aim 5

To develop an organic solvent free polymeric sustained release system for LEDGF$_{1-326}$ using supercritical (SC) CO$_2$ technology.

Hypothesis

Nanoparticle in porous microparticle (NPinPMP) sustain LEDGF$_{1-326}$ in vitro release, and hence, persistence in the vitreous humor for a few months. The tasks for this specific aim are as follows:

Task 1. To determine the effect of SC CO$_2$ on LEDGF$_{1-326}$ protein physical and chemical stability.

Task 2. To prepare and characterize LEDGF$_{1-326}$ encapsulated NPinPMP.
**Task 2.** To monitor the *in vitro* release of LEDGF$_{1-326}$ from NPinPMP.

**Task 3.** To study the persistence, delivery, and pharmacokinetics of LEDGF$_{1-326}$ NPinPMP in various ocular tissues following intravitreal injection.

**Summary**

In this project we identified a novel fragment of lens epithelium derived growth factor (LEDGF$_{1-326}$) based on the bioinformatic analysis and named it LEDGF$_{1-326}$. We evaluated its efficacy to reduce protein aggregation, a common cause of retinitis pigmentosa, using gene delivery approach and cultured retinal cells. Further, we biosynthesized, purified, and characterized LEDGF$_{1-326}$ protein and evaluated its efficacy to reduce retinal degeneration in a diseased rat model. We further developed its stable aqueous immediate release formulation using pharmaceutical additives. Two novel sustained release nanosystems were also developed to improve LEDGF$_{1-326}$ persistence in ocular tissues.
CHAPTER II

INTRODUCTION

General Introduction

Eye is a vital sensory organ in human body which gives us perception of vision. It is made up of various complex layers of tissues that work together to detect light and convert it into electro-chemical signal in neurons (Figure 2.1). Among the other layers of eye, retina is a light-sensitive delicate layer of tissue at the back of the inner eye, responsible for visual perception and essential for vision. It consists of multiple layers of neuronal cells including photosensitive (rod and cone) photoreceptors. The neuronal cells are interconnected to each other via several synapses. The photoreceptors are responsible for capturing light impulses and convert them in electro-chemical signals which are then transported via the neuronal cells to the brain.

Retinal Degeneration

Retinal degeneration (RD) or retinal degenerative disease is a collective term used for diseases that affect the retina such as age related macular degeneration (AMD) (Lim et al., 2012), retinitis pigmentosa (RP) (Ohguro et al., 2002), choroideremia (Coussa and Traboulsi, 2012), retinoschisis (Molday et al.), stargardt disease (Vasireddy et al., 2010), and usher’s disease (Bonnet and El-Amraoui, 2012). The most common form of retinal degenerative diseases are AMD, and RP. In developed countries, such as US, where cure has been found for cataract and other front of the eye disorders, retinal degeneration is the leading cause of blindness in aging population.
Figure 2.1. Schematic Diagram of Eye and Retina.
Age Related Macular Degeneration

Age related macular degeneration (AMD) is a chronic, progressive disease that causes central vision loss followed by irreversible complete blindness (Figure 2.2) (Lim et al., 2012). As name indicates, it mostly affects people above age of 50, with frequency of the disease progression increasing exponentially after age of 70 (Ambati et al., 2003). Around 1.2 million people are currently suffering from AMD and by 2020 the count is estimated to reach around 12 million (Jager et al., 2008). The underline pathology of AMD is yet to be understood completely (Ambati et al., 2003; Nowak, 2006). Complex interplay of genetic, environmental (light, UV, heat), metabolic (age), and other functional factors have been implicated to be the root cause of this disease. Recently chronic oxidative stress and inflammation (Libby and Gould) have also been found to play a major role in triggering this pathology. There are two types of AMD, “wet” and “dry”. Dry AMD also called atropic AMD, accounts for 90 % of cases of the AMD (Jager et al., 2008). Accumulation of tiny protein and fat-containing toxic “drusen” between the retinal pigmented epithelium and the Bruch’s membrane (Green, 1999) leads to progressive atrophy of retinal pigment epithelium, choriocapillaris, and photoreceptors (Fine et al., 2000; Klein et al., 2004; Kuehn, 2005) causing AMD. Wet AMD or exudative form of AMD also called choroidal neovascularization (CNV) is less frequent (10 % of the AMD cases), but advanced form of dry AMD, wherein new abnormal blood vessels develops and grow below retina (Bhutto and Lutty, 2012). These abnormal blood vessel proliferations often lead to vascular leakage, bleeding, and fluid exudation into retina, causing macular edema and irreversible vision loss. 
Retinitis Pigmentosa

Retinitis pigmentosa (RP) on the other hand is genetically inherited heterogeneous disease that causes degeneration of rods and cones (Milam et al., 1998; Ohguro et al., 2002; Stojanovic and Hwa, 2002). The disease prevalence is 1:4000 worldwide, and around 1.75 million people in US are currently suffering from this disorder (Hartong et al., 2006). Clinical manifestation of RP is night blindness, followed by peripheral vision loss (Figure 2.2). In RP, the rod photoreceptor dysfunction and degenerate followed by cone degeneration. Photoreceptor dysfunction and degeneration progressively lead to tunnel vision and sooner or later blindness. This disease can be inherited as either as autosomal-dominant (adRP) (30-40%), or autosomal-recessive (arRP) (50 -60%) or X-linked (5-15%) (Gartner and Henkind, 1982; Bunker et al., 1984). More than 50 different genes have been identified to cause RP of which rhodopsin mutation is the most prevalent gene (Berson et al., 1991; Mendes and Cheetham, 2008). Around 140 point mutation in rhodopsin gene have been implicated to cause RP (https://sph.uth.edu/retnet/), majority of which causes adRP (Dryja et al., 1990a; Dryja et al., 1990b). Mutations in rhodopsin gene have been classified based on their cellular and biochemical properties in 2 major classes (Sung et al., 1993; Stojanovic and Hwa, 2002; Mendes et al., 2005). Class I
exclusively refers to mutations that fold normally similar to wild type (WT) rhodopsin but are unable to get transported to the outer segment. On the other hand, class II mutations have folding defects, the mutant proteins are misfolded and are retained in endoplasmic reticulum (Sung et al., 1993; Mendes et al., 2005). Rhodopsin point mutation, P23H (Proline 23 → Histidine), constitutes the most common cause of adRP in North America and belongs to class II mutation (Dryja et al., 1990a; Dryja et al., 1990b; Mendes and Cheetham, 2008).

**Current Treatment for Dry AMD and RP**

Although millions of people around the world is afflicted by dry AMD and RP currently there is no drug approved by the US FDA until 2013.

The primary preventive measure to reduce dry AMD remains to be a dietary supplement PreserVision® (Bausch and Lomb Pharmaceuticals Inc.) containing beta-carotene and zinc as per the AREDS formula (Cangemi, 2007). However, high doses of beta-carotene have been identified to induce high risk of cancer in smokers (Albanes et al., 1996), and further, this therapy has not been approved by the FDA. Other oral supplements of antioxidants, anti-inflammatory, and anti-angiogenic agents such as omega-3 fatty acids (docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) are undergoing clinical trials (Damico et al.). New pharmacological treatments have emerged in recent past, targeting different pharmacological components known to be involved in pathophysiology of dry AMD. Neuroprotective drugs, including ciliary neurotropic factor (CNTF) (Neurotech Pharmaceuticals Inc), brimonidine tartrate (Allergan Inc.), tandospirone (Alcon Laboratories Inc.), and anti-amyloid β antibodies (Glatiramer acetate (Copaxone, Teva Pharmaceuticals), and (RN6G (Pfizer Inc.)) that prevent the
photoreceptor degeneration and retinal pigment epithelium cell loss are under investigation. CNTF, currently under phase II clinical trial for dry AMD, is the most studied neurotrophic factor and has been indicated to protect photoreceptor degeneration is variety of animal models of retinal degeneration including mice, rats, dogs and cats (Wen et al., 2012). It is delivered via a polymeric intravitreal implant containing live human cells (encapsulated cell technology, ECT) capable of producing human CNTF (Sieving et al., 2006). Visual cycle modifiers such as Fenretinide (ReVision Therapeutics, USA) is undergoing phase II, and ACU-4429 (Acucela, Inc., USA) is undergoing phase I trial. They delayed photoreceptors deterioration by slowing down the metabolic activity of photoreceptors and subsequently reducing the toxic accumulation of fluorophores and lipofuscin; thereby reducing photoreceptor and RPE loss. Since inflammation plays an important role in advanced dry AMD, fluocinolone acetonide (Alimera Sciences, USA), an anti-inflammatory corticosteroid is undergoing phase II trial to reduce inflammation (MAP study). Other agents including synthetic peptide POT-4 (Potentia Pharmaceuticals, USA), aptamer ARC1905 (Ophthotech Corp., USA), and monoclonal antibodies eculizumab (Alexion Pharmaceuticals, USA) and FCFD4514S (Genentech Inc., USA) are also under clinical trials as inhibitors of inflammatory complement cascade system. However, due to the multifactorial nature of the disease, and complex pathophysiology, a drug is yet to be approved for dry AMD.

With the increasing understanding of genetics and biochemical pathways involved in pathophysiology of RP, a wide spectrum of treatment modalities are undergoing research for RP. The primary preventive measure to slow the progression of RP remains daily ingestion of up to 15,000 IU of Vitamin A palmitate (Berson et al., 1993a; Berson
et al., 1993b; Li et al., 1998; Sibulesky et al., 1999). Another nutritional supplement assessed for treatment of RP is docosahexaenoic acid (DHA), an omega-3 fatty acid. Neurotropic factors including brain-derived neurotropic factor (BDNF) (LaVail et al., 1998), and others (Faktorovich et al., 1992; Delyfer et al., 2004; Leveillard et al., 2004a; Leveillard et al., 2004b; Andrieu-Soler et al., 2005; Delyfer et al., 2005) are under investigation as anti-apoptotic factors. CNTF is undergoing phase III clinical trials for RP. Gene therapy seems to be the most promising for RP treatment, however, due to enormous genetic heterogeneity of the RP, targeting every specific gene seems to be daunting. Other treatment options being evaluated are highly invasive and include Argus® II retinal prosthesis system ('Argus II') for RP (Delyfer et al., 2004), retinal transplantation, and STEM cell technology. Most of these therapies (except Argus II) are still in investigational phase and need to overcome multiple hurdles before they can be applied to humans.

**Lens Epithelium Derived Growth Factor**

Lens epithelium derived growth factor (LEDGF) was originally isolated from a cDNA library of lens epithelial cells (Singh et al., 1999). It is homologous to hepatoma-derived growth factor (HDGF), HDGF related protein-1 and protein-2 (HRP-1 and HRP-2) (Nakamura et al., 1994; Izumoto et al., 1997). It is a 60 kDa protein also known as p75 and was found to be an autocrine, and paracrine growth factor (Singh et al., 1999). LEDGF originally thought to be a growth factor was later found to be a survival and transcription factor. It stimulated growth of lens epithelial cells, keratinocytes and skin fibroblasts cultured in serum free medium (Singh et al., 1999; Singh et al., 2000). Exogenous glutathione S-transferase tagged LEDGF (GST-LEDGF) maintained
keratinocytes and fibroblasts survival to 100 % for 6 days in serum free medium while cells in absence of LEDGF had only 40 % survival. GST-LEDGF also protected lens epithelial cells and RPE cells from oxidative and thermal stresses (Singh et al., 1999; Matsui et al., 2001). In presence of 1000 ng/ml of GST-LEDGF, RPE cells grew for 1 week and survived for 3 weeks, while in absence of GST-LEDGF, RPE died slowly by week 3. LECs overexpressing LEDGF (LECs/LEDGF) had increase resistance to thermal stress (Singh et al., 1999). When LECs were cultured in medium without serum at 41°C for 7 days, LECs/LEDGF cells survived 80 % as compared to 39 % for cells not expressing LEDGF (LECs/V). When LECs were treated with different concentration of H2O2, LECs/LEDGF survived better than LECs/V. LECs/V died within 24 hours in presence of 62.5 µM of H2O2 but LECs/LEDGF survived. Further it was also found that LECs/LEDGF cells also overexpressed small heat shock proteins (sHsps) such as heat shock protein 27 (Hsp27) and αβ-crystallin, 2 to 4 fold higher then LECs/V. GST-LEDGF protected photoreceptor morphology and function in retinal degenerative rat models including light damaged and Royal College of Surgeons (RCS) rat (Machida et al., 2001). Intravitreal injection of 1 µg of GST-LEDGF survived twice the number of photoreceptors and retained significantly higher b-wave amplitude in electroretinogram of RCS rats as compared to untreated rats. Adeno-associated virus mediated LEDGF (AAV-LEDGF) delivery to RCS rats significantly protected photoreceptors up to 7 weeks after single intravitreal injection. Although LEDGF overexpressed heat shock proteins (Hsps) 27, αβ-crystallin and Hsp90 in retinal cells in vitro, no compelling evidence was indicated that LEDGF neuroprotective action is via Hsp upregulation.
LEDGF has also been specified to be a potential target for human immunodeficiency virus (HIV-1) mediated diseases (Vets et al., 2012). LEDGF has potential to bind with HIV-1 integrase at its c-terminal, and with DNA at its n-terminal. Together this binding promoted integration, transcription and replication of HIV-1 in human (Cherepanov et al., 2003; Maertens et al., 2003). Thus despite LEDGF being a potential candidate for retinal degenerative disorders, it has a risk factor associated with it. In this project we identified a novel fragment; lens epithelium derived growth factor (1-326) (LEDGF<sub>1-326</sub>), and investigated LEDGF<sub>1-326</sub> protein feasibility to treat retinal degeneration.

**Protein Drug Delivery and Formulation Development<sup>1</sup>**

**Introduction**

For people between the age of 25 and 74 in the United States, the most common cause of blindness is diabetic retinopathy (progressive damage of retina due to diabetes) (Congdon et al., 2004). For people aged 60 and older, cataract (impaired vision due to the development of cloudiness or opacity in the lens), retinitis pigmentosa (RP, a retinal disease that causes progressive peripheral loss of vision leading to central vision loss in the retina), and age related macular degeneration (AMD, degeneration of macula due to age, stress, poor nutrition, and other factors, leading to loss of vision) are the major causes of blindness. Of the above disorders, diabetic retinopathy and AMD have neovascular forms of the disease. In proliferative diabetic retinopathy and neovascular

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AMD or wet AMD, retinal neovascularization and choroidal neovascularization is evident, respectively. As discussed in this chapter, macromolecule drugs have revolutionized the treatment of wet AMD. Currently, several other macromolecules are under development for the back of the eye. Below, prior approaches to treat wet AMD along with examples of macromolecule therapeutics for this and other back of the eye disorders are discussed.

At least as early as 1971, laser photocoagulation was introduced as the primary treatment for neovascularization (new vessel growth). For laser photocoagulation, an argon, xenon or krypton laser is aimed at the new blood vessels in order to destroy them using laser generated thermal energy (May et al., 1976; Group, 1991). Since thermal laser photocoagulation may lead to reduced vision due to the destruction of photoreceptors in the targeted area of the retina, especially the fovea (a region of the macula responsible for fine vision), it is applicable when abnormal vessels do not occupy the foveal region. Clinical evidence showed a decrease in the rate of severe visual loss and prevention of further contrast sensitivity loss with laser photocoagulation. However, following laser photocoagulation, recurrence of neovascularization occurs within two years of treatment (Group, 1991; Yamaoka et al., 1994). Regardless of potential adverse events and poor clinical outcome after 2 years, laser photocoagulation remained the main treatment option for proliferative diabetic retinopathy and investigations are underway to improve the clinical outcomes with this technique (Nagpal et al., 2010).

Another treatment for neovascularization is photodynamic therapy (PDT) (Kaiser, 2005), which entails intravenous infusion of verteporfin, a photosensitizing agent, which binds to low density lipoprotein (LDL) receptors that are elevated in
abnormal endothelial vessels. Subsequent application of laser energy activates
verteporfin, which then produces free radicals, ultimately causing damage to endothelial
cells and thrombus formation. Photofrin® was approved in 1995 for the treatment of
malignant dysphagia caused by esophageal cancer and Visudyne® was approved in 2000
as the first pharmacotherapy for treating neovascular or wet AMD. Compared to thermal
laser photocoagulation, treatment with PDT is safer (Schmidt-Erfurth et al., 1998).
Photocoagulation and PDT are effective only during the proliferative stage of disease. A
clinical trial in 1998 found that leakage from choroidal neovascularization (CNV) in
majority of the patients was stabilized up to 3 months after PDT treatment, yet recurrence
of CNV was observed in 50% of the eyes after 2 years of PDT treatment (Schmidt-
Erfurth et al., 1998; Wormald et al., 2007). With the arrival of anti-vascular endothelial
growth factor (anti-VEGF) therapy, laser photocoagulation and photodynamic therapy are
finding less widespread use.

Advances in anti-VEGF drug discovery introduced promising and revolutionary
macromolecule therapeutic agents for ocular diseases. Pegaptanib (Macugen®, EyeTech),
a PEGylated aptamer (oligonucleotide ligands having selective high binding affinity for
molecular targets) is effective in preventing vision loss in patients with CNV by binding
to VEGF. Ranibizumab (Lucentis®, Genentech), a monoclonal antibody fragment, has
been shown to improve visual acuity in patients with wet AMD. Clinical trials found that
95% of patients receiving monthly ranibizumab injections maintained their visual acuity
and 34-40% had improved vision (gaining 15 or more letters in 12 months). Bevacizumab
(Avastin®, Genentech), a full length humanized anti-VEGF antibody, approved by the
FDA to prevent regrowth of vessels at tumor sites in patients with colon cancer, breast
cancer, and non-small cell lung cancer, is currently used as an off-label drug to treat wet AMD. A new VEGF analog that has received increased attention is the VEGF trap (VEGF Trap-Eye™, Regeneron), a modified soluble VEGF receptor analog protein that binds more tightly to VEGF than pegaptanib (Ng et al., 2006) and ranibizumab (Stewart and Rosenfeld, 2008). With the development of anti-VEGF therapies, visual acuity of patients suffering from wet AMD and diabetic macular edema (DME) is expected to be significantly increased. In addition, development of therapeutics that target growth factor such as ciliary neurotrophic factor (CNTF) are under development for treating retinal degenerative disorders using the NT-501 intravitreal implant (Neurotech, Inc. developed an encapsulated cell technology based implant to deliver macromolecules directly to the site of action after cellular production), which is currently undergoing clinical trials. The results thus far have shown that the implant is safe up to 1 year after injection. Thus, protein and other macromolecule therapeutics are of value in treating disorders of the eye and are currently being explored for their potential long-term effects. The primary target for the current protein therapies of the eye are tissues of the posterior segment of the eye. However, due to the presence of formidable biological barriers, therapeutic macromolecules such as pegaptanib and ranibizumab as well as implants, encapsulating cells, are typically administered in the vitreous humor in order to ensure that therapeutic concentrations of the drug reach the target site in the back of the eye.

In this chapter various routes of administration, delivery strategies, and challenges, macromolecule case studies and standard protocols for formulation development are discussed with a key focus on protein drugs. Several of the approaches discussed might be relevant to nucleic acid therapeutics as well.
**Routes of Protein Administration**

Due to the unique anatomy and physiology of the eye, ocular drug delivery is historically challenging (Lee and Robinson, 1986; Kompella et al., 2010a). Protein delivery to the eye has been evaluated for various routes of administration including topical, intracorneal, intracameral, periocular (subconjunctival, sub-tenon, retrobulbar, and peribulbar), intravitreal, subretinal, suprachoroidal, and intravenous. The route of administration directly influences the extent of drug delivery to various target sites within the eye. Topical, intracorneal, intracameral, and periocular routes deliver the highest concentration of protein and other therapeutic agents to the anterior segment as compared to the posterior segment. Whereas, intravitreal, subretinal, and suprachoroidal injections deliver the highest concentration of protein and other therapeutic agents to the posterior segment as compared to the anterior segment. On the other hand, systemic administration or delivery by the intravenous route can potentially deliver proteins and other therapeutic agents, although at low concentrations, to the anterior and/or posterior segments of the eye, provided the drug can overcome the blood-aqueous, blood-retinal, blood-brain, metabolic, and immunologic or other clearance barriers. In the following discussion, some principal routes of administration of therapeutic proteins along with some successful examples are discussed.

**Topical**

Topical administration of drugs into the inferior fornix of the conjunctiva is typically used for treating diseases of the anterior segment of the eye. Due to rapid clearance from eye surface, drugs from an eye drop cannot typically reach the posterior segment at a therapeutic level (Lee and Robinson, 1986). Topically applied drugs
undergo rapid clearance and do not reside for long durations in the precorneal area due to mixing and dilution of drug with tears, tear turnover or tear drainage (0.7 µl/min in rabbit and 1.0-2.0 µL/min in human (Owen et al., 2007)), and blinking of the eye (once per 18 min for rabbits and 4-16 times per min in human (Friedman, 1973; Owen et al., 2007)), leading to poor drug bioavailability. In addition, the tight junctions of the corneal and conjunctival epithelial layers further restrict the drug from entering the eye.

Formulations such as suspensions, ointments, and gels may be used to prolong the preconeal drug residence. In-situ forming gels such as Gelrite™ were designed to overcome the precorneal elimination problem to a certain extent (Carlfors et al., 1998). Upon instillation, these drops undergo sol-gel transition in the cul-de-sac of the eye in the presence of mono- or di-valent cations of the lacrimal fluid. A formulation of indomethacin using Gelrite sustained drug release for 8 hours in vitro and was efficacious in treating uveitis in a rabbit model (Balasubramaniam et al., 2003). Topically applied drugs may be able to reach the posterior segment of the eye to a greater extent if the formulation has enhanced precorneal drug retention.

Interestingly, few protein drugs have been reported to permeate to the back of the eye following topical eye drop instillation. In a recent study, tumor necrosis factor (TNF)-alpha inhibitory single-chain antibody fragment (scFv; 26 kDa) (ESBA105) when administered as topical drop at high frequency followed by persistent opening of the eyes, showed absorption and distribution to various compartments of the eye as opposed to an intravenous injection of an equivalent dose (Furrer et al., 2009). In this study, rabbits were divided into three groups: two groups received ESBA105 topically as drops and one group received ESBA105 via intravenous administration. In Group one, ESBA105 was
administered topically as one drop every hour for 10 hr, up to 5 mg/day for one single day (after each administration, the eyes were kept still for 30 sec). Group two was given one topical drop of ESBA105 five times a day for six days up to 15 mg/6 days. Group three received an intravenous bolus injection of 5 mg of ESBA105 one time through the marginal ear vein. Drug concentrations were recorded in the following tissues: aqueous humor, vitreous humor, neuroretina, retinal pigmented epithelial (RPE)-choroid and serum. In Group one, the tissue levels of ESBA105 were: 12, 295, 214, 263, and 0.5 ng/ml in the aqueous humor, vitreous humor, neuroretina, RPE-choroid and serum, respectively, after a single day administration. Interestingly, vitreous humor levels were nearly 25 times higher than aqueous humor levels. In Group three, the following drug levels were obtained: 175, 63, 66, 2690, and 89,284 ng/ml in the aqueous humor, vitreous humor, neuroretina, RPE-choroid and serum, respectively after intravenous administration. Interestingly, rabbits given multiple topical doses in one day (Group one) had 4.7 times higher vitreous humor levels of ESBA105 as compared to rabbits given a single intravenous dose (Group three). In addition, the aqueous humor levels of ESBA105 after intravenous administration (Group three) was nearly 15 times higher than after topical administration (Group one). RPE-choroid drug levels were approximately 10 times higher after intravenous administration (Group three) than after multiple topical doses (Group one). In summary, the vitreous humor and neuroretina drug levels were nearly five times higher after multiple topical doses in one day than after a single bolus intravenous injection of the same dosage, yet aqueous humor levels were 15 times higher after intravenous bolus administration as compared to topical administration. After a single drop of ESBA105, the concentration reached 98 ng/ml in the vitreous humor. The
half-life of ESBA105 after multiple topical doses in a single day as well as after a single intravenous administration was significantly longer in vitreous, neuroretina and RPE-choroid as compared to aqueous humor and serum. Although the half-life of ESBA105 after intravenous administration was 1.5 times higher (24 hrs vs. 15 hrs) in the vitreous than multiple topical doses in one day, the neuroretinal half-life after multiple topical doses was 1.2 times (27 vs. 23 h) higher than intravenous administration. These results confirm the presence of the ESBA105 in specific locations within the back of the eye up to twenty seven hours after topical administration. Group two (topical administration for multiple days) showed a continuous rise in ESBA105 levels in all tissues and reached a steady state concentration of above 300 ng/ml in retina and above 500 ng/ml in vitreous humor. For both groups that received topical administration of ESBA105 either multiple doses in a single day or multiple doses over multiple days, the systemic exposure of ESBA105 was minimal compared to group three, given a single intravenous administration. The results from this study indicated that daily multiple topical doses of a protein drug may deliver therapeutic quantities to the posterior segment of the eye depending on the protein characteristics and concentration needed for a therapeutic effect.

In another study, eye drops of vasostatin, an endogenous angiogenesis inhibitor containing N-terminal fragments (CGA1-76 and CGA1-113) of chromogranin A, with an apparent molecular weight of 7 to 22 kD, have been shown to reduce CNV lesion area for at least up to day 35 following eye drop dosing for 20 days (Sheu et al., 2009). In this study, rats were dosed topically with 1 µg/ml of vasostatin in PBS, three times daily for 20 days after induction of CNV lesions by laser photocoagulation. On day 21, CNV lesions decreased to $3.5 \pm 1.11 \text{ mm}^2$ for vasostatin treated eyes as compared to $7.01 \pm$
1.07 mm$^2$ and 6.87 ± 2.03 mm$^2$, respectively, for untreated and vehicle (PBS) treated eyes. On day 28, the lesion sizes were 5.27 ± 1.06, 10.34 ± 1.3, and 8.99 ± 2.03 mm$^2$, respectively, in vasostatin, untreated, and vehicle treated groups. Although the CNV lesion areas did increase in all groups by day 35, the rate of increase was much slower in the vasostatin treated eye. The CNV lesion areas were 6.11 ± 1.33 mm$^2$, 11.03 ± 0.72 mm$^2$, and 9.75 ± 1.62 mm$^2$, respectively, for vasostatin, untreated, and PBS treated groups on day 35.

Insulin is currently administered only by systemic injection. However, earlier efforts have demonstrated that insulin administered as an eye drop can also reduce blood glucose levels (Nakamura et al., 1994) and further, the effects of insulin are enhanced when an absorption enhancer such as glycocholate or fusidic acid are included in the insulin formulation at pH 8.0 (Xuan et al., 2005). When 50 µl drops of 0.5% insulin (either pH 3.5 or pH 8.0) were administered into rabbit eyes topically, the blood glucose level was significantly reduced as a therapeutic effect of insulin. The blood glucose level was reduced to 65% when insulin was formulated at pH 8.0 (0.5% concentration), whereas the blood glucose level was reduced to 80% when the same concentration insulin was formulated at pH 3.5. Further, when 1% insulin drops were administered, the blood glucose level decreased to 30% and 70% for pH 8.0 and pH 3.5 formulations, respectively. When either 0.5% or 1% glycocholic acid was added to 0.125% insulin at pH 8.0, the blood glucose level decreased to 60% as compared to 80% with 0.125% insulin at pH 8.0 without glycocholic acid. Further, addition of either 0.25% or 0.5% fusidic acid to 0.125% insulin at pH 8.0 reduced the glucose level to 55% and 35%, respectively, as compared to 80% with 0.125% insulin at pH 8.0 without fusidic acid.
This study demonstrates that it is possible to reduce blood glucose levels by administering insulin as eye drops in the presence of an absorption enhancer at pH 8.0. The results from the studies discussed above demonstrate that protein therapeutics can potentially exert therapeutic effects in tissues of the back of the eye after topical administration. Since all of the above studies were conducted in animals, it is still uncertain if therapeutic proteins or other therapeutic agents can reach the posterior segment of the eye after topical administration in humans. Although drug is capable of reaching the posterior segment of the eye after topical administration, it is expected that drug levels in the posterior segment of the eye will be much less than if the drug was administered by intravitreal injection.

**Intracameral**

Intracameral injections either into the anterior chamber or aqueous humor are commonly used for delivering anti-infective agents or anti-inflammatory agents during eye surgery (Lee and Robinson, 2001; Karalezli et al., 2008). This route is inefficient in delivering therapeutic agents to the posterior segment of eye, and therefore, it might not be suitable for treating diseases such as retinal degeneration (Lee and Robinson, 2001). For instance, Lee and Robinson compared the vitreous and aqueous humor drug levels after intracameral and subconjunctival injections of $^{14}$C-mannitol into albino rabbits (Lee and Robinson, 2001). After 50 µL of 1.82 mM $^{14}$C-mannitol subconjunctival injection, the vitreous and aqueous drug concentrations were 18.7 nM and 261 nM, respectively, after 0.5 hr post-administration. However, when 10 µL of 1.82 mM $^{14}$C-mannitol was injected intracameraly, the vitreous and aqueous drug concentrations were 0.348 µM and 2.57 nM, respectively, after 0.5 hr. At five times the concentration of the intracameral
injected dose, the subconjunctival dose had 133 times less mannitol concentration in the aqueous humor and about 7.3 times higher concentration of mannitol in the vitreous humor. Thus, the ratio of aqueous to vitreous humor drug level was much higher for intracameral injections as compared to subconjunctival injections (13600 vs 14). Therefore, while intracameral route is efficient in distributing hydrophilic macromolecules to the aqueous chamber, subconjunctival route is more efficient in the relative distribution of drugs to the vitreous humor of the eye.

Interestingly, a separate study reported that intraocular injections (both intravitreal and intracameral) of bevacizumab was more effective in treating corneal neovascularization than subconjunctival injections in mice (Dratviman-Storobinsky et al., 2009). Mice were administered 25 mg/ml of intracameral, intravitreal, or subconjunctival injections of bevacizumab and the neovascularization areas were measured. The relative areas of corneal neovascularization after 10 days were: 19.86±1.23%, 24.20±14.87%, and 39.73±14.51% for intracameral, intravitreal, and subconjunctival administrations, respectively, compared to the untreated mice (50.62±24.74%). Similarly on day two, four, and eight, a similar trend was seen. That is, intracameral and intravitreal administrations resulted in lower corneal neovascularization areas as compared to subconjunctival route. These differences might be due to more rapid protein clearance from the subconjunctival space as opposed to slower clearance from the vitreous; and due to exposure of high peak concentrations to the cornea following intracameral injections. However, all three administrations had lower corneal neovascularization areas as compared to untreated group. Thus, in the treatment of anterior ocular illnesses such as corneal neovascularization, intracameral injections may be more appropriate than
subconjunctival. However, the focus of this chapter is on protein delivery to the posterior segment of the eye and thus far, intracameral injection of protein therapeutics has not been successful in delivering therapeutic quantities to the posterior segment of the eye.

Intravitreal

In 1940, intravitreal injections of penicillin were first injected into the posterior segment of the affected eye in combination with sulfadiazine to treat ocular infections of *P. aeruginosa* (Spencer, 1953; Baum et al., 1982). Since then, off-label intravitreal injections have found a wide range of clinical applications for the treatment of various diseases of the posterior segment of the eye. Macromolecules are relatively well suited for this route of administration as they are retained for a long period of time within the vitreous cavity after intravitreal injection, and this leads to an increase in the duration of pharmacological action. Within the vitreous, high molecular weight species were found to have a much longer half-life than low molecular weight species (Durairaj et al., 2009). Intravitreal injections are desirable as they inject the drug directly into the vicinity of the targeted tissue (i.e., the posterior segment of the eye, which is difficult to reach by other routes of administration) and in addition, they are relatively safe although several rare complications have been observed. After a single intravitreal injection of 1.25 mg (0.05ml) of bevacizumab administered to rabbit eyes, its concentration was maintained in the vitreous at >10 μg/ml for 30 days. The half-life of bevacizumab in the vitreous was reported to be 4.32 days in rabbits with a peak concentration of 37.7 μg/ml in the aqueous humor after only three days of administration (Bakri et al., 2007b). However, in serum, the maximum concentration of bevacizumab was 3.3 μg/ml after eight days and fell below 1 μg/ml after 29 days of injection. In the untreated contralateral eye, negligible
amount of drug was found (0.35 ng/ml after day one and 11.17 ng/ml after four weeks). This route of administration provides high concentrations of therapeutic agents within the proximity of target tissues of the back of the eye. Therefore, for high efficacy in treating retinal disorders, the intravitreal route of administration is the preferred route for macromolecules. However, repeated intravitreal injections can result in various complications including cataracts, retinal detachment, and endophthalmitis (Raghava et al., 2004). In addition, depending on the location of the target tissue, macromolecules may experience difficulty in permeating cellular membranes as well as intercellular junctions due to their extremely large molecular size and surface charge.

**Periocular (Transscleral)**

A potential solution to the many safety limitations of intravitreal injections is to use a transscleral delivery system, which is less invasive to the globe and hence, it might reduce the incidence of cataract, retinal detachment, and endophthalmitis. The transscleral route includes periocular routes of delivery such as subconjunctival, sub-tenon, posterior juxtascleral, and retrobulbar injections (Olsen et al., 1995; Ambati et al., 2000; Raghava et al., 2004). Depending on the site of action, the drug may have to cross the sclera, choroid and/or the RPE to exert its effects in the posterior segment of the eye. Since drugs are much more permeable across the sclera than the cornea (Prausnitz and Noonan, 1998), many investigators have assessed the periocular route of administration. Although high molecular weight compounds up to 150 kDa (fluorescein isothiocyanate-rabbit immunoglobulin) are able to permeate the sclera with a permeability coefficient of \(1.34 \pm 0.88 \times 10^{-6}\) cm/sec, this value is much lower compared to \(84.5 \pm 16.1 \times 10^{-6}\) cm/sec observed for a 376 Da (sodium fluorescein) compound.
(Ambati et al., 2000). Although small molecules as well as macromolecules permeate across the sclera, the permeability decreases with an increase in size (Prausnitz and Noonan, 1998; Ambati et al., 2000).

Periocular injections of an adenoviral vector expressing either FLT-1.10 (soluble VEGF receptor 1) or TGF-β.10 (transforming growth factor beta) or PEDF.11 (Pigment epithelium derived factor) showed similar concentration levels in both the sclera and choroid, but the retinal level was relatively low (8.6% of the peak level in the choroid) compared to choroidal level, which indicates that the protein could easily penetrate the sclera and choroid, but not the RPE to reach the neuroretina (Demetriades et al., 2008). This indicates that proteins of a size similar to PEDF can penetrate well into the choroid, but not into the retina. Thus, periocular injections of proteins might be more effective in treating choroidal neovascularization as opposed to retinal neovascularization.

**Suprachoroidal**

An emerging route of drug delivery is the suprachoroidal route of ocular administration. In this mode of administration drug is administered below the sclera and above choroid (Olsen et al., 2006). Early studies indicate that the drug solution can freely move into the suprachoroidal region exposing the drug to various parts of the eye. Unique needles, such as micro-needles, with a defined needle length are needed to inject drugs precisely below sclera and into the suprachoroidal space. Biodegradable poly (ortho esters) (POEs) polymers have been developed as a sustained delivery system to deliver proteins and oligomers to the back of the eye (Einmahl et al., 2002). POEs form a viscous ointment like material. Different formulations including POE alone, 1% sodium hyaluronate, 1% magnesium hydroxide (MG) with POE, 1% dexamethasone sodium...
phosphate (DEX) in POE were injected into the suprachoroidal site of rabbit eyes. Drugs were injected into the suprachoroidal space after separating the sclera from the choroid by making an incision (5-6 mm) on the sclera using a solid curved cannula. Eyes were monitored after operation on a regular interval by fundus photography, ultrasonography, fluorescein angiography, and histology. Fundus photography did not reveal any significant subretinal or choroidal hemorrhage. Fluorescein angiography showed some choroidal coloration and punctuated mask effect, but overall there was no visible detachment and destruction of RPE, which was further confirmed by ultrasonography. Histology revealed disorganization of RPE in case of sodium hyaluronate including focal loss. In the case of POE alone or with DEX or MG, vacuoles of different sizes were observed in the tissue near the injection site; however, neither inflammation nor any major retinal disorganization was observed and the neuroretina was intact except for some mild atrophy in both retina and choroid. Thus, POEs do provide for a safe and easy delivery system, but the advantage of this route and this biomaterial in terms of safety and efficacy has yet to be established for delivering proteins and other macromolecules for treating various back of the eye diseases.

Subretinal

Subretinal injections (injection between RPE and neuroretina) are commonly used in surgical procedures. Although subretinal injection leads to temporary retinal detachment, it is an excellent route for delivering drug to the retina while escaping most of the delivery barriers such as membrane and enzymatic degradation barriers (Johnson et al., 2008).
Goat immunoglobulin (goat-IgG) adsorbed on gold nanoparticles was successfully delivered to photoreceptor cells as well as RPE when injected subretinally (Hayashi et al., 2009). Rabbit sclera was punctured using a 25 gauge needle and a subretinal cannula was inserted. Goat-IgG (0.15-0.2 ml) either adsorbed on nanoparticles and suspended in PBS or directly dissolved in PBS was injected into the subretinal space using a 28 gauge needle. Nanoparticles successfully delivered the goat-IgG locally to photoreceptor cells as well as RPE as confirmed by immunohistochemistry and transmission electron microscopy; however, one week after injection, there was extensive retinal degeneration. It was argued that the retinal degeneration was specific to rabbits because rabbit retina is mostly avascular, in which case the retina largely depends upon the choroid for its nourishment. One of the major side effects of subretinal injection is temporary retinal detachment, which may in fact initiate retinal degeneration. The same may not be true for species with extensive retinal blood supply and hence, this route may still be promising to deliver drug locally to the retina.

A novel cell penetrating peptide for ocular delivery (POD) was found to be an effective delivery system for delivering green fluorescent protein (GFP) to the photoreceptor and RPE cells via subretinal injections (Steele et al., 1993). Approximately 8.5 µg of POD-GFP was injected in the subretinal space of mice and the eyes were enucleated after 6 hrs. The presence of POD-GFP was examined by immunocytochemistry. POD-GFP was detected in both photoreceptor cells and in RPE cells. Electroretinography (ERG) showed no significant toxicity. This route of administration may be promising for delivering proteins and macromolecules, but great
care has to be taken in the injection procedure to prevent complications associated with injection technique such as retinal detachment and degeneration.

**Systemic**

Systemic route involves injection of drug subcutaneously or intramuscularly or intraperitoneally or directly into the vein (intravenously). Although this route is safe and has better patient compliance in comparison to intravitreal or other routes of administration, this route fails to deliver therapeutic concentrations of drug in efficient manner to the back of the eye due to the presence of blood-aqueous, blood-retinal, and enzymatic barriers. Protein or other therapeutic agents delivered via systemic route, reach the back of the eye in very limited quantity (Furrer et al., 2009). After systemic administration of radio labeled chimeric protein IL-2 and pseudomonas exotoxin PE40, a very high level of drug was detected in blood, liver, and spleen; however, there was an undetectable quantity of drug in ocular tissues (BenEzra et al., 1995). Systemic delivery of bevacizumab for neovascular AMD was evaluated in an uncontrolled clinical study SANA (systemic bevacizumab (Avastin) therapy for neovascular age-related macular degeneration) (Moshfeghi et al., 2006). Bevacizumab was given as an intravenous infusion of 5 mg/kg twice or thrice every 2 weeks to patients suffering from neovascular AMD. On average there was increase in the visual acuity by 14 letters, while there was decrease in retinal thickness by 112 µm after 24 weeks. Bevacizumab was well tolerated and there were no significant systemic adverse effects. Although the study proved that systemic delivery is effective, it was inconclusive as to whether there was a possibility for systemic side effects in patients suffering from illness or disease. Thus, this route may
be of interest only if a targeted drug delivery system is developed that can deliver proteins or macromolecules specifically to ocular tissues and reduce the systemic distribution to other parts of the body such as liver, kidney and heart. Singh et al. (Singh et al., 2009) demonstrated that nanoparticles functionalized on their surface with peptide/protein ligands for integrin and transferrin receptors enhance nanoparticle delivery to the neovascular region of the choroid following intravenous administration, without enhancing nanoparticle uptake by various other organs. Table 2.1 summarizes various routes of administration for protein drug delivery to the back of the eye.

**Advantages and Challenges of Protein Delivery**

Proteins and peptides have unique tertiary structures that fold into unique conformations that allow for the protein to have selective interactions with its target. Many proteins function as enzymes, transcription factors and membrane receptors. Since proteins are biologically active molecules, they have been naturally designed to work at specific physiological conditions (pH, temperature, and salt conditions). In addition, the body has designed specific mechanisms to generate mature proteins and degrade them as necessary. The body has also developed a mechanism to recognize and destroy foreign macromolecules including proteins (the immune system). The biological function of a protein not only makes it a strong candidate to treat ocular diseases, but it is intrinsically flawed due to its instability, degradation by proteolytic enzymes, rapid excretion, and immunogenicity. Proteins are highly sensitive to their environment and therefore, are extremely unstable outside of their optimal physiological conditions. Protein properties can change with the pH, temperature, and ion concentration of the solvent, exposure to proteases, oxygen, or heavy metals. Freezing and thawing can also change the physical
### Table 2.1. Routes of Macromolecule Administration

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<td>Intracameral</td>
<td>Injection directly into the anterior chamber of the eye</td>
<td>Cataract surgery/ anterior segment diseases (neovascular iris rubeosis)</td>
<td>Bevacizumab</td>
<td>Little or no drug is expected to reach the posterior segment</td>
<td>Raghuram et al., 2007</td>
</tr>
<tr>
<td>Transscleral (Subconjunctival)</td>
<td>Injection underneath the conjunctiva</td>
<td>Corneal neovascularization</td>
<td>Bevacizumab</td>
<td>Regression of corneal vessels; not as effective as intracameral injection in reducing corneal neovascularization area</td>
<td>Raghuram et al., 2007; Furrer et al., 2009</td>
</tr>
<tr>
<td>Routes</td>
<td>Site of administration</td>
<td>Disease targeted</td>
<td>Therapeutic macromolecule delivered</td>
<td>Outcome/comment</td>
<td>References</td>
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<tr>
<td>Transscleral (Sub-Tenon)</td>
<td>Injection below the Tenon’s capsule</td>
<td>Inflammation and autoimmune eye disease</td>
<td>Chimeric protein IL2-PE40</td>
<td>IL2PE40 reaches the optic nerve in high quantities</td>
<td>(BenEzra et al., 1995)</td>
</tr>
<tr>
<td>Transscleral (Retrobulbar)</td>
<td>Injection in the conical compartment within the four rectus muscle and their intermuscular septa beyond the posterior segment of eye globe</td>
<td>Neurotrophic keratouveitis</td>
<td>PEDF (in rats)</td>
<td>Prevention of capsaicin-induced neurotrophic keratouveitis and peripheral vitreoretinal inflammation</td>
<td>(Feher et al., 2009)</td>
</tr>
<tr>
<td>Transscleral (Peribulbar)</td>
<td>Injection beyond the posterior segment of eye globe external to four rectus muscle and their intermuscular septa</td>
<td>Anesthesia; drug therapy</td>
<td>Lidocaine, triamcinolone acetonide (TA), a small molecule</td>
<td>Anesthesia before bilateral cataract surgery, peribulbar TA is effective in treating Graves’ ophthalmopathy</td>
<td>(Bordaberry et al., 2009; Budd et al., 2009)</td>
</tr>
<tr>
<td>Routes</td>
<td>Site of administration</td>
<td>Disease targeted</td>
<td>Therapeutic macromolecule delivered</td>
<td>Outcome/comment</td>
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<tr>
<td>Intravitreal</td>
<td>Injection or placement directly into the vitreous chamber</td>
<td>Age related macular degeneration, chronic non-infectious uveitis</td>
<td>Ranibizumab, Bevacizumab, Pegaptanib, Fluocinolone acetonide (Retisert&lt;sup&gt;TM&lt;/sup&gt;)</td>
<td>Complications associated with injection procedure, delivers the drug directly to site of action</td>
<td>(Landa et al., 2009)</td>
</tr>
<tr>
<td>Suprachoroidal</td>
<td>Injection between the sclera and choroid</td>
<td>Age related macular degeneration, choroidal neovascularization</td>
<td>Delivery systems such as POEs show reasonable tolerance and safety; macromolecules might clear more rapidly than from vitreous</td>
<td></td>
<td>(Einmahl et al., 2002)</td>
</tr>
<tr>
<td>Subretinal</td>
<td>Injection between the RPE and photoreceptor cells, that is, neuroretina</td>
<td>Retinitis pigmentosa, age related macular degeneration</td>
<td>POD system tested with green fluorescent protein</td>
<td>POD-GFP reaches the photoreceptor and RPE substantially</td>
<td>(Steele et al., 1993)</td>
</tr>
<tr>
<td>Routes</td>
<td>Site of administration</td>
<td>Disease targeted</td>
<td>Therapeutic macromolecule delivered</td>
<td>Outcome/comment</td>
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<tr>
<td>Intravenous</td>
<td>Systemic infusion</td>
<td>Age related macular degeneration</td>
<td>Bevacizumab</td>
<td>Decrease in the macular thickness and lesion but systemic side effects like arterial hypertension and thrombosis exist</td>
<td>(Schmid-Kubista et al., 2009)</td>
</tr>
</tbody>
</table>
conditions of protein (Simpson, 2010). In addition, proteins have poor shelf lives due to their instability and sensitivity to altered environments. Protein formulations are extremely sensitive to temperature. Generally the storage temperature preferred is below 4 °C; however, depending upon the nature of protein, the storage temperature may be as low as -70 °C. For example, the accelerated stability testing of bevacizumab at 30 °C showed changes in size exclusion chromatography, indicating change in the molecular size of the protein; ion-exchange chromatography indicated changes in the ionic variants of the protein as well as 20-30% decrease in potency (www.ema.europa.eu). (LaVail et al., 1975) Protein based therapeutics have often developed immunogenicity in patients leading to rapid drug clearance and even life threatening side effects such as anaphylactic shock. For example, muromonab-CD3, an immunosuppressant has been reported to cause immunogenicity in about 50% of patients; however, recent advancements in protein formulation have a lower frequency of immunogenicity. For example, the clinical trial for ranibizumab detected immunogenicity in only 1 to 8% of patients (Yoon et al., 2010).

**Current Development Strategies**

**Pure Protein**

The delivery of protein in its unmodified form is currently the most common approach due to the prolonged half-life of proteins in the vitreous humor, unlike in the plasma (Kompella and Lee, 1991), which allows administration once in several weeks. For instance, ranibizumab, FDA approved anti-VEGF therapy for treating CNV associated with wet AMD, is administered in its native form into the vitreous cavity. This
protein has a vitreal half-life of 3 days and allows intravitreal administration of 0.5 mg of drug in the clinic once every 6 weeks. Bevacizumab, another FDA approved drug for metastatic colorectal cancer is also administered intravitreally (off-label use) for treating CNV, central retinal vein occlusion, and proliferative diabetic retinopathy. Its vitreal half-life is found to be 4.32 days in a rabbit model following intravitreal administration of 1.25 mg in 50 µl. Concentrations above 10 µg/ml were maintained in the vitreous humor for bevacizumab for up to 30 days following intravitreal injection (Bakri et al., 2007b). When ranibizumab was administered at a 0.5 mg dose in 50 µl volume in the same rabbit model, its vitreal half-life was found to be only 2.88 days, but the concentration of 10 µg/ml of ranibizumab was detectable in the vitreous humor similar to bevacizumab for 29 days (Bakri et al., 2007a). Phase I clinical trails of VEGF Trap-Eye in twenty one patients suffering from neovascular AMD (NVAMD) were followed. Intravitreal injection of VEGF-trap up to 4 mg showed no ocular inflammation (Nguyen et al., 2009). There was a mean decrease in excess foveal thickness for all patients up to 104.5 mm at 6 weeks, and the visual acuity increased to 4.43 letters. Although the intravitreal half-life of VEGF Trap-Eye is unknown, it was predicted to be 4-5 days in primate eye based on its molecular weight. Further, based on the molecular model it was predicted that significant intravitreal VEGF-binding activity comparable to that of ranibizumab was maintained for 10 – 12 weeks after single injection of 1.15 mg of VEGF Trap-Eye (Stewart and Rosenfeld, 2008). Thus, relatively high retention rates have been observed after intravitreal delivery of protein therapeutics in their native form and these agents proved to be efficacious in treating several diseases.
PEGylation

PEGylation (attachment of polyethylene glycol, PEG) is one approach to enhance the half-life of protein drugs, thereby increasing their duration of action. PEG moieties are long chain amphiphilic hydrocarbons having repeated units of ethylene glycol (linear or branched) with a hydrophobic chain and hydrophilic ends. Some PEG containing pharmaceutical products are approved by the FDA for internal use. PEGs are generally considered as inert and possess very low toxicity. For example, PEG 300 showed acute oral toxicity in rats at LD$_{50}$ (lethal dose that kills half of the rats) of 27500 mg/kg (Lee and Robinson, 1986). For PEGs with higher molecular weight such as PEG 9000, the LD$_{50}$ can be as high as 50,000 mg/kg (Kompella et al., 2010b). PEGs can be branched or linear structures having different molecular weights ranging from 200 to 40,000 Da. PEGylation can affect the conformation, electrostatic binding and hydrophobicity of the protein molecule (Harris and Chess, 2003). It is hypothesized to reduce protein immunogenicity by coating the protein and preventing its recognition by the immune system (Harris and Chess, 2003). PEG-1900 and PEG-5000 covalently attached to bovine serum albumin increased its size almost 2 times and 4 times respectively, producing a substantial change in the physical and chemical properties of the protein such as solubility and movement in acrylamide gel during electrophoresis (Abuchowski et al., 1977). Antiserum against PEG-1900-albumin and albumin alone were raised in rabbits. Antiserum against PEG-1900-albumin did not react with PEG-1900-albumin, which is a clear indication that PEG-1900-albumin does not elicit an immune response. When this antiserum was tested against either PEG-5000-albumin or albumin in immunodiffusion plates, it showed no reaction with either PEG-5000-albumin or albumin alone. Further,
antiserum against native albumin was tested against albumin modified with increasing amount of PEG-5000. It was found that the higher the PEGylation of amino group of albumin with PEG-5000, the lower the antiserum binding. PEGylation of albumin to approximately 42% and higher led to complete loss of its reactivity with the anti-serum. Thus, PEGylation seems to be an efficient way to mask the immunogenic responses during protein therapy, potentially leading to reduced incidence of immunotoxicity.

PEG is soluble in both water and organic solvents, and has high hydration and hydrodynamic volume, resulting in reduction of systemic clearance and alteration in pharmacokinetic and pharmacodynamic properties of the protein (Delgado et al., 1992; Israelachvili, 1997; Mehvar, 2000; Harris and Chess, 2003). The plasma half-life of PEG is highly dependent on its molecular weight; the half-life ranges from 18 minutes to 1 day with an increase in PEG molecular weight from 6000 to 190,000 (Yamaoka et al., 1994). PEGylation can mask the protein from various enzymes (e.g., proteases). This not only reduces the proteolysis, but also toxicity which might occur due to the formation of unwanted metabolites. Thus, PEGylation increases protein/macromolecule stability and prolongs tissue retention time.

Despite its advantages, PEGylation causes partial loss in biological activity, especially when it is non-specifically conjugated. The attachment of PEG molecules to new investigational protein drugs is becoming a routine exercise due to the potential to increase drug retention time and reduce immunogenicity. However, as is the case for a new investigational protein drug, trichosanthin (a type I ribosome inactivating protein drug is currently under investigation for the treatment of HIV-1), PEGylation often reduces protein activity (Veronese et al., 2001). When 20 kDa PEG was non-specifically
conjugated to trichosanthin, the ribosome inactivating activity of the protein decreased by 20-30 fold as characterized by the decrease in half maximal inhibitory concentration (IC$_{50}$) (Wang et al., 2004). However, the loss in biological activity can be in most cases compensated by the substantial increase in the circulatory half-life of the molecule, which ultimately makes the molecule more efficient in comparison to the non-PEGylated moiety. N-terminal PEGylation or other site specific PEGylation have also been recently developed to overcome this problem in part (Israelachvili, 1997; Veronese et al., 2001; Harris and Chess, 2003; Nie et al., 2006; Veronese and Mero, 2008). The protein’s N-terminal amino group is the most exploited moiety for such conjugation. PEGs are available in various active derivatives with functional groups such as carbonate, ester, and aldehyde. These functional groups can react with free amino groups within the protein (e.g., lysine residues) to form a covalent bond. Controlling the pH of the reaction media has resulted in site specific PEGylation (Lee et al., 2003). N-terminal mono PEGylation has been successfully achieved for epidermal growth factor (EGF) by PEGylating EGF with PEG-propionaldehyde derivatives (Lee et al., 2003).

Aptamers, short oligonucleotides or peptides, 15 to 60 bases in length (Jarosch et al., 2006), have very short half-lives (Nimjee et al., 2005) due to the presence of nuclease in the body that rapidly degrade nucleic acid aptamers. PEGylation of aptamer was successfully used in an ophthalmic formulation to increase the half-life of the parent molecule. The half-life of the unmodified aptamer was found to be just 108 seconds in serum as compared to 10 ± 4 days for pegaptanib (PEGylated aptamer available as Avastin®) (Ng et al., 2006). Intravitreal injection of this molecule proved to be of therapeutic value in scavenging VEGF and treating wet AMD.
Micro- and Nano- Particles

Due to the limitations of current delivery systems and the problems associated with delivering macromolecules and small molecules to the posterior segment of the eye, nano- and micro-particles are being investigated. Nanoparticles are spherical particles of lipids, proteins, carbohydrates or polymers having a diameter within the nanometer range. Microparticles are polymeric particles with a size ranging from one micrometer to several microns. Poly (lactic acid), poly (glycolic acid), and related biodegradable and biocompatible polymers and copolymers are the most widely used polymers to synthesize sustained release micro- and nano-particles, which have promising applications in the eye.

Poly(lactic acid) (PLA) microparticles and nanoparticles were assessed for their ability to sustain retinal delivery of budesonide, a corticosteroid capable of inhibiting VEGF, following posterior subconjunctival administration (Kompella et al., 2003). PLA nanoparticles encapsulated with budesonide initially released approximately 25% of the loaded drug by day 1 and then the rate of drug release slowly declined to 0% after 10 days. However, PLA microparticles loaded with budesonide showed no initial burst and maintained a release rate of ~7 µg/day for 25 days. Assessment of retinal delivery in vivo in a Sprague Dawley rat model indicated prolonged retinal drug delivery with microparticles compared to nanoparticles. A single periocular injection of biodegradable poly(lactide-co-glycolide) microparticles loaded with celecoxib was more effective than plain drug suspension and alleviated vascular leakage associated with diabetic retinopathy (Amrite et al., 2006). Following intravitreal injection, PLA microparticles
sustained the intravitreal delivery of anti-angiogenic low molecular weight drug for at least 3 months (Shelke et al., 2011). Poly(lactide-co-glycolide) microparticles are also useful in sustaining peptide drug delivery (Koushik and Kompella, 2004). Functionalized nanoparticles capable of rapidly entering and crossing corneal and conjunctival barriers might be useful in enhancing delivery of macromolecules to the anterior as well as posterior segment eye tissues (Kompella et al., 2006).

**Liposomes**

Liposomes are closed spherical bilayers entrapping an aqueous core. The lipid bilayer can be comprised of phospholipids, cholesterol and other lipid moieties such as cardiolipin, which is similar to the composition of biological membranes. Macromolecules can be entrapped either in the lipid bilayer or within the aqueous core of the liposome depending on the lipophilicity of the protein or peptide drug; lipophilic agents prefer the lipid bilayer, while hydrophilic agents prefer the aqueous core. The residence time of the liposomal formulation of bevacizumab was significantly increased in the vitreous of rabbit eye compared to bevacizumab alone (Abrishami et al., 2009). The vitreous concentration of the liposome formulation of bevacizumab in a rabbit model was two-fold (48 vs. 28 µg/ml) compared to bevacizumab alone after 28 days and almost five times higher after 48 days (16 vs. 3.3 µg/ml), as monitored by an enzyme-linked immunosorbent assay.

**Stem Cells**

Once the visual acuity of patients has been reduced due to the loss of photoreceptors and retinal neurons, vision loss cannot be reversed with the use of anti-VEGF therapies or by PDT. Introducing stem cells that resemble retinal neurons and
photoreceptors may be the key to regain visual acuity. Depending on the retinal disease, neuroretinal cells (photoreceptors, bipolar cells, ganglion cells, and glial cells), retinal pigment epithelial cells, and vascular endothelial cells may be replaced by generating stem cells and introducing the mature cells into the retina. Genetically modified embryonic stem cells have been shown to survive for a prolonged period of time when administered intravitreally into the retinal tissue (Gregory-Evans et al., 2009). Mouse embryonic stem (mES) cells were genetically engineered to overexpress glial cell-derived neurotrophic factor (GDNF). Four microliters of cell suspension (50,000 cells/µl) was injected into the vitreous of rat eyes of the test group; control group received either unengineered mES cell suspension or PBS buffer. A statistical increase in the photoreceptor cells were observed in the test group as compared to the control group. Adverse events included retinal detachment, endophthalmitis, and lens opacity. In another study, retinal progenitor cells (RPC) were isolated from human placental alkaline phosphatase (hPAP)-positive embryonic day 17 (E17) rat retina and were transplanted in the subretinal space of transgenic rats via transscleral route (Qiu et al., 2005). The morphology of the cells in the transplant was similar to those in the normal rat eye. The RPC cells were found to be immunoreactive to a variety of antibodies like calbindin, rhodopsin and protein kinase C, suggesting that the cells were well integrated into the retina of the rat eye. The RPC stem cells were observed one month later in the rat eye using an immunohistochemistry assay. There were no retinal hemorrhages or detachments, vitreous opacities, or other signs of intraocular inflammation or clinical toxicity at week two and four after transplantation as seen under ophthalmoscope and
confirmed by optical coherence tomography (OCT). Stem cell research is currently underway to quantify the gain in visual acuity as well as to assess the stem cell transplant efficiency.

**Implants**

Implants are regularly used in ophthalmic diseases for providing sustained drug delivery. In 2005, Retisert® (Bausch and Lomb) was approved by the FDA for the treatment of noninfectious uveitis. It is a non-biodegradable implant comprised of fluocinolone acetonide (active ingredient) in a silicone/polyvinyl alcohol polymer coating situated on a polyvinyl suture that can effectively release fluocinolone acetonide for as long as two and half years (Jaffe et al., 2006). The disadvantage of this system is that it must be inserted and removed by a surgeon. The advantage is that patients are not subjected to frequent injections and doctor visits.

NT-501 (Neurotech, USA), a polymer capsule implant for intravitreal injection that is currently in clinical trials involves the use of encapsulated cell technology (ECT). This implant works by culturing a cell to secrete certain proteins or peptides in a slow release fashion. A phase III trial of ECT is currently underway involving genetically engineered cells capable of secreting ciliary neurotrophic factor (CNTF) for treating RP and non-neovascular AMD. In the phase I study (Tao et al., 2002; Sieving et al., 2006), capsules of cells transfected with CNTF were surgically implanted in the right eye of ten participants suffering from retinal neurodegeneration. Six months later when the implants were removed, the implant contained viable cells and the CNTF level was found to be still at a therapeutic level for treating retinal degeneration in RC1 dogs. Implants may be an alternative approach to obtain sustained release of macromolecules such as proteins.
The cell reservoir within an implant may be an efficient mechanism to slowly release the drug over prolonged periods. However, the long-term safety of any other factors released by these implants has yet to be ascertained.

**Case Studies**

Vascular endothelial growth factor (VEGF) is a vasoactive cytokine capable of increasing blood vessel permeability and proliferation. The VEGF family consists of peptides VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PIGF); all have similar structural domains, but each have different biological and physical properties (Ferrara, 2004). Alternative exon splicing of the VEGF-A gene generates eight isoforms and the predominant isoforms are VEGF$_{121}$, VEGF$_{165}$ (most predominant isoform), VEGF$_{189}$, and VEGF$_{206}$. VEGF plays a key role in angiogenesis and vasculogenesis (Ferrara and Gerber, 2001; Takahashi and Shibuya, 2005) during embryonic and early postnatal stage. It acts as a survival factor, vasodilator (Ferrara and Gerber, 2001) and has a role in glomerulogenesis, renal glomerular capillary function (Eremina et al., 2003) and the female reproductive cycle (Ferrara and Gerber, 2001; Takahashi and Shibuya, 2005). In pathological conditions, it plays an important role in wound healing (Nissen et al., 1998) including proliferative diabetic retinopathy (PDR) and diabetic macular edema (DME).

**Pegaptanib**

Pegaptanib (Macugen®; Eyetech Pharmaceuticals, New York, USA) is a PEGylated ribonucleic acid (RNA) aptamer that binds and neutralizes human VEGF$_{165}$ (Gragoudas et al., 2004). It is made up of single stranded nucleic acid that is synthesized
chemically and PEGylated with two 20 kDa polyethylene glycol molecules attached at each end. Its molecular weight is approximately 50 kDa. It has a unique three-dimensional structure that allows it to selectively inhibit VEGF$_{165}$, which has role in pathological conditions such as wet AMD. In 2004 pegaptanib was approved by the US FDA for the treatment of all forms of neovascular AMD. It is supplied in a single dose prefilled syringe containing 0.3 mg of the drug in 90 µl of injectable volume. Intravitreal injection of pegaptanib in human patients of 0.3 mg every six weeks for two years showed delayed neovascular AMD progression in comparison to controlled patients (Gragoudas et al., 2004; Donati, 2007). Its vitreous half-life is approximately four days and the therapeutic level is maintained for six weeks after single injection. Pegaptanib injection, every six weeks, significantly reduced mean visual acuity loss by approximately 50% in patients having subfoveal CNV as confirmed by the VEGF inhibition study in ocular neovascularization (VISION). Patients with AMD were reported to gain visual benefits when 0.3 mg of pegaptanib was administered intravitreally every six weeks for two years; that is, visual acuity was maintained compared to those patient who received standard of care or were discontinued from therapy (Chakravarthy et al., 2006). A clinical study confirmed that pegaptanib is systemically and ocularly safe (Cunningham et al., 2005). However, adverse effects associated with pegaptanib included anterior chamber inflammation, conjunctival hemorrhage and ocular discomfort, but these adverse effects were mild to moderate and were often due to injection procedure and not due to the drug itself. Further, a rise in intraocular pressure (IOP) was reported due to the intravitreal injection, but the IOP
diminished within an hour of injection. Other adverse effects included about 1% incidence of endophthalmitis, retinal detachment and iatrogenic traumatic cataract, and about 15% incidence of retinal arterial and venous thrombosis (2006). Despite these side effects, pegaptanib is clinically safe up to 0.3 mg dose (Apte et al., 2007).

Bevacizumab

Bevacizumab (Avastin®; Genentech, California, USA) is a full length humanized antibody (148 kDa) from mouse monoclonal antibody expressed in Chinese hamster ovary cells. It can inhibit all forms of VEGF (Ferrara et al., 2004). Its vitreal half-life is 5.6 days (Bakri et al., 2007b). It has been approved for systemic delivery of colorectal cancer in 2004, but it is also used off-label for the treatment of AMD-related CNV (Hurwitz et al., 2004). Typically it is administered as intravenous infusion with a dose ranging from 5 to 10 mg/kg body weight (infusion time: 1 hr) every two weeks in combination with other drugs such as 5-flurouracil for treating metastatic colorectal cancer. It blocks all forms of VEGF and hence, it might impair both physiological and pathological neovascularization, especially after systemic administration. Intravitreal injections of bevacizumab (1.25 mg in 0.05 ml every month) are capable of preventing ocular angiogenesis (Rosenfeld et al., 2005; Avery et al., 2006; Iliev et al., 2006; Spaide et al., 2006). Patients with neovascular AMD reported a gain in visual acuity by 2.2 lines in six months when treated with three intravitreal injections of 1.0 mg of bevacizumab every 4 weeks (Weigert et al., 2008). Similar to other anti-VEGF therapies, bevacizumab is also associated with ocular complications such as uveitis (0.09%), endophthalmitis (0.16%), and retinal detachments (0.16%). Further it can result in acute systemic blood
pressure elevation (0.59%) and even death (0.4%) (Arevalo et al., 2007). Triple therapy with verteporfin photodynamic therapy (PDT), bevacizumab, and dexamethasone also showed a significant improvement in the visual acuity after a single cycle of treatment in patients with CNV (Augustin et al., 2007).

**Ranibizumab**

Ranibizumab (Lucentis®; Genentech, California, USA), a recombinant humanized antigen Fab fragment (48 kDa) of mouse monoclonal antibody, has one binding site for VEGF (Ferrara et al., 2006). It is a globulin G1K isotype monoclonal antibody fragment. It is expressed in the *Escherichia coli* expression system. It blocks all forms of VEGF and hence it might impair both physiological and pathological neovascularizations, resulting in increased risk for systemic side effects. In 2006 it was approved for treatment of wet AMD by the US FDA. Its vitreal half-life is 2.88 days in rabbit eye (Bakri et al., 2007a). It is three to six fold more potent at inhibiting VEGF than bevacizumab (Chen et al., 1999; Heier et al., 2006a). A phase III clinical trial to was done to evaluate the safety and efficacy of intravitreal injections of ranibizumab (Rosenfeld et al., 2006). A dose of 0.5 mg in 0.05 ml was given monthly to patients with classic CNV secondary to AMD. Ranibizumab effectively increased the visual baseline. Another clinical trial ANCHOR (the anti-VEGF antibody for the treatment of predominantly classic choroidal neovascularization in age related macular degeneration) was conducted in 2006 and it confirmed that ranibizumab was moderately able to prevent the vision loss in neovascular AMD (Rosenfeld et al., 2006). Repeated intravitreal injections of ranibizumab were determined to be well tolerated by three clinical trials.
MARINA (Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the treatment of Neovascular Age-Related Macular Degeneration), PIER (phase I AMD, Multi-Center, Randomized, Double-Masked, Sham Injection-Controlled Study of the Efficacy and Safety Ranibizumab), and ANCHOR. Transient subconjunctival hemorrhage, minor intraocular inflammation and transient elevated intraocular pressure are few of the minor side effects that have been reported (Heier et al., 2006a; Rosenfeld et al., 2006).

**VEGF Trap-Eye**

VEGF Trap-Eye (Aflibercept®, Regeneron Pharmaceuticals, New York, USA) is a 110 kDa fusion protein composed of an extra cellular VEGF receptor sequence (VEGF1 and VEGF2) fused with a fragment crystallizable (Fc) region of human IgG1 (Saishin et al., 2003). It binds to all VEGF isoforms more tightly than any other anti-VEGF agent (about 140 times higher than ranibizumab). It also has a longer intravitreal half-life compared to ranibizumab due to its larger size. It is predicted to exhibit a longer duration of activity than ranibizumab at similar doses (Stewart and Rosenfeld, 2008). It is predicted that VEGF Trap-Eye requires less frequent administration and hence, there will be multiple advantages including lower medicinal cost, less frequent injections, improved patient compliance and fewer physician appointments (Stewart and Rosenfeld, 2008). Intravenous infusion of VEGF Trap-Eye seems to decrease the retinal thickness in a dose-dependent manner (Nguyen et al., 2006). A single intravitreal infusion of VEGF Trap-Eye (1.0 mg/kg body weight, infusion time - 1 hr) improved the visual acuity or at least stabilized the visual acuity in 95% of patients after 6 weeks (Nguyen et al., 2006). In
addition, a significant reduction in central retinal thickness was seen after eight weeks of injection. Currently phase III trials are being conducted.

**Ciliary Neurotrophic Factor**

Ciliary neurotrophic factor (CNTF) is a 64 kDa protein hormone, nerve growth factor and is also a survival factor for neurons. It has recently been shown to effectively reduce tissue attack during inflammatory response and it is currently in clinical phase II and III trial for the treatment of retinal neurodegenerative diseases including retinal pigmentosa (RP), caused by the loss of retinal photoreceptor cells (Sieving et al., 2006). CNTF has been found to effectively retard retinal degeneration in several animal models including rhodopsin knockout mice (Liang et al., 2001) and Q334ter rhodopsin transgenic mice (LaVail et al., 1998). The formulation of CNTF in the clinical phase I trial was delivered by retinal pigment epithelium cells that are transfected with the human CNTF gene and encapsulated in a capsule that is surgically placed in the vitreous (Sieving et al., 2006). The outer layer of the implant is permeable to CNTF and allows it to secrete from the implant once the encapsulated cells have produced the protein. Two cell lines (NTC-201-10 and NTC-201-6A) were used in the trial and they released 250 and 800 ng protein per 1 million cells *in vitro*, respectively. Three out of seven individuals showed an increase of 10-15 letters after two months of injection and their visual acuity was maintained for six months. *In vivo*, release rates of CNTF from the implant device remained constant for both the low and high output implant devices at 0.287 ± 0.7 ng/day and 1.53 ± 0.54 ng/day, respectively. Encapsulation of CNTF in an implant filled with RPE cells may be an effective method to deliver sufficient amounts of protein over a long period of time to treat retinal neurodegenerative diseases.
Other Growth Factors

Retinal diseases such as RP and AMD are caused by apoptotic cell death (Travis, 1998; Dunaief et al., 2002). Impediment of apoptosis (i.e., retinal cell death) is one of the promising fields, which can have a major impact on blindness. A variety of neurotrophic growth factors have shown great potential in inhibiting retinal degeneration in several animal models (Wenzel et al., 2005). Basic fibroblast growth factor (bFGF) was shown to delay photoreceptor degeneration in Royal College of Surgeons (RCS) rat (Faktorovich et al., 1990). Brain-derived neurotrophic factor (BDNF) (LaVail et al., 1998), ciliary neurotrophic factor (CNTF) (LaVail et al., 1998; Thanos et al., 2004), glial-derived neurotrophic factor (GDNF) (Andrieu-Soler et al., 2005; Buch et al., 2006), lens epithelium derived growth factor (LEDGF) (Machida et al., 2001), and rod derived cone viability factor (RdCVF) (Leveillard et al., 2004b) have been shown to inhibit retinal degeneration in various animal models. Pigment epithelium derived factor (PEDF) is a neuro-protective factor preventing neovascularization by protecting the retina and retinal pigmented epithelium and by inhibiting angiogenesis (Steele et al., 1993; Cayouette et al., 1999; Mori et al., 2002). Adenoviral vector delivery of complimentary DNA encoding human PEDF (AdPEDF.11; GenVec, Gaithersburg, MD, USA) has successfully inhibited ocular neovascularization (Mori et al., 2002). A phase I trial has shown that there are no adverse events or dose dependent toxicities associated with PEDF in patients with neovascular AMD (Campochiaro et al., 2006). Table 2.2 summarizes clinical trials of several macromolecule drugs in the eye. Table 2.3 summarizes promising protein drugs useful in treating diseases of the back of the eye.
<table>
<thead>
<tr>
<th>Therapeutic agent</th>
<th>Target disease</th>
<th>Clinical trial</th>
<th>Observation</th>
<th>Clinical level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pegaptanib</td>
<td>CNV</td>
<td>VISION (Chakravarthy et al., 2006)</td>
<td>Risk of ≥3 lines vision loss reduced to 67% in 1 year</td>
<td>FDA approved</td>
</tr>
<tr>
<td></td>
<td>DME</td>
<td>MDRS- Phase II (Cunningham et al., 2005)</td>
<td>In 1 year, a gain of 18% vision</td>
<td>Phase-III ongoing</td>
</tr>
<tr>
<td>Ranibizumab</td>
<td>CNV</td>
<td>ANCHOR MARINA, PIER, PrONTO (Takeda et al., 2007; Regillo et al., 2008)</td>
<td>In 2 years, there was a gain of 6.6 letters</td>
<td>FDA approved</td>
</tr>
<tr>
<td></td>
<td>DME</td>
<td>READ-2-Phase II (Hayashi et al., 2009)</td>
<td>Reasonable safety profile</td>
<td>Phase III- for DME- ongoing</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>CNV</td>
<td>Case series (Avery et al., 2006; Bashshur et al., 2006; Rich et al., 2006; Spaide et al., 2006)</td>
<td>Visual improvement of 15–30 letters</td>
<td>Phase-III ongoing</td>
</tr>
<tr>
<td></td>
<td>DME</td>
<td>Case series (Haritoglou et al., 2006; Arevalo et al., 2007)</td>
<td>In 1 year, visual improvement of 7 letters</td>
<td>Phase-III ongoing</td>
</tr>
<tr>
<td>VEGF-trap</td>
<td>CNV</td>
<td>CLEAR IT-1 (Nguyen et al., 2006)</td>
<td>In 6 weeks, visual improvement of 4.8 letters</td>
<td>Phase-II ongoing</td>
</tr>
<tr>
<td></td>
<td>DME</td>
<td>DAVINCI –Phase II (Ferrara et al., 2006)</td>
<td>Visual acuity gain of 8.6 to 11.4 letters depending on dose</td>
<td>Phase II Completed</td>
</tr>
<tr>
<td>Therapeutic agent</td>
<td>Target disease</td>
<td>Clinical trial</td>
<td>Observation</td>
<td>Clinical level</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------</td>
<td>----------------</td>
<td>-------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Wet AMD</td>
<td>Phase II</td>
<td>5.3 mean letter gain in visual acuity in 52 weeks</td>
<td>Phase-III- VIEW-1 and VIEW-2 ongoing</td>
<td></td>
</tr>
<tr>
<td>RP</td>
<td>Phase I (Einmahl et al., 2002)</td>
<td>Reasonable safety profile</td>
<td>Phase I Completed</td>
<td></td>
</tr>
<tr>
<td>CNTF (NT-501)</td>
<td>Dry AMD</td>
<td>Phase II (Ehrlich et al., 2008)</td>
<td>Stabilized best corrected visual acuity (BCVA) in 12 months</td>
<td>Phase II Completed</td>
</tr>
<tr>
<td></td>
<td>Phase II/III (Feher et al., 2009)</td>
<td>Not available</td>
<td>Phase II/III ongoing</td>
<td></td>
</tr>
</tbody>
</table>

CNV = Choroidal neovascularization; DME = Diabetic macular edema; AMD = Age related macular degeneration; RP = Retinitis pigmentosa; ANCHOR = Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in Age-Related Macular Degeneration; CLEAR = Clinical Evaluation of Antiangiogenesis in the Retina; DA VINCI= DME And VEGF Trap-Eye: Investigation of Clinical Impact; ETDRS = Early Treatment for Diabetic Retinopathy Study; FAIS = Fluocinolone Acetonide Implant Study; MARINA = Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration; MDRS = Macugen Diabetic Retinopathy Study; PIER = Phase I AMD, Multi-Center, Randomized, Double-Masked, Sham Injection-Controlled Study of the Efficacy and Safety Ranibizumab; PrONTO = Prospective Optical Coherence Tomography Imaging of Patients with Neovascular AMD Treated with Intra-Ocular Ranibizumab (Lucentis); READ = Ranibizumab for Edema of the Macula in Diabetes VEGF = vascular endothelial growth factor; VIEW = VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet age related macular degeneration; VISION = VEGF Inhibition Study in Ocular Neovascularization.
Table 2.3. Growth Factors for the Treatment of Retinal Degenerative Diseases.

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Target Disease</th>
<th>Species Tested</th>
<th>Delivery Approach</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fibroblast growth factor (bFGF)</td>
<td>Retinal degeneration</td>
<td>Rat</td>
<td>Subretinal injection</td>
<td>(Faktorovich et al., 1990)</td>
</tr>
<tr>
<td>Brain-derived neurotrophic factor (BDNF)</td>
<td>Retinal degeneration slow (RDS), nervous (NR), and Purkinje cell degeneration (PCD)</td>
<td>Mouse</td>
<td>Intravitreal injection</td>
<td>(LaVail et al., 1998)</td>
</tr>
<tr>
<td>Ciliary neurotrophic factor (CNTF)</td>
<td>Retinal degeneration</td>
<td>Rabbit</td>
<td>Encapsulated cell therapy (ECT)-based NT-501 device implant</td>
<td>(Thanos et al., 2004)</td>
</tr>
<tr>
<td>Glial-derived neurotrophic factor (GDNF)</td>
<td>Retinal degeneration</td>
<td>Mouse</td>
<td>PLGA- microspheres, Intravitreal injection</td>
<td>(Andrieu-Soler et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Retinal degeneration</td>
<td>Rat</td>
<td>Mouse Embryonic stem cells (mES)</td>
<td>(Gregory-Evans et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Glaucoma</td>
<td>Rat</td>
<td>Biodegradable microspheres, Intravitreal injection</td>
<td>(Jiang et al., 2007)</td>
</tr>
<tr>
<td>Lens epithelium derived growth factor (LEDGF)</td>
<td>Retinal degeneration</td>
<td>Rat</td>
<td>Intravitreal injection</td>
<td>(Machida et al., 2001)</td>
</tr>
<tr>
<td>Growth Factor</td>
<td>Target Disease</td>
<td>Species Tested</td>
<td>Delivery Approach</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>---------------------------------</td>
<td>----------------</td>
<td>-----------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Pigment epithelium-derived growth factor (PEDF)</td>
<td>Retinal degeneration, Retinal degeneration slow (RDS)</td>
<td>Mice</td>
<td>Intravitreal injection</td>
<td>(Cayouette et al., 1999)</td>
</tr>
<tr>
<td>Rod derived cone viability factor (RdCVF)</td>
<td>Retinitis pigmentosa</td>
<td>Mice</td>
<td>Subretinal injection</td>
<td>(Leveillard et al., 2004b)</td>
</tr>
</tbody>
</table>
Ophthalmic Protein Formulation Development

Ophthalmic protein formulation development is a complicated process as proteins are sensitive and easily perturbed by changes in their surroundings. Conformational stability of proteins, which is maintained by weak physical interactions and disulfide linkages, can be compromised by changes in pH and ionic strength (Saishin et al., 2003).

The three-dimensional structure of proteins can also be disrupted by a number of variables that are encountered during the development of suitable formulations. One of the major concerns while formulating proteins is the humidity of the surroundings. A “low humidity” environment in most manufacturing units will be around 20% relative humidity. However, this can be too high for proteins, which have an inherent nature to absorb large amounts of water leading to degradation during storage or distribution. Changes in protein structure can not only negatively impact its therapeutic effect, but can also trigger adverse immune reactions in the body (Hermeling et al., 2004).

Effective formulations must, therefore, safeguard a protein's structural integrity, while achieving the desired therapeutic effect. In order to maintain the protein’s efficacy, the formulation developed must be resistant to both physical degradation, such as aggregation and denaturation, as well as chemical degradation, such as oxidation and deamination.

Table 2.4 lists four macromolecule formulations that were either approved (ranibizumab and pegaptanib) or used off-label (bevacizumab and infliximab) for administration to the vitreous humor of the eye. Of these, all formulations are protein based, except pegaptanib, which is an aptamer. While the off-label use of bevacizumab is
widely undertaken with no known serious adverse events, off-label use of infliximab has been associated with retinal toxicity and immunogenicity (Giganti et al.).

**Protein Biosynthesis**

The first step in protein formulation is to genetically engineer a cell to produce therapeutic protein. For instance, ranibizumab is produced in *Escherichia coli* cells. The genetic information encoding the protein (DNA) provides the cell with the complete instructions to produce (generate) the protein. Typically the cells are engineered to express the protein in the cell and then depending on the nature of the protein it might either be secreted or retained within the cell. Genetically engineered cells are kept frozen as stock for future use in a manufacturing process. At the time of use, these cells are thawed and allowed to grow in a culture medium. The medium properties and growth parameters adopted during this step are crucial since they can drastically affect the cell growth and consequently the protein output. Once the cells have grown to a significant number, they are transferred to a larger tank (e.g., 1000 liter capacity), wherein their growth is continued. The cell medium is separated and if the protein is secretory in nature, the media is subjected to additional steps wherein any possible contaminants including cell debris, salts or unwanted proteins are removed. When the protein is retained in the cell, the cell is disrupted either by sonication or lysis and the protein is separated from the cellular debris. Bioburden within the manufacturing room should be controlled during the processing. Also, bacterial endotoxins in the end product should be eliminated or minimized as per regulatory guidelines. A pure protein devoid of contaminants prepared as above is used in further development.
Table 2.4. Macromolecule Formulations used for Intravitreal Administration in the Clinic.

<table>
<thead>
<tr>
<th>Product Brand Name (Generic name)</th>
<th>Dose; Route of Administration</th>
<th>pH of the Formulation</th>
<th>Excipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lucentis® (Ranibizumab)</td>
<td>0.05 ml of a 10 mg/ml solution; intravitreal injection</td>
<td>pH 5.5</td>
<td>10 mM histidine HCl, 10% α, α-trehalose dihydrate, 0.01% polysorbate 20, q.s. water for injection</td>
</tr>
<tr>
<td>Avastin® (Bevacizumab)</td>
<td>4 ml or 16 ml of a 25 mg/ml solution; intravenous injection</td>
<td>pH 6.2</td>
<td>Each 100 ml solution contains, 240 mg α, α-trehalose dehydrate, 23.2 mg of sodium phosphate monobasic monohydrate, 4.8 mg of sodium phosphate dibasic anhydrous, 1.6 mg polysorbate 20, q.s. water for injection</td>
</tr>
<tr>
<td>Macugen® (Pegaptanib sodium)</td>
<td>0.3 mg/90µl; intravitreal injection</td>
<td>pH 6-7</td>
<td>Each 90µl contains, 0.069 mg sodium phosphate monobasic monohydrate, 0.11 mg of sodium phosphate dibasic heptahydrate, 0.8 mg sodium chloride, q.s. water for injection</td>
</tr>
<tr>
<td>Remicade® (Infliximab)</td>
<td>100 mg /10 ml; intravenous injection</td>
<td>pH 7.2</td>
<td>Each 10 ml contains, 500 mg sucrose, 0.5 mg polysorbate 80, 2.2 mg monobasic sodium phosphate monohydrate, 6.1 mg dibasic sodium phosphate dihydrate, q.s. water for injection</td>
</tr>
</tbody>
</table>
Preformulation Studies

Development of a stable protein formulation is one of the crucial steps in developing a protein as a therapeutic moiety. The first step in developing a formulation is the selection of a dosage form for the delivery of the protein. Most of the formulations available today are in the form of freeze dried powders since freeze drying results in a stable formulation with good shelf-life (Tang and Pikal, 2004; Tsinontides et al., 2004). Selection of the dosage form also helps in selecting the vehicle and process parameters. Chang and Hershenson have summarized a list of strategies useful in designing formulation studies. Information such as clinical indications, dose requirement, and drug interactions can help in narrowing down the formulation and dosage form (Chang and Hershenson, 2002).

The second step of preformulation studies comprises identification of different mechanisms that lead to degradation of the protein. Mechanisms of degradation are determined by subjecting the protein to conditions that protein might encounter during processing and final formulation (Table 2.5). Preformulation studies help in making rational decisions about excipients and conditions to be used in formulation studies and also give relevant information about handling and storage of the protein. A reproducible stability indicating assay is also developed during preformulation studies to assess the loss of protein integrity and activity during testing. Reversed phase HPLC and mass spectrometry are good techniques for development of stability indicating assays to identify amino acid modifications, sequence variations and degradation products (Hoffman, 2000; Srebalus Barnes and Lim, 2007).
<table>
<thead>
<tr>
<th>Conditions for preformulation</th>
<th>Condition range &amp; limits</th>
<th>Changes to monitor</th>
<th>Instruments needed to monitor change in conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>0-50°C</td>
<td>Increase in aggregates</td>
<td>Size exclusion chromatography –HPLC</td>
</tr>
<tr>
<td>Light</td>
<td>&gt; 1.2 million lux hours &amp; 200 watt hours/square meter UV light</td>
<td>Structural changes (secondary and tertiary) Identification of the degradation mechanism</td>
<td>Analytical centrifuge</td>
</tr>
<tr>
<td>Freezing and thawing</td>
<td>Freeze at -20°C and thaw at room temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidation</td>
<td>Peroxide treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical stresses</td>
<td>Agitation, stirring using a mixer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>Protein subjected to pH range of 3-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionic strengths</td>
<td>Different ionic strengths of formulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffers</td>
<td>Based on desired pH, different buffers to be tried</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Based on information provided in Chang et al. (Chang and Hershenson, 2002)
Selection of Excipients

The third step in developing a formulation involves selection of various excipients that can potentially be used for preserving and stabilizing the formulation. In addition, a set of formulations are identified at this stage for stability and process compatibility studies. The different excipients that are to be optimized are given below. Excipients listed below are mostly of pharmacopoeial grade (United States Pharmacopoeia) and generally regarded as safe (GRAS). Use of new excipients requires additional studies, time, and cost to meet regulatory standards.

Buffer Ingredients

pH is the most significant parameter to be stabilized as it is a major cause of degradation of proteins and therefore, buffers should be selected judiciously (Khosravi and Borchardt, 2000; Liu et al., 2008). Akers and DeFelippis have listed the buffering agents that are useful for protein formulations (Table 2.6). Based on the optimum pH selected during preformulation, the pH range is defined and the buffer ingredients are selected accordingly.

Table 2.6. Examples of Buffers Useful in Preparing Protein Formulations.

<table>
<thead>
<tr>
<th>Buffer system</th>
<th>Effective pH range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>2.5-6.5</td>
</tr>
<tr>
<td>Citrate</td>
<td>3.0-8.0</td>
</tr>
<tr>
<td>Phosphate</td>
<td>3.0-8.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.0-7.8</td>
</tr>
<tr>
<td>Glycinate</td>
<td>6.5-7.5</td>
</tr>
<tr>
<td>Tris</td>
<td>6.8-7.7</td>
</tr>
</tbody>
</table>

Prepared based on Akers and Defelippes (Akers, 2000)
Stabilizing Agents

As most of the protein formulations are in the form of a lyophilized cake, the protein has to be protected from degradation due to the stresses encountered during freezing and dehydration of the lyophilization cycle. Lyophilization is a process by which bound water is removed from the protein, thus breaking hydrogen bonds, which is the main cause of protein aggregation (Kim et al., 2003). Stabilizing agents such as sucrose and trehalose are added to the formulation to minimize protein aggregation. It has been observed that sugars prevent unfolding during dehydration because they form hydrogen bonds with protein in the place of lost water molecules (Carpenter et al., 1997).

Surfactant

Surface active agents help in reducing air-water or water-solid interactions of proteins (Saishin et al., 2003). Surfactants are amphiphilic molecules, meaning they have both a hydrophobic group and hydrophilic group, and therefore, can preferentially interact with surfaces (where the hydrophilic portion interacts with the solution and the hydrophobic interaction interacts with air). This prevents the protein from interacting with the air or solid, which is in close vicinity of the protein, ultimately preventing protein unfolding. Nonionic surfactants such as polysorbate 20 and 80 are commonly used to protect proteins from unfolding at interfaces. Surfactants also protect proteins from surface induced denaturation during freezing, which is encountered during lyophilization (Chang et al., 1996).

Antioxidants

Some amino acids are sensitive to oxidation and therefore, exposure to oxygen, light or free radical initiators can result in oxidation of the protein and subsequent loss of
activity (Chang and Hershenson, 2002). Oxidation of cysteine and methionine leads to disulphide bond formation and loss of activity. Other oxidation prone amino acids are the ones with ring structures such as tryptophan, phenylalanine and tyrosine (Manning et al., 1989). Ascorbic acid and salts of sulphurous acid are the most frequently used antioxidants and a concentration of 0.1 – 1.0 % can typically be used to prevent oxidation (Hovorka and Schoneich, 2001).

**Preservatives**

Other than the excipients listed above, particular situations demand the use of some special excipients. If the formulation is packed in multiple dosages instead of a single unit dosage, preservatives are a mandatory regulatory requirement. Parabens, cresol, and benzyl alcohol are some common preservatives used and can be added to the formulation to stabilize against microbial agents that affect the formulation once it is exposed to air.

**Optimization of Process Variables**

Formulation development involves the use of different processes to achieve the final formulation. Below is a list of process variables that are needed to be optimized during formulation development.

**Temperature**

Incubating protein formulations at abnormal temperatures can cause irreversible denaturation due to aggregation (Saishin et al., 2003). A range of temperatures need to be assessed during formulation development to determine an optimum temperature range that does not affect the protein. As protein can denature at both high and low temperatures, temperatures that are both higher and lower than the protein’s optimum
temperature need to be assessed. The temperature study has to be done with the formulation that was finalized by selecting suitable excipients from the above list.

*Agitation During Manufacturing (Shaking and Stirring)*

Studying protein stability at different agitation rates is necessary to predict the behavior of the formulation during shipping and transport. Aggregation can occur during the mechanical stresses encountered, thereby causing protein unfolding (Wang, 1999). Agitation studies need to be done at temperature extremes to determine the effect of temperature on agitation induced aggregation.

*Freeze Thawing*

During lyophilization and also during shipping of the formulation, the product is subjected to intermittent freezing and melting. During freezing, the fall in temperature can perturb protein’s secondary and tertiary structures. This can lead to aggregation due to structural perturbation (Chang et al., 1996). The formulation has to be subjected to freezing and thawing cycles to study the loss of protein during temperature fluctuations.

*Photodegradation*

During formulation preparation, proteins are subjected to light exposure either during purification via UV based column chromatography or during fill finish operations wherein inspection of filled vials is performed under light. Light can cause damage to the protein if an amino acid such as methionine that is prone to oxidative degradation in presence of light is present in the protein structure (Hovorka and Schoneich, 2001). Typically, light exposure of 1.2 million lux hours and 200 Watt hours/square meter of ultra violet (UV) light is needed to induce degradation of proteins.
**Container Closure System**

Container closure system entails the entire packaging that protects and contains the product. The final immediate pack of the formulation is of critical importance because of the direct interaction that occurs between the protein and container material. The material that the container is made of may contaminate the formulation, which can contribute to the degradation of the protein. Decisions regarding the choice of material for the container are taken at an early stage of the development cycle. Some inputs are expected from the marketing department (based on the market appeal of a pack) and some inputs are put forth by the formulation scientist (based on the interaction study of the container material with the protein and also based on the physical strength of the pack to withstand the stress applied during filling, packing, and transport). Most lyophilized formulations are supplied in glass vials made of borosilicate glass type I. Glass is able to withstand stress and is also physically appealing. Prefilled syringes can also be used to improve patient compliance if self-administration is an option.

**Specifications and Regulatory Guidelines**

After optimizing the formulation, product specifications have to be finalized by the formulation scientist. Product specifications are used to monitor the product for reproducible manufacturing and also for stability. Specifications include physical and chemical parameters of the formulation with acceptable limits and analytical methods to carry out the tests for these parameters (Figures 2.3 and 2.4). Guidelines have been issued by the FDA for the Chemistry, Manufacturing and Controls for a therapeutic recombinant DNA-derived product. Other significant guidance has also been instituted to make the
review of manufacturing changes more reliable (Nakamura et al., 2000; Shashidharamurthy et al., 2005).

Drug substance and drug product specifications are to be made based on the information gathered during protein manufacturing and development of protein formulation. These will help in determining the identity, purity, and potency of the product.

**Identity**

Tests for identity are meant to be specific and capable of uniquely identifying the protein in the formulation (or as a substance). These tests are not necessarily meant to be quantitative. Tests like color and pH also offer simple assays to characterize the formulation.

**Purity**

No single method can be relied upon for the measure of purity of a protein. A combination of methods is usually used to assess purity. Chromatographic methods such as reversed phase HPLC and mass spectroscopy can be combined to assess the purity of the protein in the formulation. It is of paramount importance that the method selected to judge the purity of the protein is able to quantify the protein from its degradation products.

**Potency**

Potency assays are intended to mimic the specific biological activity of the protein. For many products, *in vivo* assays have been developed, to measure the biological activity of the protein. For example, the human growth hormone is tested by measuring the daily weight gain in rats which are given a daily injection of the hormone (Hoffman, 2000).
Figure 2.3. Sample Tests Conducted on Drug Substance to Assure its Identity, Purity and Potency. Stability studies, substance development studies, and routine batch analysis are used to finalize the tests and the acceptance criteria.
Figure 2.4. Sample Tests Performed on Drug Products to Assure that the Drug Product is Suitable for Release. Tests are a combination of physical, chemical, biological, and microbiological tests that help in confirming the identity, purity, and potency of drug products.
The International Conference on Harmonization of Technical Requirements (ICH) is a joint effort by the regulatory authorities of Europe, Japan and the United States and experts from the pharmaceutical industry in the three geographical regions to develop technical guidelines and registration processes for pharmaceutical products. Many guidelines are provided at the official website of ICH (www.ich.org), which can be accessed to understand the process of product development. Listed below are a few ICH and FDA (www.fda.gov) guidelines that are important for biotechnological products.

1. Q5C Quality of Biotechnological Products: Stability Testing of Biotechnological /Biological Products. ICH.
2. Q5E Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process. ICH.
3. Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological /Biological Products. ICH.
4. Drug Administration: Guidance for Industry for the Submission of Chemistry, Manufacturing, and Controls Information for a Therapeutic Recombinant DNA-Derived Product or a Monoclonal Antibody Product for In Vivo Use. FDA.
5. Guidance Concerning Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-Derived Products. FDA.
8. Guidance for Industry - Changes to an Approved Application for Specified Biotechnology and Specified Synthetic Biological Products. FDA.

**Conclusions**

Visual acuity is sacrificed in millions of patients suffering from a variety of retinal diseases including diabetic retinopathy and macular degeneration. Macromolecule-based anti-VEGF pharmaceuticals have been recently approved by the FDA to treat neovascularization in patients with wet age-related macular degeneration. When designing a protein pharmaceutical for ophthalmic applications, in addition to ensuring that the drug recognizes a specific target, it is important to ensure that the protein drug can be delivered to its site of action in its active form. Delivery to the site of action may be hindered by ocular barriers such as the blood-retinal barriers and vascular clearance mechanisms present at a particular site of administration. Due to differences in these barriers, various routes of ocular administration deliver different quantities of protein drug to the back of the eye. Novel delivery approaches such as PEGylation, protein-secreting implants, and nanoparticles can potentially overcome the drug clearance mechanisms, localize the drug near the target, and maintain the drug in an effective form. Finally, the design of any pharmaceutical product must consider formulation development challenges to ensure that the drug is not inactivated during the formulation process and storage, by carefully selecting the excipients, process parameters, and storage conditions. With novel delivery systems such as implants and nanoparticles, chronic or noninvasive delivery of proteins to the posterior segment of the eye will likely be realized in the near future. Once novel approaches are developed to enhance and sustain delivery
of protein drugs to the posterior segment of the eye, more impetus for the discovery of novel protein-based treatments for back of the eye diseases is anticipated.

Acknowledgements

This work was supported by the NIH grants R01EY018940, R01EY017533, and RC1EY020361.
CHAPTER III

LEDGF<sub>1-326</sub> DECREASES P23H AND WILD TYPE RHODOPSIN AGGREGATES AND P23H RHODOPSIN MEDIATED CELL DAMAGE IN HUMAN RETINAL PIGMENT EPITHELIAL CELLS<sup>2</sup>

Abstract

Purpose

P23H rhodopsin, a mutant rhodopsin, is known to aggregate and cause retinal degeneration. However, its effects on retinal pigment epithelial (RPE) cells are unknown. The purpose of this study was to determine the effect of P23H rhodopsin in RPE cells and further assess whether LEDGF<sub>1-326</sub>, a protein devoid of heat shock elements of LEDGF, a cell survival factor, reduces P23H rhodopsin aggregates and any associated cellular damage.

Methods

ARPE-19 cells were transiently transfected/cotransfected with pLEDGF<sub>1-326</sub> and/or pWT-Rho (wild type)/pP23H-Rho. Rhodopsin mediated cellular damage and rescue by LEDGF<sub>1-326</sub> was assessed using cell viability, cell proliferation, and confocal microscopy assays. Rhodopsin monomers, oligomers, and their reduction in the presence

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of LEDGF<sub>1-326</sub> were quantified by western blot analysis. P23H rhodopsin mRNA levels in the presence and absence of LEDGF<sub>1-326</sub> was determined by real time quantitative PCR.

**Results**

P23H rhodopsin reduced RPE cell viability and cell proliferation in a dose dependent manner, and disrupted the nuclear material. LEDGF<sub>1-326</sub> did not alter P23H rhodopsin mRNA levels, reduced its oligomers, and significantly increased RPE cell viability as well as proliferation, while reducing nuclear damage. WT rhodopsin formed oligomers, although to a smaller extent than P23H rhodopsin. Further, LEDGF<sub>1-326</sub> decreased WT rhodopsin aggregates.

**Conclusions**

P23H rhodopsin as well as WT rhodopsin form aggregates in RPE cells and LEDGF<sub>1-326</sub> decreases these aggregates. Further, LEDGF<sub>1-326</sub> reduces the RPE cell damage caused by P23H rhodopsin. LEDGF<sub>1-326</sub> might be useful in treating cellular damage associated with protein aggregation diseases such as retinitis pigmentosa.

**Introduction**

Intracellular protein aggregation has been linked to many degenerative diseases including retinitis pigmentosa (RP) (Milam et al., 1998; Stojanovic and Hwa, 2002). Rhodopsin, a protein present in retinal cells is one such protein, which forms aggregates upon cellular accumulation. Some mutations of rhodopsin such as the point mutation P23H (Proline 23 → Histidine) result in greater aggregation (Sung et al., 1993; Mendes et al., 2005). These aggregates cause progressive degeneration of retinal cells, leading to blindness in RP. The P23H point mutation constitutes one of the most common causes of autosomal dominant RP in North America. P23H rhodopsin forms protein aggregates and
accumulates as aggresomes in the cytosol, leading to the death of human embryonic kidney cells (Illing et al., 2002; Mendes et al., 2005). P23H rhodopsin also exerts a dominant negative effect on the biosynthesis of normal wild type (WT) rhodopsin and induces formation of WT/P23H rhodopsin aggregates as well P23H/P23H rhodopsin aggregates (Colley et al., 1995; Kurada and O'Tousa, 1995; Saliba et al., 2002). While numerous studies have investigated P23H rhodopsin aggregation including its localization, morphology, as well as its effect on the expression of WT rhodopsin (Sung et al., 1993; Kaushal and Khorana, 1994; Lem et al., 1999; Mendes et al., 2005), there is a distinct lack of methods for potentially reducing the pathological effects of this mutation that may be applied to the clinical setting.

In this study we investigated the ability of LEDGF$_{1-326}$, a fragment of lens epithelium derived growth factor (LEDGF), to prevent cellular damage mediated by P23H rhodopsin and WT rhodopsin. LEDGF, a 530 amino acid (aa) protein originally isolated from a cDNA library of lens epithelial cells (Singh et al., 1999), is a transcription factor that confers cellular resistance to oxidative and thermal stresses and increases cell survival. Once in the nucleus, it binds to stress response elements and/or heat shock elements present in the promoters of stress associated genes, and thereby upregulates their transcription and expression (Singh et al., 2001; Kubo et al., 2003; Sharma et al., 2003; Fatma et al., 2004). LEDGF was also isolated in a screen for proteins that interact with the HIV integrase (Cherepanov et al., 2003) and appears to be essential for nuclear targeting of the integrase in human cells (Maertens et al., 2003).

Bioinformatic analysis of the structural organization of LEDGF predicted various domains of LEDGF (Singh et al., 2006). LEDGF contains an N-terminal DNA binding
domain spanning from amino acid aa5 to aa62 (Figure 3.1). This amino acid region contains a proline-tryptophan-tryptophan-proline (PWPP) motif that has been shown to bind to stress response elements (STRE; A/TGGGGA/T; A=Adenine, T=Thymine, G=Guanine) of DNA and transactivate stress related genes. A nuclear localization signal spans aa148 to aa156 (GRKRKAEEKQ; R=Arginine, K=Lysine, E=Glutamic acid, Q=Glutamine). An AT hook-like domain at aa178 to aa197, a looped structure at aa178 to aa250, and a coiled coil domain rich in lysine from aa216 to aa343, are also present in LEDGF. The C-terminus of LEDGF contains two helix-turn-helix (HTH) domains at aa421 to aa442 and aa471 to a492 that are capable of binding to heat shock elements (HSE) within the promoters of various genes. The minimum requirement for LEDGF activity appears to be a nuclear binding domain to up regulate various stress related proteins, a nuclear localization sequence to translocate LEDGF fragment to the nucleus, and a stretch of lysine rich residues to assist binding to the DNA (Singh et al., 2006). While heat shock proteins are known to act like chaperones in reducing protein aggregation (Itoh et al., 1999; Treweek et al., 2000), the ability of LEDGF or its derivatives in reducing protein aggregation are unknown. Considering all the requirements for LEDGF activity, we designed LEDGF\textsubscript{1-326}, a fragment of LEDGF, which contained all the functionally important residues of LEDGF, minus the heat shock elements and tested its ability to reduce rhodopsin aggregates and cell damage.

Retinal pigmented epithelial (RPE) cells play an important role in the generation and maintenance of photoreceptors (Strauss, 2005). They are located between the choroid and the photoreceptors of the neural retina. They supply nutrients to the neural retina and phagocytose dead photoreceptors. They also maintain the visual function of
photoreceptors. With aging, RPE cells in human eyes are known to accumulate rod outer segments containing rhodopsin, due to a deficiency of rhodopsin degrading enzymes including alpha-mannosidase (Wyszynski et al., 1989). Given the propensity of rhodopsin to aggregate, it is possible that rhodopsin accumulation contributes to RPE loss. Indeed, mice expressing rhodopsin mutations such as P23H lose RPE at a much accelerated rate (Nishikawa and LaVail, 1998). Since RPE degeneration is an integral part of RP, we chose in this study to more directly examine the effects of rhodopsin aggregates (both P23H and WT) on RPE survival. Another objective of this study was to assess whether LEDGF\textsubscript{1-326} reduces rhodopsin aggregates as well as the cellular damage induced by rhodopsin proteins in RPE cells leading to decreased survival. Towards these objectives, we performed in vitro transient transfections of P23H/WT rhodopsin and LEDGF\textsubscript{1-326} plasmids in a human retinal pigmented epithelial cell line (ARPE-19). Subsequently, we
performed trypan-blue assay for cell viability, BrDu assay for cell proliferation, confocal microscopy to visualize the proteins and their localization, western blot analysis to quantify rhodopsin monomers and aggregates, and real time quantitative PCR to measure the mRNA level of P23H rhodopsin in the presence and absence of LEDGF\textsubscript{1-326}.

Our work has shown that both P23H and WT rhodopsin form aggregates in retinal pigment epithelial cells and LEDGF\textsubscript{1-326} is capable of reducing these protein aggregates. Further, LEDGF\textsubscript{1-326} decreases P23H rhodopsin mediated cell damage in RPE cells.

Materials and Methods

Plasmid pP23H-CFP-Rho was a gift from Dr. Ron R. Kopito, (University of Stanford, Stanford, CA) and pCMV5 was a gift from Dr. David W Russell (University of Texas Southwestern Medical Center, Dallas, Texas). Rabbit polyclonal anti-cyan fluorescent protein (anti-CFP) antibody, goat polyclonal horse peroxidase linked anti-rabbit antibody and CFP protein was purchased from BioVision (Mountain View, CA). ARPE-19 cells were obtained from ATCC (Manassas, VA). DMEM/F12 cell culture medium, fetal bovine serum, LF2000 (Lipofectamine\textsuperscript{®} 2000), and nucleic acid staining dye TO-PRO-3 iodide was obtained from Invitrogen (Carlsbad, CA). All other chemicals, unless specified otherwise, were purchased from Sigma- Aldrich (St. Louis, MO).

Plasmid Construction

pP23H-Rho was cloned from the pP23H-CFP-Rho by cutting out CFP using Ecor1 and Not1 restriction enzymes. The cloned plasmid was ligated and then transformed in E. coli DH5\textalpha. The plasmid construct was confirmed for molecular weight by gel electrophoresis and for gene sequence by sequencing.
For point mutation of pP23H-CFP to pRho-CFP and pP23H-Rho to pWT-Rho, the primers used were 5’GGGTGTGGTACGCAGGCCCTTTCCGTAGTCACCACAG3’ and 5’CGTGGGTACTCGAAGGGCGTCCGTACCACACC 3’. The mutation was done using the Quick Change kit (Stratagene, La Jolla, CA) as per manufacturer’s protocol. The mutation was confirmed by sequencing the gene.

**Cell Transfection**

ARPE-19 cells were cultured in DMEM/F12 (1:1) medium containing 10% (v/v) fetal bovine serum(FBS), 2% (v/v) L-glutamine (200 mM), and 1% (v/v) penicillin-streptomycin (10,000 units/ml of penicillin G sodium mixed with 10,000 μg/ml of streptomycin sulphate) in a cell incubator maintained at 37°C and 5% carbon dioxide as per ATCC protocol. For transient transfection, about 10^5 cells were plated in 12-well plate and incubated. After 24 h, the medium was aspirated out and the cells were transfected/cotransfected with pP23H-CFP-Rho/ pLEDGF_1-326 (this plasmid contained GFP tag at the N-terminus of LEDGF_1-326)/ pCMV5 (empty vector) using LF2000 in serum free DMEM/F12 medium. Plasmids of different ratios (e.g., increasing pLEDGF_1-326, with decreasing pCMV5 plasmid, with a constant total plasmid level) were used a) to keep the lipofectamine level in various groups constant and b) to control plasmid transfection efficiency, since an increase in plasmid level can saturate the transfection process. Cells transfected with LF2000 alone (control) were also used. After 8 h of incubation, the transfection medium was removed and the cells were further incubated for 24 h in normal serum containing medium. Thereafter, cells were treated as per individual experiments. All the transfection reagents including cells and plasmids were up scaled to five times for studies done in 60 mm plates according to LF2000 transfection protocol.
All figures are labeled to represent the dose exposed as the level used per 10,000 cells. Similar transfection was done for WT rhodopsin.

**Phase Contrast Microscopy**

For phase contrast microscopy, after transfection, cells were kept on ice and live cells were imaged using an inverted light microscope (Nikon Eclipse TE300). Hoffman Modulation Contrast images were taken using 10x optical lens. The images were captured using Image pro® software (Nikon).

**Cell Viability Assay**

After transfection, cells were trypsinized using 0.25% trypsin-EDTA and collected in Eppendorf tubes. They were then centrifuged at 1000 g for 5 min to form a pellet. The supernatant was discarded and the pellet was resuspended in PBS. To this 0.4 % of trypan blue was added to stain the dead cells. Unstained viable cells were thereafter counted using Bright-Line haemocytometer (Hausser Scientific, Horsham PA). The percentage viability of cells in individual group was calculated with respect to the LF2000 group.

**Cell Proliferation Assay**

The proliferation assay of ARPE-19 cells was done using bromodeoxyuridine (BrDU) assay kit (Calbiochem, San Diego, CA) as per manufacturer’s protocol. BrDU, a thymidine analogue, gets incorporated into newly synthesized DNA strands as the cells proliferate. Thus, a reduction in percentage of BrDU incorporation is indicative of reduction in percentage of cells proliferating. The number of cells and the transfection reagents were scaled down ten times, as compared to 12-well plate, for transfection in 96-well plate. BrDU label (20 μl) was added to each well along with the serum containing
medium after 8 h of transfection. Two types of negative controls were maintained: blank (BrDU label but no cells) and background (untransfected cells with no BrDU label). The cells were further incubated for 24 h and thereafter fixed, permeabilized, and the DNA was denatured. The cells were incubated with mouse anti-BrDU antibody for 1 hour. After washing the unbound antibody, goat anti-mouse antibody conjugated with horseradish peroxidase was added and incubated for 30 min and then tetra-methylbenzidine (TMB), a chromogenic substrate was added and the absorbance of color developed was measured at dual wavelengths of 450 nm and 540 nm using a microplate reader. The absorbance at 450 nm minus the absorbance at 540 nm indicated the proliferation. The percentage proliferation of individual group was then calculated with respect to LF2000 group.

**Confocal Microscopy**

For confocal microscopy, ARPE-19 cells were grown on cover slips. After transfection, the cells were fixed with 4% buffered formalin for 20 min, and permeabilized with 0.2 % Triton X-100 for 10 min. They were then treated with RNase (100 µg/ml) for 20 minutes to prevent staining of RNAs with TO-PRO-3 iodide. The nucleus (nuclear DNA) was then stained with TO-PRO-3 iodide (1µM) for 15 min. All treatments were done at 37°C unless specified and were followed by three washes with PBS. The cover slip was mounted on a glass slide using Supermount® (Biogenex, San Ramon, CA) mounting media to prevent rapid loss of fluorescence. The slides were allowed to dry for 20 min at room temperature and fluorescence was visualized using confocal microscope (Nikon Eclipse C1) at 60x optical zoom. The excitation-emission wavelengths used for CFP, GFP, and TO-PRO-3 iodide were 408-450/35, 488-515/30,
and 637-605/75 nm, respectively. Images were captured using Nikon EZ-C1 software version 3.40. For nuclei count, images from three independent studies were taken and the number of distinct nuclei per frame in each image was counted and the mean number of distinct nuclei per group was plotted against the level of pP23H-CFP-Rho transfected.

For WT-CFP rhodopsin visualization, Zeiss LSM 510 NLO laser scanning confocal microscope was used at 63x optical zoom. The excitation-emission wavelengths used for CFP, GFP, and TO-PRO-3 iodide were 800 nm (2-photon excitation)-435/485, 488-505 (long pass), 633-650/710 nm, respectively. Images were analyzed using Zen 2000 light edition software (Carl Zeiss, Thornwood, NY).

**Western Blotting**

For western blotting, cells were cultured in 60 mm dishes. The number of cells and the transfection reagents were scaled up five times. After transfection, the cells were lysed for 30 min using 200 µl of lysis buffer (5mM EDTA, 1 % Triton X-100, protease inhibitor (Complete Mini, Roche Diagnostic, IN) in PBS) under ice-cold conditions. The lysed cells were collected and centrifuged at 13000g for 10 min. The supernatant was collected (named as detergent soluble fraction) and the pellet was further redissolved into 50 µl of PBS containing 1% SDS at room temperature for 10 min. Then 150 µl of lysis buffer was added and samples were sonicated for 20 sec using a probe sonicator (Mesonix 3000) set at 3 Watts. The cells were recentrifuged and the supernatant (named as detergent insoluble fraction) was collected. Protein estimation of both the detergent soluble and the insoluble fraction was done using BCA protein assay reagent (Pierce, Rockford, IL). For gel electrophoresis the samples were mixed with 4x loading dye, however, boiling was avoided to prevent heat induced aggregation of rhodopsin. For
detergent soluble fraction 40 µg and for detergent insoluble fraction 30 µg of protein was loaded for each experimental group. CFP protein was loaded for positive control. Samples were run on 4-20 % gradient SDS-PAGE gel (Bio-Rad, Hercules, CA) and then transferred to nitrocellulose membrane. The membrane was then immunoblotted using rabbit polyclonal anti-CFP antibody (1:1000) as primary antibody and anti-rabbit antibody (1:10000) as secondary antibody. Protein bands were visualized using enhanced chemiluminescence ECL™ detection kit (GE Healthcare, Piscataway, NJ), and high performance chemiluminescence films (GE Healthcare, Piscataway, NJ). Thereafter, the membrane was stripped with stripping buffer containing 2% SDS, and 0.1 M beta-mercaptoethanol at 50° C for 15 min and reprobed for β-actin protein using mouse monoclonal anti-actin antibody as primary antibody (1:1000) and sheep anti-mouse antibody (1:10000) as secondary antibody. Similarly, WT rhodopsin immunoblotting was also done.

Densitometry analysis was done using Quantity One 1-D analysis software from Bio-Rad. Equal regions from individual lanes of blots were selected and the mean intensity of each region was measured and normalized for β-actin. Thereafter, the mean intensity was subtracted from the corresponding region of the untransfected group to reduce the background noise. The mean intensity of corresponding lane and protein fraction was then plotted for rhodopsin transfected groups.

**Real-Time Quantitative PCR**

ARPE-19 cells were transfected as above and thereafter RNA was isolated from cultured cells using the RNeasy kit (Qiagen, Valencia CA) according to the manufacturer’s protocol. To remove contaminating genomic DNA, 10 µg RNA from each
sample was treated with DNase using the Turbo DNA free kit (Ambion, Austin, TX) as per user’s manual to degrade any DNA present in the sample. Thereafter, first strand of DNA was synthesized from the mRNA using the high capacity RNA to DNA kit (Applied Biosystems, Inc., Carlsbad, CA). Then, PCR was performed on an ABI 7500 PCR machine to amplify the DNA. The number of thermal cycle required to reach threshold was obtained. Rhodopsin mRNA level was calculated from this threshold cycle. Similarly GAPDH mRNA (mRNA commonly present in all cells) level was detected and rhodopsin mRNA level was normalized to GAPDH mRNA to represent the mRNA level of rhodopsin present in each group.

**Statistical Analysis**

All statistical analysis was done using SPSS, ver. 19; (SPSS, Chicago, IL). The data is represented as the mean ± SD, Independent samples student’s t-test was done for statistical comparisons between two experimental groups. To compare between multiple experimental groups one-way ANOVA followed by the Tukey’s post hoc analysis was used. The results were considered statistically significant at p<0.05.

**Results**

**P23H Rhodopsin Decreases RPE Cell Viability and Proliferation**

To determine the effect of P23H rhodopsin expression in ARPE-19 cells, a trypan-blue cell viability assay was done (Figure 3.2B). Before cells were trypsinized, Hoffman Modulation Contrast (HMC) images were taken of the live cells, using phase contrast microscopy (Figure 3.2A). The untransfected and LF2000 (Lipofectamine® 2000) transfected (control) groups showed confluent cells. As the transfection level of pP23H-Rho increased, the number of cells per frame of the image decreased.
In our cell viability assay (Figure 3.2B), cells transfected with LF2000 alone were indistinguishable from cells transfected with, low levels (<0.25 µg) of pP23H-Rho. As the level of transfected pP23H-Rho was increased, we observed a significant decrease in the percentage of viable cells (p<0.001). At transfection level of 1.0 µg, the cell viability was 25 ± 5 %, whereas at 2.0 µg, the cell viability was only 15 ± 1 %, indicating a significant loss in cell viability (p<0.001). A similar trend was seen when cells were transfected with pP23H-CFP-Rho (P23H rhodopsin tagged with cyan fluorescent protein). Transfection of 1.0 µg of P23H resulted in a cell viability of 34 ± 4 %, whereas at 2.0 µg, the cell viability was only 15 ± 2 %, indicating a significant loss in cell viability (p≤0.001).

Similar to P23H rhodopsin, as the transfection level of pWT-Rho increased, the number of cells per frame of the image decreased (Figure 3.2A), but the change was not as prominent as pP23H-Rho transfected groups. While there was no significant difference at 0.25, 0.5, and 0.75 µg doses, there was a significant difference between the cell viability of pWT-Rho and pP23H-Rho/pP23H-CFP-Rho groups at 1 and 2 µg doses. At the 2 µg dose for instance, the viability with pWT-Rho was 72 ± 11 % as opposed to 15 ± 1 % with pP23H-CFP.

To further understand whether the loss of viability of cells is due to a decrease in cell proliferation, a BrDU assay was performed (Figure 3.2C). pP23H-Rho decreased proliferation of ARPE-19 cells in a dose-dependent manner when compared to LF2000 controls, with the % proliferation being 50 ± 6 % at 2 µg, the highest dose tested. Although there appeared to be a decline in cell proliferation with pWT-Rho, the differences were not statistically significant. Cells transfected with pP23H-Rho showed
Figure 3.2. P23H Rhodopsin is Toxic to RPE Cells.
Figure 3.2. P23H Rhodopsin is Toxic to RPE Cells. A) For HMC images, cells were visualized using Nikon inverted light microscope at 10x magnification. Representative images from one of the three independent studies have been shown. B) For cell viability, the cells were trypsinized, collected and resuspended in PBS. Total number of viable cells was determined in each group using trypan blue dye and percent viability was calculated with respect to the group transfected with LF2000 (Lipofectamine® 2000) alone. C) For cell proliferation cells were treated with BrDU for 24 h and then detected using anti-BrDU antibody. The percentage proliferation was calculated with respect to LF2000 transfected group. Data is expressed as mean ± S.D. for N=3. *, p< 0.01 as compared to corresponding pWT-Rho transfected group. *, p<0.05 compared to LF2000 group.
significantly lower proliferation when compared to cells transfected with pWT-Rho at all
doses. Although there was no dose dependent decline in cell viability with pWT-Rho, the
decline in viability was significant with transfections of 0.75 and 1µg DNA, when
compared to controls.

**LEDGF<sub>1-326</sub> Decreases RPE Cell Viability Loss Induced by P23H Rhodopsin**

To demonstrate the ability of LEDGF<sub>1-326</sub> to rescue cells from rhodopsin induced
damage, ARPE-19 cells were cotransfected with a constant dose of pP23H-CFP-Rho/pP23H-Rho/pWT-Rho and increasing doses of pLEDGF<sub>1-326</sub>. An empty plasmid (pCMV5) was introduced to keep the total transfected plasmid constant in the various
treatment groups. Trypan-blue cell viability assays (Figure 3.3B) and BrDU cell
proliferation assays (Figure 3.3C) were then performed. Further, HMC images (Figure
3.3A) were taken in those groups with fluorescently tagged proteins. Figure 3.3A
represents images from one of the three independent studies. The HMC images indicated
a significant loss in number of cells in groups transfected with pP23H-CFP-Rho alone.
As the cotransfection level of pLEDGF<sub>1-326</sub> was increased, cell loss commensurably
decreased.

The percentage viability of ARPE-19 cells in the group transfected with pP23H-CFP-Rho alone was 40 ± 5 % as compared to the LF2000 transfected group (Figure
3.3B). In the presence of pLEDGF<sub>1-326</sub>, cell viability increased in a dose dependent
manner. As the level of cotransfected pLEDGF<sub>1-326</sub> was increased from 0 to 3 µg, the
percentage cell viability increased from 40 ± 5 to 72 ± 8 %, that is, about ~1.7 times (p<
0.01). However, cell viability could not be restored to 100% even at the highest dose of
pLEDGF<sub>1-326</sub>. 

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Figure 3.3. LEDGF$_{1-326}$ Increases the Cell Viability and Proliferation in a Dose-Dependent Manner in RPE Cells Expressing P23H Rhodopsin.
Figure 3.3. LEDGF$_{1-326}$ Increases the Cell Viability and Proliferation in a Dose-Dependent Manner in RPE Cells Expressing P23H Rhodopsin. A) For HMC images, cells were visualized using Nikon inverted light microscope at 10x magnification. Representative images from one of the three independent studies have been shown. B) For cell viability, cells were trypsinized, collected and resuspended in PBS. Total number of viable cells was determined in each group using trypan blue assay and percent viability was calculated with respect to the group transfected with LF2000 (Lipofectamine® 2000) alone. C) For cell proliferation assay, cells were treated with BrDU for 24 h and then detected using anti-BrDU antibody. The percentage proliferation was calculated with respect to LF2000 transfected group. Data is expressed as mean ± S.D. for N=3. p<0.01 compared with LF2000 transfected (control) group.
In the BrDU cell proliferation assay (Figure 3.3C), cell proliferation in the group transfected with pP23H-Rho was low (46 ± 6 %) as compared to the LF2000 transfected group. When pLEDGF\textsubscript{1-326} was cotransfected with pP23H-Rho, there was an increase in cell proliferation in a dose dependent manner. The % proliferation of pP23H-Rho transfected cells increased from 46 ± 6 to 77 ± 2 % at the highest dose of pLEDGF\textsubscript{1-326}. On the other hand, pWT-Rho transfected groups did not show any significant changes in the proliferation in the absence or presence of pLEDGF\textsubscript{1-326}.

**P23H Rhodopsin Disrupts RPE Nuclear Shape and Content**

We assessed the effect of P23H rhodopsin expression on ARPE-19 cells histologically by confocal microscopy. ARPE-19 cells were cultured on cover slips and transiently transfected with increasing level of pP23H-CFP-Rho. To visualize the nucleus, cells were stained with red TOPRO-3 iodide dye, while P23H rhodopsin expression was represented by blue fluorescence emitted by P23H rhodopsin tagged with CFP (Figure 3.4A). Representative images from one of the three independent studies are shown. As the level of pP23H-CFP-Rho was increased there was an increase in blue fluorescence. When <0.5 µg of pP23H-CFP-Rho was used for transfection, the nucleus was distinct and well formed (Figure 3.4A (a, and b)). This distinct nuclear structure (defined as well formed ovals) started disappearing as the transfection level of pP23H-CFP-Rho was increased (Figure 3.4A (c, d, and e)). When cells were visualized at higher magnification (Figure 3.4A, Row 4), disrupted nuclear material was observed (Figure 3.4A (s, and t)). The LF2000 treated control group (Figure 3.4A, p), in contrast, did not show any such structural damage. HMC images were taken at 10x magnification (Figure 3.4A, Row 5). As the transfection level of pP23H-CFP-Rho was increased, the number of
Figure 3.4. P23H Rhodopsin Disrupts the Nuclear Material and Aggregates in RPE Cells.
Figure 3.4. P23H Rhodopsin Disrupts the Nuclear Material and Aggregates in RPE Cells. A) For confocal images, cells were fixed with 4% buffered formalin, permeabilized with 0.2% Triton X-100, and treated with RNase. The nucleus was then stained with TO-PRO-3 iodide. Confocal images were taken using the excitation/emission wavelengths of 637-605/75 and 408-450/35 nm for TO-PRO-3 iodide and CFP, respectively. Representative images from one of the three independent studies have been shown. For HMC images, the cells were visualized using Nikon inverted light microscope at 10x magnification. B) The number of distinct nuclei was counted in each confocal image from three independent studies and was plotted against the pP23H-CFP-Rho transfection level. Data is expressed as mean ± S.D. for N=3. *, p<0.01 compared with LF2000 transfected (control) group.
cells present in a given area decreased, indicating loss of cells. This result correlates both with our cell viability and proliferation data (Figure 3.2).

Further, to understand the significance of the confocal data, the numbers of distinct nuclei visible in each confocal image were counted from images from three independent studies (Figure 3.4B). The number of distinct nuclei was significantly reduced in groups transfected with 2.0 µg of pP23H-CFP-Rho as compared to LF2000 transfected control group (p<0.01). Thus, P23H rhodopsin expression disrupts nuclear structure.

**LEDGF<sub>1-326</sub> Rescues RPE Cells from Nuclear Damage Induced by P23H Rhodopsin**

To further investigate the role of LEDGF<sub>1-326</sub> in reducing aggregation, confocal microscopy was performed on cotransfected ARPE-19 cultures (Figure 3.5A). Nuclei were stained red as previously described, LEDGF<sub>1-326</sub> expression was visualized by green fluorescence emitted by tagged GFP protein with LEDGF<sub>1-326</sub>, and P23H rhodopsin was visualized as blue. The LF2000 transfected control group (Figure 3.5, Column 1) indicated clear red nuclei staining and the nuclei exhibited well-formed oval structures. The group transfected with pLEDGF<sub>1-326</sub> (Figure 3.5, Column 2) showed clear green fluorescence colocalized with the red labeled nuclei, indicating the presence of LEDGF<sub>1-326</sub> primarily in the nucleus. The group transfected with pP23H-CFP-Rho (Figure 3.5, Column 3) showed blue fluorescence and once again indicated disrupted nuclear material as before (Figure 3.4A). However, in the presence of cotransfected pLEDGF<sub>1-326</sub>, disrupted nuclear material (broken, non-uniform red pattern) was decreased and the intact nuclear structure was restored (Figure 3.5, Column 4). Interestingly, while P23H rhodopsin expression was clearly visible near the perinuclear region of the cell in the
absence of LEDGF$_{1-326}$ (Figure 3.5, Row 3), it was diffused and far less visible in the presence of LEDGF$_{1-326}$ (Figure 3.5, Column 4).

WT rhodopsin expression was similarly visualized similarly (Figure 3.6). Unlike P23H rhodopsin, WT rhodopsin was expressed evenly in the cytoplasm. The plasma membrane was clearly marked by its expression leading us to believe that WT rhodopsin migrated to the plasma membrane after its expression. No disrupted nuclear material was evident due to the expression of WT rhodopsin.

**LEDGF$_{1-326}$ Reduces Rhodopsin Aggregates**

Aggregation of P23H rhodopsin was monitored by western blotting in the absence and presence of LEDGF$_{1-326}$. Figure 3.7A represents blots from one of four independent experiments. The band at 50-55 kDa represented the monomeric form of P23H rhodopsin, whereas the dimers and trimers were represented by smears at about 100–120 kDa and 180-200 kDa, respectively. The oligomers represented all higher molecular weight ($\geq$ 200 kDa) species of P23H rhodopsin.

A high molecular weight smear (representing oligomers) of P23H rhodopsin was seen within the detergent insoluble fraction in all groups transfected with pP23H-CFP-Rho (Figure 3.7A (i), Lane 2, 4-7). This dark smear gradually diminished as the cells were cotransfected with increasing doses of pLEDGF$_{1-326}$, while keeping pP23H-CFP-Rho constant. At a transfection ratio of 1:1 of pP23H-CFP-Rho to pLEDGF$_{1-326}$, there was slight increase in total P23H rhodopsin. Interestingly we also observed a decrease in the oligomers and an increase in the monomers at this ratio. However, at transfection ratio of 1:2 and 1:3, total P23H rhodopsin was seen to decrease with increasing levels of pLEDGF$_{1-326}$ for both detergent insoluble and soluble portion.
**Figure 3.5. LEDGF<sub>1-326</sub> Reduces P23H Rhodopsin Mediated Nuclear Damage.** Cells were fixed with 4% buffered formalin, permeabilized with 0.2% Triton X-100 and treated with RNase. The nucleus was then stained with TO-PRO-3 iodide. Confocal images were taken using the excitation/emission wavelengths of 408-450/35, 488-515/30, and 637-605/75 nm for CFP, TO-PRO-3 iodide, and GFP, respectively. Images were analyzed using EZ-C1 3.20 FreeViewer software. Data presents representative images from one of the three independent studies.
Figure 3.6. Diffuse Fluorescent Signal of WT Rhodopsin in RPE Cells. Cells were fixed with 4% buffered formalin, permeabilized with 0.2% Triton X-100 and treated with RNase. The nucleus was then stained with TO-PRO-3 iodide. WT rhodopsin visualization was done using Zeiss LSM 510 NLO laser scanning confocal microscope with 63x optical zoom. The excitation-emission wavelengths used for CFP, GFP, and TO-PRO-3 iodide were 800 nm (2-photon excitation)-435/485, 488-505 (long pass), 633-650/710 nm, respectively. Images were analyzed using Zen 2000 light edition. Data presents representative images from one of the three independent studies.
For P23H rhodopsin, the percentage of oligomers in the detergent insoluble fraction decreased from 39 ± 2 to 27 ± 9 % in a dose proportionate manner in the presence of LEDGF$_{1-326}$ (Figure 3.7B(i(b))) (p < 0.05). The percentage of monomers increased from 18 ± 1 to 25 ± 4 %, but did not attain statistical significance. The P23H rhodopsin oligomer percentage in the detergent insoluble fraction was significantly (p<0.05) higher than the monomer percentage in controls (Figure 3.7B(i(b)), Group1); but with increasing dose of LEDGF$_{1-326}$, this difference diminished. The monomer to oligomer ratio increased with increasing dose of LEDGF$_{1-326}$ (Figure 3.7B(i(c))). In the detergent insoluble fraction (Figure 3.7B), it was apparent that the total P23H rhodopsin abundance increased when the transfection ratio of pP23H-CFP-Rho and pLEDGF$_{1-326}$ was 1:1, but decreased in the presence of higher levels of pLEDGF$_{1-326}$ (Figure 3.7B (i(a)). However, these changes were not statistically significant. For the detergent soluble fraction, similar, but less evident trends were seen (Figure 3.7B (ii)).

To further investigate whether LEDGF$_{1-326}$ had a similar effect on WT rhodopsin, western blot studies were repeated with pWT-CFP-Rho (wild type rhodopsin tagged with cyan fluorescent protein). Figure 3.8A represents blots from one of four independent studies. The detergent insoluble (Figure 3.8A (i)) and detergent soluble fraction (Figure 3.8A (ii)) indicated a ladder of WT rhodopsin.

For WT rhodopsin in the detergent soluble fraction, the percentage of monomer was higher than the oligomer and this difference increased due to a decline in oligomer percentage and an increase in monomer percentage and became statistically significant (p<0.05) with increasing dose of LEDGF$_{1-326}$ (Figure 3.8B(ii(b))). There was a decrease in the WT rhodopsin oligomer percentage from 12 ± 5 to 9 ± 2 % and an increase in the
Figure 3.7. LEDGF\textsubscript{1-326} Decreases the Oligomers of P23H Rhodopsin in RPE Cells in a Dose-Dependent Manner.
Figure 3.7. LEDGF<sub>1-326</sub> Decreases the Oligomers of P23H Rhodopsin in RPE Cells in a Dose-Dependent Manner. A) For western blotting cells were lysed using 1% Triton X-100 lysis buffer and the detergent soluble fraction (ii) was collected after centrifugation. Thereafter, the detergent insoluble pellet was sonicated using a probe sonicator in 1% SDS buffer, the supernatant was collected by centrifugation and described as detergent insoluble fraction (i). SDS-PAGE was run for both fractions on 4-20% gradient gel. The protein was then transferred onto nitrocellulose membrane and then immunoblotted using rabbit polyclonal anti-CFP antibody and goat polyclonal anti-rabbit. β-actin was probed after stripping the membrane and then immunoblotting with mouse monoclonal anti-actin antibody and sheep polyclonal anti-mouse antibody. The protein was detected using enhanced chemiluminescence film detection method. Representative blot from one of the four independent studies has been shown. B) For densitometry analysis equal regions were selected from each lane corresponding to each fraction (monomer, dimer, trimer, and oligomer). Total rhodopsin mean density was calculated by adding all the fractions and then mean density was normalized to β-actin. Data is expressed as mean ± S.D. for N=4. *, ( p<0.05), indicates significant difference from oligomer in the same group.
Figure 3.8. LEDGF<sub>1-326</sub> Decreases the Oligomers of WT Rhodopsin in RPE cells in a Dose-Dependent Manner.
Figure 3.8. LEDGF<sub>1-326</sub> Decreases the Oligomers of WT Rhodopsin in RPE cells in a Dose-Dependent Manner. A) For western blotting cells were lysed using 1% Triton X-100 lysis buffer and the detergent soluble fraction (ii) was collected after centrifugation. Thereafter, the detergent insoluble pellet was sonicated using a probe sonicator in 1% SDS buffer, the supernatant was collected by centrifugation as detergent insoluble fraction (i). SDS-PAGE was run for both fractions on 4-20% gradient gel. The protein was then transferred onto nitrocellulose membrane and immunoblotted using rabbit polyclonal anti-CFP antibody and goat polyclonal anti-rabbit. β-actin was probed after stripping the membrane and then immunoblotting with mouse monoclonal anti-actin antibody and sheep polyclonal anti-mouse antibody. The protein was detected using enhanced chemiluminescence film detection method. Representative blot from one of the three independent studies has been shown. B) For densitometry analysis equal regions were selected from each lane corresponding to each fraction (monomer, dimer, trimer, and oligomer). Total rhodopsin mean density was calculated by adding all the fractions and then mean density was normalized to β-actin. Data is expressed as mean ± S.D. for N=4. *, (p<0.05), indicates significant difference from oligomer in the same group.
monomer percentage from 24 ± 6 to 28 ± 7% in presence of LEDGF1-326 at the highest dose. Further, the monomer to oligomer ratio in the detergent soluble fraction increased with increasing dose of LEDGF1-326 (Figure 3.8B(ii(c))). In the detergent insoluble fraction, although not statistically significant, the WT rhodopsin oligomer percentage was slightly higher than the monomer percentage in control (Group1) and this difference decreased with increasing dose of LEDGF1-326 due to an increase in the monomer percentage. Specifically, there was a decrease in the WT rhodopsin oligomer percentage from 23 ± 7 to 21 ± 4% and an increase in the monomer percentage from 17 ± 7 to 24 ± 7% in the presence of LEDGF1-326 (Figure 3.8B(i(b)). The monomer to oligomer ratio increased with increasing dose of LEDGF1-326 (Figure 3.8B(i(c))). Although statistically not significant, densitometry analysis indicated that, similar to P23H rhodopsin, there was a decrease in the total WT rhodopsin in both detergent insoluble as well as in soluble fraction (Figure 3.8B(i(a)), and (ii(a))), especially at the highest dose of LEDGF1-326. Compared to P23H rhodopsin, the oligomer percentage of WT rhodopsin in each individual group was less (compare Figure 3.7B(i(b))) and 3.8B(i(b)))).

Apart from rhodopsin smears, another band was seen at about 65-70 kDa in all blots (Figure 3.7, and 3.8). Since this band was well separated from the rhodopsin smears and was visualized only in those groups transfected/cotransfected with pLEDGF1-326, it was assumed to be representing LEDGF1-326, possibly due to cross reactivity of anti-CFP antibody with GFP tag of LEDGF1-326.

**LEDGF1-326 has No Effect on Transcription of P23H Rhodopsin**

To determine if LEDGF1-326 had an effect on P23H rhodopsin transcription, we examined the levels of P23H rhodopsin mRNA (Figure 3.9). Rhodopsin mRNA was
measured by qRT-PCR (quantitative real time polymerase chain reaction). No significant
difference in the mRNA levels was found between the group transfected with pWT-Rho,
pP23H-Rho, and pP23H-CFP-Rho. Further, the mRNA level of P23H-CFP rhodopsin
was seen to have no significant change in the absence and presence of LEDGF$_{1-326}$,
indicating that LEDGF$_{1-326}$ has no effect on the transcription of P23H-CFP rhodopsin.
Thus, the LEDGF$_{1-326}$ mediated decrease in total P23H-CFP rhodopsin abundance is not
due to decreased P23H transcription.

Discussion

The principal findings of this study are: 1) P23H rhodopsin and WT rhodopsin
form aggregates in RPE cells 2) LEDGF$_{1-326}$ decreases both P23H and WT rhodopsin

![Plasmid (μg) : LF2000 (μg) = (1:3)]

**Figure 3.9. LEDGF$_{1-326}$ Does Not Alter the Transcription Level of P23H Rhodopsin.**
mRNA was isolated from transfected ARPE-19 cells using the RNeasy kit. To remove
contaminating genomic DNA, 10 μg RNA from each sample was treated with DNase
using the Turbo DNA free kit. First strand synthesis was done using the high capacity
RNA to DNA. PCR was performed to amplify the DNA on an ABI 7500 PCR machine.
The threshold thermal cycle was used to calculate the mRNA level of rhodopsin and was
normalized to GAPDH mRNA level. Data is expressed as mean ± S.D. for N=3. Data
was considered significant at p<0.01 compared with pWT-Rho transfected group.
aggregates; 3) P23H rhodopsin disrupts nuclei and decreases the viability and proliferation of RPE cells; and 4) LEDGF$_{1-326}$ decreases the cellular damage caused by P23H rhodopsin. Thus, LEDGF$_{1-326}$ might be a suitable therapeutic agent for reducing rhodopsin aggregates and preventing cellular degeneration in diseases like retinitis pigmentosa. Others have shown that P23H rhodopsin mediated aggregation is not limited to photoreceptors, but rather is a fundamental property of the mutant protein (Illing et al., 2002). Our data is consistent with this observation. P23H rhodopsin, when expressed in RPE cells, formed insoluble aggregates and decreased cell viability. P23H rhodopsin likely induced cell death via aggregation mediated stress. RPE cells play an important role in the development and maintenance of photoreceptor cells. They are closely associated with photoreceptor outer segments and phagocytose the constantly shed segments throughout the life of the organism. Additionally, they play an active role in photoreceptor metabolism, taking up waste products and supplying essential metabolic intermediates. Given that RPE take up large quantities of shed outer segments, they are almost certainly exposed to the P23H rhodopsin mutant.

LEDGF$_{1-326}$ partially rescued RPE cultures from P23H rhodopsin mediated cell death as measured by cell viability (Figure 3.3). LEDGF$_{1-326}$ also appeared to rescue cells from P23H rhodopsin mediated damage as measured by confocal microscopy (Figure 3.5). In multiple independent experiments, P23H rhodopsin expression resulted in a distinct reduction in the number of nuclei and disruption of nuclear shape and content, indicating nuclear damage (Figure 3.4, and 3.5). Cotransfection of pLEDGF$_{1-326}$ appeared to rescue cells from this damage (Figure 3.5). Compared to pP23H-CFP-Rho transfected group, the group cotransfected with both plasmids indicated a higher number of distinct,
normal nuclei and more viable cells. Further, P23H rhodopsin was localized near the perinuclear region, consistent with protein aggregation, as reported by others (Illing et al., 2002). Conversely, we observed a significant diffused fluorescence of P23H rhodopsin when the cells were cotransfected with pLEDGF_{1-326}. WT rhodopsin, on the other hand, showed diffuse fluorescence pattern in the absence and presence of pLEDGF_{1-326} (Figure 3.6).

Our western blot analysis indicated that although both P23H and WT rhodopsin aggregated in ARPE-19 cells, the percentage of WT rhodopsin oligomers were less as compared to P23H rhodopsin in both detergent insoluble and soluble fractions (Figure 3.7 and 3.8). Further, LEDGF_{1-326} can reduce the aggregates of rhodopsin in RPE cells. As the level of pLEDGF_{1-326} cotransfection was increased, there was a dose-dependent decrease in the oligomers of P23H as well as WT rhodopsin. At low doses, LEDGF_{1-326} reduced P23H and WT rhodopsin oligomers and increased their monomers, without affecting the total rhodopsin. However, at the higher doses of pLEDGF_{1-326} there was also a decrease in the total rhodopsin protein indicating possibly degradation of rhodopsin. This indicates that LEDGF_{1-326} function is complex, apart from deaggregation, it might be assisting the aggregated rhodopsin to degrade. Although LEDGF_{1-326} was present in detergent insoluble fraction, it was not present in oligomeric forms, indicating it is not aggregation prone. Since LEDGF_{1-326} was found primarily in the nucleus (Figure 3.5), it might be tightly bound to DNA and the initial mild lysis condition (1% Triton-x 100) may not have been sufficient to fully extract LEDGF_{1-326} into the soluble fraction. Thus, LEDGF_{1-326}, a protein that may not aggregate by itself, reduces the intracellular aggregates of rhodopsin.
Since our western blots were performed with CFP tagged rhodopsin, there may be a concern that CFP might show aggregation behavior. However, although CFP was tagged to both P23H rhodopsin as well as to WT rhodopsin, the aggregation behavior and cellular damage was more prominent with P23H rhodopsin (Figures 3.7 and 3.8). Further, both P23H rhodopsin and P23H rhodopsin tagged with CFP resulted in similar loss in cell viability (Figure 3.2B). Hence, it appears that CFP itself may have minimal effects, if any, on the observed aggregation behavior of rhodopsin proteins and cytotoxicity of P23H rhodopsin. Further, it was previously demonstrated that CFP fusion at the C-terminal of rhodopsin has no discernable effect on its folding or intracellular distribution (Michaelson and Philips, 2006).

Given that LEDGF has transcriptional activity, it is theoretically possible that coexpression of LEDGF<sub>1-326</sub> may alter the expression of rhodopsin in our co-transfection experiments. Since the qPCR study (Figure 3.9) showed no change in the mRNA level of P23H rhodopsin in the presence and absence of LEDGF<sub>1-326</sub>, it appears that LEDGF<sub>1-326</sub> does not play a role in the transcription of P23H rhodopsin. However, whether LEDGF<sub>1-326</sub> is acting directly on P23H rhodopsin or exerting its effect via the upregulation of various stress related proteins is unknown at this time.

A previous study conducted using LEDGF protein showed that there was no rescue of b-waves in the electroretinogram (ERG) of P23H transgenic rat model with rapidly degenerating photoreceptors (Line 1), indicating that LEDGF cannot rescue P23H mediated photoreceptor damage in the rat model, when dosed intravitreally on day 10 at a dose of 1 μg (Machida et al., 2001). However, the same study showed that LEDGF can rescue photoreceptors in light-damaged and RCS rats. Another study employing AAV
vectors capable of expressing LEDGF also showed photoreceptor rescue in RCS rats but not P23H transgenic (Line 1) rat model (Raz-Prag et al., 2009). While the in vivo studies in P23H rats would seem to contradict our findings, it is possible that once the aggregates of P23H rhodopsin are formed to a certain extent, LEDGF cannot prevent further damage. Dosing at an earlier stage than the previous study and at a higher level might be necessary in order to obtain significant benefits with LEDGF or its derivatives in animal models. Thus, LEDGF$^{1-326}$ is of potential value in treating retinal degenerations associated with P23H rhodopsin.

We have found that the deletion of the HSE from LEDGF has not abrogated its ability to promote cell survival or protein deaggregation, indicating that the HSE domain is not essential for this function. The effect of this deletion on the ability of LEDGF to activate gene expression has not been directly assessed. Deletion studies in which somewhat larger sections of the C-terminus were removed resulted in significant decreases in the transcriptional activity of the construct (Singh et al., 2006). Additionally, LEDGF constructs consisting only of N-terminal sequence retained much of their ability to drive the transcription of a reporter gene, indicating that the N-terminus of LEDGF provides domains important for transcription. These data would suggest that LEDGF$^{1-326}$ may be transcriptionally impaired. However, this has not yet been explicitly tested. Further investigations are warranted in this context. Since LEDGF$^{1-326}$ reduced rhodopsin aggregates in RPE cells, we speculate that this contributes at least in part to its ability to reduce nuclear damage and cell death in RPE cells.

RPE degeneration contributes to vision loss in human RP. Our data demonstrates that P23H can be toxic to RPE, suggesting that the uptake of toxic photoreceptor
components may play an important role in RP pathogenesis. The degree to which phagocytosed P23H rhodopsin contributes to RPE degeneration will be dependent on the ability of the RPE to process phagocytosed material; an important and underappreciated issue requiring further investigation.

Acknowledgements

This work was supported in part by NIH grants R21EY17360 and RC1EY020361.
Abstract

Purpose

For vision-threatening retinitis pigmentosa (RP) and dry age related macular degeneration (dry AMD), there are no FDA approved treatments. We identified, biosynthesized, purified, and characterized lens epithelium derived growth factor fragment (LEDGF$_{1-326}$) as a novel protein therapeutic.

Results

LEDGF$_{1-326}$ was produced at about 20 mg per liter of culture when expressed in *E.coli* system, with about 95% purity and aggregate free homogenous population with a mean hydrodynamic diameter of 9 ± 1 nm. The free energy of unfolding of LEDGF$_{1-326}$ was 3.3 ± 0.5 kcal mol$^{-1}$ and melting temperature was 44.8 ± 0.2 °C. LEDGF$_{1-326}$ increased human retinal pigment epithelial cell (RPE) viability from 48.3 ± 5.6 to 119.3 ± 3

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21.1% in the presence of P23H mutant rhodopsin mediated aggregation stress. LEDGF\textsubscript{1-326} also increased RPE FluoSphere uptake to 140 ± 10%. Eight weeks after single intravitreal injection in Royal College of Surgeon (RCS) rats, LEDGF\textsubscript{1-326} increased the b-wave amplitude significantly from 9.4 ± 4.6 to 57.6 ± 8.8 μV for scotopic electoretinogram (ERG) and from 10.9 ± 5.6 to 45.8 ± 15.2 μV for photopic ERG. LEDGF\textsubscript{1-326} significantly increased the retinal outer nuclear layer thickness from 6.34 ± 1.6 to 11.7 ± 0.7 μm.

**Conclusions**

LEDGF\textsubscript{1-326} is a potential new therapeutic agent for treating retinal degenerative diseases.

**Introduction**

Retinal degenerations including retinitis pigmentosa (RP) and dry age related macular degeneration (dry AMD) are a group of heterogeneous, chronic, progressive, and blinding diseases (Congdon et al., 2004). RP is a genetically inherited disease (Ohguro et al., 2002) caused by more than 50 different gene mutations (Dryja et al., 1990b) including P23H mutant rhodopsin (Berson et al., 1991), which result in endoplasmic stress and protein aggregation (Mendes and Cheetham, 2008). Around 1.5 million people worldwide currently suffer from RP (Hartong et al., 2006). For dry AMD, although the cellular mechanism are not fully understood, it has been associated with chronic oxidative stress and inflammation (Libby and Gould), leading to deposition of tiny protein and fat-containing toxic “drusen” and loss of photoreceptors and ultimately irreversible blindness (Fine et al., 2000; Klein et al., 2004; Kuehn, 2005). Currently, about 1.75 million individuals are suffering from AMD in the United States and by 2020
this number is expected to reach 3 million (Friedman et al., 2004). Dry AMD accounts for 90% of AMD cases (Stuen, 2003).

Despite the widespread prevalence of RP and dry AMD, there is no treatment approved by the US FDA (Nowak, 2006; Nowak and Bienias, 2007). The primary preventive measure to slow the progression of RP remains daily ingestion of up to 15,000 IU of Vitamin A palmitate (Berson et al., 1993a; Berson et al., 1993b; Li et al., 1998; Sibulesky et al., 1999). Other treatment options include a recently approved Argus® II Retinal Prosthesis System ('Argus II') for RP (Delyfer et al., 2004). For dry-AMD, a nutrition supplement (Ocuvite PreserVision®) containing beta-carotene and zinc is recommended (Cangemi, 2007). However, high doses of beta-carotene can increase the risk of cancer in smokers (Albanes et al., 1996). Currently there is no FDA approved drug therapy for dry AMD or RP.

Lens epithelium derived growth factor (LEDGF) is a transcription factor in human lens epithelial cells (Singh et al., 1999). Glutathione S-transferase tagged LEDGF (GST-LEDGF) protein promotes retinal cell growth under serum starvation and thermal stress, and protects the photoreceptors in rats receiving light damage and in Royal College of Surgeon (RCS) rats (Nakamura et al., 2000; Machida et al., 2001; Matsui et al., 2001). However, full length LEDGF has two major drawbacks. First, full length LEDGF has been associated with human immunodeficiency virus (HIV-1) mediated diseases (Vets et al., 2012). The n-terminal (aa1-324) of LEDGF acts as chromatin tether and binds LEDGF to DNA, while the c-terminal (aa325-530) binds to the HIV-1 integrase and promotes transcription and replication of HIV (Cherepanov et al., 2003; Maertens et al., 2003). In vivo administration of C-terminal LEDGF\textsubscript{325-530} potently
inhibited HIV replication by competing with endogenous LEDGF for binding to HIV integrase (Vets et al., 2012). Second, purification of full length LEDGF in bulk quantities has never been reported, possibly due to inherent instability. Since GST-LEDGF protein degrades to smaller fragments during its biosynthesis and purification (Fatma et al., 2000), attempts were made to stabilize GST-LEDGF with heparin. In the presence of 71 mg/ml of heparin in the culture media, the full-size GST-LEDGF in purified protein fraction increased to 56 % from 32 % in controls. In a previous study, we identified that LEDGF₁-₃₂₆, an n-terminal fragment of LEDGF, can reduce P23H rhodopsin aggregation and promote cell survival (Baid et al., 2011a). Due to the absence of c-terminal domain of LEDGF in LEDGF₁-₃₂₆, the possibility of HIV-1 integration is expected to be minimized with LEDGF₁-₃₂₆.

In this study we cloned, synthesized and purified LEDGF₁-₃₂₆ protein for the first time in stable and non-degraded form and scaled up its production to large quantities. Further, we established its biophysical properties of LEDGF₁-₃₂₆ and assessed its ability to reduce in vitro P23H rhodopsin aggregation mediated retinal cell damage. Finally, the ability of LEDGF₁-₃₂₆ to reduce retinal degeneration in the RCS rat model for dry AMD and RP was determined.

**Materials and Methods**

**Materials**

Forward primer 5’AGTAGTGGATCCATGACTCGGATTTCAAAC3’ and reverse primer 5’AATAATAGCTTTCACTGCTCAGTTTCCATTTGTC3’ were obtained from Integrated DNA Technologies (Coralville, IA). DNA polymerase I, T4
DNA ligase, and restriction enzymes were obtained from New England BioLabs Inc. (Ipswich, MA). QIAquick gel extraction kit, QIAprep spin miniprep kit, and QIAGEN plasmid mini kit were obtained from Qiagen (Valencia, CA). XK 16/20 column, S-200 gel filtration column, SP sepharose beads were obtained from GE Life sciences Healthcare (Piscataway, NJ). ARPE-19 cells were obtained from ATCC (Manassas, VA). DMEM/F12 cell culture medium, fetal bovine serum, Lipofectamine 2000, LB medium, and agarose were obtained from Invitrogen (Carsbad, CA). All other materials unless specified were obtained from Sigma-Aldrich (St. Louis, MO).

**Preparations of LEDGF<sub>1-326</sub> DNA Construct**

Gene encoding LEDGF<sub>1-326</sub> protein was designed to clone into pET-28a (+) vector (Novagen, Madison, WI). Briefly, LEDGF<sub>1-326</sub> gene was amplified from the pEGFP-LEDGF plasmid using the forward primer 5’AGTAGTGGATCCATGACTCGCGATTTCAAAC3’ and reverse primer 5’AATAATAAGCTTTCACTGCTCAGTTTCCATTTGTTC3’. Thereafter, the purified LEDGF<sub>1-326</sub> gene and pET-28a (+) vector were digested using HindIII and BamHI restriction enzymes and ligated overnight at 4 °C using T4 DNA ligase. Competent *Escherichia coli* DH5α cells were transformed with the ligation product as per the manufacturer’s protocol. Insertion of LEDGF<sub>1-326</sub> in pET-28a (+) vector (named as pLEDGF<sub>1-326</sub>) was confirmed by PCR screening, restriction digest, and finally by DNA sequencing. Purity and the size of the recombinant DNA was analyzed using 2 % agarose gel. The colony showing positive PCR signal and correct sequence was cultured further and the bacterial glycerol stock was made and stored at -80 °C for all future use.
**Cloning and Expression of LEDGF<sub>1-326</sub>**

For protein biosynthesis, pLEDGF<sub>1-326</sub> plasmid was isolated from *Escherichia coli* DH5α colony and transformed in *Escherichia coli* BL21(DE3) as per manufacturer’s protocol. LEDGF<sub>1-326</sub> was expressed and purified in shake flask culture under controlled addition of isopropyl-β-D-thio-galactoside (IPTG). Cells were harvested, lysed, and crude LEDGF<sub>1-326</sub> was collected as soluble cell lysate.

**Purification of LEDGF<sub>1-326</sub> using Fast Protein Liquid Chromatography (FPLC)**

LEDGF<sub>1-326</sub> was solely expressed in soluble fraction as determined by SDS-PAGE. LEDGF<sub>1-326</sub> was purified using two step fast protein liquid chromatography (FPLC), first based on charge (cation exchange) and then based on size (gel filtration). Briefly, cation exchange SP sepharose beads were packed in XK 16/20 column and the soluble cell lysate was loaded. The column was washed with buffer A (25 mM Tris-HCl pH 7.0, 1 mM EDTA, 1 mM PMSF, and 5 % sucrose). The non-specifically and loosely bound impurities were eluted using a gradient of NaCl. Fractions containing high protein amount were pooled together, dialyzed using dialysis buffer (25 mM Tris pH 7.0, and 0.1 % sucrose) and lyophilized for 48 hours. The lyophilized protein was resolubilized in 2 ml of D.I. water and further purified using prepacked S-200 gel filtration column in buffer B (25 mM Tris-HCl pH 7.0, and 100 mM NaCl). Fractions containing the pure LEDGF<sub>1-326</sub> were pooled together and dialyzed in the dialysis buffer (25 mM Tris-HCl pH 7.0, and 0.1 % sucrose) for 48 hours at 4 °C with three buffer exchanges. The dialyzed LEDGF<sub>1-326</sub> was lyophilized, aliquoted and stored at -80°C for all future purposes.
**Bioinformatic Analysis**

LEDGF$_{1-326}$ amino acid sequence was submitted to ExPASy bioinformatics resource portal and the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life of LEDGF$_{1-326}$ were computed. Post translational modification of LEDGF$_{1-326}$ was predicted using NetNGlyc 1.0, NetOGlyc 3.1, and NetCGlyc 1.0 server.

**Physical Characterization**

**Size Exclusion Chromatography (SEC-HPLC)**

The lyophilized protein was dissolved in deionized water to final concentration of 500 µg/ml and filtered through 0.22 µm polyvinylidene difluoride (PVDF) filters. The protein was size separated in Agilent Bio SEC-3 column using 25 mM Tris buffer containing 1 mM CaCl$_2$, pH 7.0 at 25 °C with a flow rate of 1 ml/min. Retention time was averaged from 4 chromatograms.

**Matrix-assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry**

Protein homogeneity and molecular weight was confirmed by 4800 Plus MALDI TOF/TOF$^\text{TM}$ (AB Sciex, Framingham, MA) mass spectrometry. The protein sample was dissolved into a solution of standard MALDI matrix sinnapinic acid, spotted and dried onto the metal target plate. Data were collected as total ion current (TIC) from 1000 laser shots of 5900 intensity.

**Dynamic Light Scattering (DLS)**

The homogeneity and size of the LEDGF$_{1-326}$ protein was analyzed using Nano ZS (Malvern, Westborough, MA). Briefly, lyophilized protein sample was dissolved in
D.I. water to get final protein concentration of 1 mg/ml. The hydrodynamic diameter of LEDGF₁₋₃₂₆ using the dynamic light scattering technique with data collection at 173° backscatter angle was obtained. Measurement was an average of 13 scans. Data represents mean of N=8.

**Biophysical Characterization**

For biophysical characterization the protein was extensively dialyzed in 25 mM phosphate buffer pH 7 to remove Tris-HCl and sucrose and filtered through 0.22 µm PVDF syringe filter. Spectra obtained were analyzed using either Origin® 8.5 (OriginLab Corp., Northampton, MA) or SigmaPlot 11.0 (Systat Software, Inc, Chicago, IL). The data was fitted using equations 1 and 2, defined by Scholtz et.al. as below to determine the ΔG, m-value, and [urea]₁/₂ (Scholtz et al., 2009).

\[
y = \frac{\left( y_F + m_F \text{ [urea]} \right) + \left( y_U + m_U \text{ [urea]} \right) \times e^{-\frac{\Delta G(H_2O) - m \text{ [urea]}}{RT}}}{1 + e^{-\frac{\Delta G(H_2O) - m \text{ [urea]}}{RT}}} 
\]

\[
\Delta G = \Delta G(H_2O) - m \text{ [urea]} 
\]

where \( y_F \) and \( y_U \) are the intercepts, \( m_F \) and \( m_U \) are the slopes of the pre- and post-transition phase baselines, and m-value is the slope of the transition phase. ΔG is the free energy change at any particular urea concentration and it varies linearly with urea concentration, and is used to estimate ΔG(H₂O). ΔG(H₂O) is defined as the Gibbs free energy of a protein in the absence of urea at 25 °C. R is universal gas constant and T is the temperature of the sample. [urea]₁/₂ is the concentration of urea at which LEDGF₁₋₃₂₆ is 50 % unfolded. Data represents mean of duplicate studies.
Circular Dichroism (CD)

To determine the secondary structures of LEDGF<sub>1-326</sub> and its conformational stability parameters, far-UV CD spectra were recorded. Briefly, 500 μg/ml of protein sample was placed in 1 mm quartz cuvette and spectra was recorded at a scan speed of 0.5 sec per time point, step size of 1 nm and the bandwidth of 4 nm from 200 to 280 nm using Chirascan® CD instrument (Applied Photophysics Ltd, UK). All scans were done in triplicate. The native LEDGF<sub>1-326</sub> spectrum thus obtained was deconvulated using CDNN 2.1 software (Dr. Gerald Bohm, Martin-Luther-University at Halle, Wittenberg, Germany, UK) to get the percentage of secondary structures present in native LEDGF<sub>1-326</sub> protein.

LEDGF<sub>1-326</sub>, chemical denaturation was performed at various urea concentrations. Briefly, 300 μg/ml of protein was incubated with 0 to 6 M urea in 25 mM phosphate buffer pH 7.0 for 24 hours. CD signal was recorded as mentioned above. The conformational stability parameters of LEDGF<sub>1-326</sub> were determined by plotting the CD signal at 230 nm as a function of urea concentration as we obtained the maximum CD signal difference between the folded and unfolded protein spectrum at this wavelength. Similarly, to investigate the thermal stability of LEDGF<sub>1-326</sub>, 500 μg/ml of LEDGF<sub>1-326</sub> was subjected to heat denaturation from 25º C to 90 ºC in smooth ramp mode at ramp rate of 1º C per min. Since major changes were seen at 222 nm, the CD signal at this wavelength was used to determine the melting point (T<sub>m</sub>).

Fluorescence Spectroscopy

The steady state fluorescence spectroscopy was done to determine the tertiary structure perturbation. The protein sample (final concentration 300 μg/ml) was incubated
with various concentration of urea solution (0 to 6 M) in 25 mM phosphate buffer pH 7.0 for 24 hours. The intrinsic tryptophan fluorescence spectra were recorded from 300 to 400 nm, at 280 nm excitation wavelength, with an increment of 1 nm using Spectramax M5 (Molecular Devices, Downingtown, PA). The conformational stability parameters of LEDGF$^{1-326}$ were determined by plotting the fluorescence intensity ratio at 340/356 nm as a function of urea concentration. All intensity values were corrected for buffer effects and inner filter effects.

Functional Characterization

In Vitro Assay

ARPE-19 cells were maintained as described earlier (Baid et al., 2011a). For cell viability assay, 10000 cells were plated in 96-well plate and incubated for 24 hours. After 24 hours, the serum containing medium was aspirated out. The test groups (pP23H-Rho+ LEDGF$^{1-326}$) were transiently transfected with pP23H-Rho plasmid (1 µg/ml) using 1:3 ratio of lipofectamine 2000 (LP-2000) in serum free medium as per manufacturer's protocol. After six hours of transfection, the medium were aspirated out and cells were treated with increasing amount of LEDGF$^{1-326}$. No cells (just the medium), cells with no LP-2000 and cells with LP-2000 were also maintained as control.

MTT Assay

After 48 hours, the medium was aspirated out and 200 µl of fresh serum free medium was added. 20 µl of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, 5 mg/ml in PBS pH 7.4) was added to each well and further incubation was done for 3 hours at 37 °C. The MTT containing medium was aspirated out and the formazan crystals formed were dissolved in 200 µl of DMSO. The
absorbance of the color developed was measured at 570 nm using Spectramax M5. The percentage viability of groups was calculated with reference to the control group containing cells with no LP-2000. All groups were repeated with N=4.

**Live/Dead Cell Count Assay**

ARPE-19 cells were treated similar to MTT assay as stated above. At the end of LEDGF$_{1-326}$ treatment period, cells were washed with PBS. The cells were labeled with a combination of plasma membrane permeant (Hoechst 33342), a plasma membrane impermeanble molecule (BOBO$^\text{TM}$ 3), and a nuclear dye (4',6-diamidino-2-phenylindole, dihydrochloride; DAPI). Hoechst 33342 labeled cell nucleii, whereas BOBO$^\text{TM}$ 3 labeled dying or dead cells. The cells were visualized using Operetta$^\text{®}$ high content imaging system. Cell count was obtained using automated software tool in the Operetta$^\text{®}$ instrument. Number and percentage of live cells were calculated by subtracting the dead cell count from “all cell” count.

**Phagocytic Assay**

ARPE-19 cells were seeded in 24-well plates and transfected with 20 pM/ml of MERTK siRNA (Santa Cruz Biotechnology Inc., Dallas, TX), using siRNAtansflecting agent (Santa Cruz Biotechnology Inc., Dallas, TX) for 6 hours. The transfecting medium was removed and cells were further incubated in serum free medium for 24 hours. Cells transfected only with the transfecting medium and no MERTK siRNA were maintained as control. Cells were washed once and treated with 0.05, 0.5, or 5 µg/ml of LEDGF$_{1-326}$ for 24 hours and then phagocytosis of 2 µm particles were monitored. Briefly, 100 µg/ml of 2 µm blue FluoSpheres (Life Technologies, Grand Island, NY) was incubated with cells for 3 hours. Thereafter, cells were washed twice with cold PBS pH 7.4, followed by
two washes of cold PBS pH 5.0 to remove adherent FluoSpheres. Cells were lysed using 1% Triton-x, and the fluorescence of the particles in the cell lysate was measured using 350 nm excitation and 430 nm emission. Cells transfected with only transfecting agent without siRNA was taken as control for particle uptake. Cells with no particle treatment were used for background fluorescence measurements.

In Vivo Efficacy Assay

Animal Maintenance

Homozygous RCS rat breeders were generously gifted by Dr. Jeffrey Olson (University of Colorado Anschutz Medical Campus, Aurora, CO). Thereafter, RCS rat colony was maintained in the animal facility of University of Colorado Anschutz Medical Campus and with approval of IUCAC. The experiments were carried as per the ARVO statement for the Use of the Animals in Ophthalmic and Vision Research.

Electroretinography

At 4 weeks age, rats were dark adapted for at least 30 min. Thereafter, the animal was prepared for ERG under dim red light. Briefly, rat was anaesthetized with intraperitoneal injection of mixture of 80 mg/kg of ketamine and 12 mg/kg of xylazine. The pupil was dilated with 0.5 % tropicanamide (Akorn, Lake Forest, IL) and was kept moist using 2.5 % hypromellose (Akorn, Lake Forest, IL). Thereafter, the animal was placed on a heated water jacket stabilized at 37 °C. A reference electrode (LKC Technologies Inc., Gaithersburg, MD) was inserted into the animal’s tail and cheek. A DTL plus electrode (LKC Technologies Inc., Gaithersburg, MD) was placed across the cornea of each eye. Animal was exposed to brief flashes of 0.4 log cd–s/m² with interval of 10 secs between each flash and scotopic eletroretinograms (ERGs) were recorded.
Thereafter, the animal was light adapted for 3 min with a background light of 30 cd/m². Photopic ERG was recorded at same intensity flash but with background light on. At least three ERGs were averaged to get a single ERG for each animal. Sterile filtered, 2 µl of 0.25, 0.5, or 2.5 mg/ml of LEDGF₁₋₃₂₆ was given intravitreally in one eye and vehicle in contralateral eye. ERGs were recorded every two weeks for eight weeks after intravitreal injection, i.e. till 12 week age of rats. Data is presented for N=3 or 4.

**Histology**

At the end of the study i.e., on 12th week, eyes were enucleated after ERG measurements and fixed in Davidson’s fixative (2 % of 37-40 % formaldehyde, 35 % ethanol, 10 % glacial acetic acid, and 53 % distilled water) for 24 hours at room temperature. The eyes were then stored in 70 % ethanol for subsequent serial dehydration and embedment in paraffin. Three vertical sections of 6 µm thick were cut from the nasal to the temporal side at the optical nerve (500 µm apart) on a standard microtome. Gross retinal morphology was assessed by light microscope following hematoxylin/eosin staining of tissue sections. The thickness of outer nuclear layer (ONL) and inner nuclear layer (INL) was measured methodically using Aperio ImageScope software v11.1.2.760. Since the photoreceptor cell protection may be uneven across the retina, every 500 µm from the superior edge to the inferior edge in each section was analyzed and average of three sections was done for each point. Data represent average of three eyes.

**Immunofluorescence**

For immunofluorescence, after removal of paraffin, eye sections were processed through the following sequential steps at room temperature, unless otherwise indicated. Antigen was retrieved by boiling the sections at 80 °C for 15 min. After blocking the
nonspecific binding, sections were incubated with mouse anti-rhodopsin (1D4) primary antibody at 4 °C overnight followed by 30 min incubation with Alexa Fluor® 594 conjugated donkey anti-mouse IgG and DAPI. Finally, eye sections were washed and mounted by Supermount H (Biogenex, San Ramon, CA) mounting medium to prevent rapid loss of fluorescence. The fluorescence was visualized using confocal microscope (Nikon Eclipse C1) at 20X optical zoom. The excitation-emission wavelengths used for DAPI and, Alexa Fluor were 408–450/35, and 637–605/75 nm, respectively. Images were captured using Nikon EZ-C1 software version 3.40.

Statistical Analysis

All data are represented as the mean ± SD. An independent-samples student’s t-test or one-way ANOVA followed by Tukey’s post hoc analysis (SPSS, ver.11.5; SPSS, Chicago, IL) was performed for comparisons between the two or multiple experimental groups, respectively. The differences were considered statistically significant at p ≤ 0.05.

Results

Cloning, Expression, and Purification of LEDGF1-326

DNA gel electrophoresis of the PCR amplification product during cloning indicated a positive band of 1000 bp of LEDGF1-326 (Figure 4.1A). A positive band, at about 40 kDa, in the supernatant fraction, indicated expression of LEDGF1-326 as a soluble protein in E.coli BL21 (DE3) (Figure 4.1B). Cation exchange (Figure 4.1C) of LEDGF1-326 preparation removed all negatively charged proteins and eluted LEDGF1-326 along with other lower molecular weight positively charged proteins (Figure 4.1B, Lane 4). On further purification using gel filtration (Figure 4.1D), LEDGF1-326 was eluted as a
strong positive band of ~ 40 kDa (Figure 4.1B, Lane 5). Protein estimation indicated that about 20 mg of protein was obtained per liter of the shake flask culture.

**Bioinformatic Analysis of LEDGF\textsubscript{1-326}**

Bioinformatics analysis of LEDGF\textsubscript{1-326} sequence using SIB ExPASy portal (Gasteiger et al., 2003) indicated its theoretical molecular weight to be 36.9 kDa. The computed isoelectric point (pI) of LEDGF\textsubscript{1-326} was 9.23, with 73 positively charged (arginine and lysine) and 63 negatively charged (aspartic acid and glutamic acid) amino acid residues. The theoretical molar extinction coefficient was 15470 M\textsuperscript{-1} cm\textsuperscript{-1} at 280 nm in water. Based on its N-terminal amino acid methionine, its half-life in mammalian cells was predicted to be 30 hours. The N-, O-, and C- glycosylation of LEDGF\textsubscript{1-326} was predicted using NetNGlyc 1.0, NetOGlyc 3.1, and NetCGlyc 1.0 servers, respectively.

According to the output of NetNGlyc, LEDGF\textsubscript{1-326} has only one potential site for N-glycosylation at 103 aa. However, the probability of this site to be glycosylated is 0.6, indicating low likelihood of N-glycosylation. According to NetOGlyc 3.1 and NetCGlyc 1.0 server outputs, LEDGF\textsubscript{1-326} is unlikely to be O- or C- glycosylated. Thus, bacterial biosynthesis of protein may not alter LEDGF\textsubscript{1-326} potency due to differences in glycosylation status.

**LEDGF\textsubscript{1-326} is purified to Near Homogeneity as a Random Coiled Protein**

The purity of LEDGF\textsubscript{1-326} protein, determined by size exclusion chromatography (SEC-HPLC) (Figure 4.2A) indicated 95% pure LEDGF\textsubscript{1-326} with a retention time of 10.63 ± 0.06 min. To further investigate whether LEDGF\textsubscript{1-326} self-associates to form any higher molecular weight oligomers, dynamic light scattering (DLS) was performed.
Figure 4.1. LEDGF<sub>1-326</sub> Cloning, Biosynthesis, and Purification. A) DNA Gel Electrophoresis- Lane 1: PCR amplified LEDGF<sub>1-326</sub> gene from pEGFP-LEDGF, Lane 2: linearized BamHI digested pET-28a(+), Lane 3: linearized BamHI digested pLEDGF<sub>1-326</sub>, Lane 4: linearized HindIII digested pLEDGF<sub>1-326</sub>, Lane 5: BamHI and HindIII double digested pLEDGF<sub>1-326</sub>, Lane 6: PCR amplified LEDGF<sub>1-326</sub> gene from pLEDGF<sub>1-326</sub>. B) SDS-PAGE – reducing conditions: UN: uninduced cell lysate, IN: IPTG induced cell lysate, LY: Soluble fraction of lysate, CEX: LEDGF<sub>1-326</sub> obtained from cation exchange column, GFC: Pure LEDGF<sub>1-326</sub> obtained from gel filtration column. C) FPLC-CEX (cation exchange)- Red: absorbance of eluted protein at 280 nm, Blue: conductance of the elution buffer, D) FPLC-GF (gel filtration): Absorbance of eluted protein at 280 nm.
(Figure 4.2B). DLS indicated a homogeneous population of LEDGF\textsubscript{1-326}, with a mean hydrodynamic diameter of 9 ± 1 nm.

The molecular weight of LEDGF\textsubscript{1-326} was confirmed by matrix assisted laser desorption/ionization (MALDI-TOF) mass spectrometry. The major peak obtained in MALDI-TOF spectrum was at 40314.32 and 80663.19 m/z (mass to charge) ratio (Figure 4.2C). MALDI-TOF indicated that LEDGF\textsubscript{1-326} has a molecular weight of 40.314 kDa, which was equivalent to its theoretical molecular weight, indicating that the protein is not glycosylated. However, a second peak at 80663 m/z was also seen, which indicated that LEDGF\textsubscript{1-326} may exist as a dimer. To investigate the existence of the dimers, SDS-PAGE of LEDGF\textsubscript{1-326} was run under reducing and non-reducing conditions (Figure 4.2D). Under non-reducing (NR) conditions (right lane), there was an upward shift of the LEDGF\textsubscript{1-326} band to 95-105 kDa size, indicating that LEDGF\textsubscript{1-326} may exist in dimeric form. Under reducing/denaturing (R) conditions (left lane) the dimers dissociated into monomers.

To investigate the secondary structure of LEDGF\textsubscript{1-326}, far-UV circular dichroism (CD) spectrum of the native LEDGF\textsubscript{1-326} was analyzed (Figure 4.2E). The CD signal remained negative from 280 to 200 nm. A very low ellipticity above 210 nm and negative band below 200 nm indicated that LEDGF\textsubscript{1-326} may be primarily composed of random coils (Gokce et al., 2005). To further dissect the secondary structure of LEDGF\textsubscript{1-326}, the CD spectrum was deconvoluted using CDNN 2.1 software (Table 4.1).

Assuming that the spectrum obtained is the linear combination of the individual spectrum of the component secondary structure elements and the noise due to the aromatic chromophores and prosthetic groups, LEDGF\textsubscript{1-326} was predicted to be 45.1 %
Figure 4.2. LEDGF<sub>1-326</sub> Characterization.
Figure 4.2. LEDGF<sub>1-326</sub> Characterization. A) Size exclusion chromatography (SEC-HPLC): LEDGF<sub>1-326</sub> was eluted primarily as single peak at 11.5 min with about 5% higher molecular weight species. B) Dynamic light scattering (DLS): LEDGF<sub>1-326</sub> had a monodisperse population of ~ 9 nm diameter, with the absence of aggregates. C) MALDI-TOF: LEDGF<sub>1-326</sub> has a molecular weight of 40 kDa, and may exist as a dimer. D) SDS-PAGE: LEDGF<sub>1-326</sub> was size separated on a 4-15% SDS-PAGE gel under reducing and non-reducing conditions. The non-reducing gel indicated the presence of dimers of LEDGF<sub>1-326</sub>. E) Circular dichroism (CD): Presence of the strong negative signal at 200 nm indicated that LEDGF<sub>1-326</sub> is primarily a random coiled protein.
Table 4.1. Estimation of Secondary Structures of LEDGF<sub>1-326</sub>.

<table>
<thead>
<tr>
<th></th>
<th>200-260 nm (%)</th>
<th>205-260 nm (%)</th>
<th>210-260 nm (%)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Helix</td>
<td>14.00</td>
<td>20.00</td>
<td>16.30</td>
<td>16.77</td>
</tr>
<tr>
<td>β-Sheets Antiparallel</td>
<td>20.80</td>
<td>12.20</td>
<td>15.90</td>
<td>16.30</td>
</tr>
<tr>
<td>β-Sheets Parallel</td>
<td>15.30</td>
<td>14.60</td>
<td>15.10</td>
<td>15.00</td>
</tr>
<tr>
<td>β-Turn</td>
<td>22.90</td>
<td>19.60</td>
<td>21.10</td>
<td>21.20</td>
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<tr>
<td>Random Coil</td>
<td>42.40</td>
<td>44.60</td>
<td>48.30</td>
<td>45.10</td>
</tr>
<tr>
<td>Total</td>
<td><strong>115.4</strong></td>
<td><strong>111</strong></td>
<td><strong>116.7</strong></td>
<td><strong>114.37</strong></td>
</tr>
</tbody>
</table>

The native LEDGF<sub>1-326</sub> spectrum was deconvoluted using CDNN 2.1 software to determine the percentage of secondary structures present in LEDGF<sub>1-326</sub>. LEDGF<sub>1-326</sub> primarily contains random coils.

randomly coiled. The β-turn was about 21.2 %, there were 15 % parallel β-sheets and 16 % antiparallel β-sheets. The contribution from the α-helix was about only 16 %. The three dimensional structure of LEDGF<sub>1-326</sub> native protein was predicted to be predominantly a random coil, using I-Tasser (Iterative Threading Assembly Refinement) protein modeling server (Figure 4.2F). LEDGF<sub>1-326</sub> predicted model had the confidence score (C-score) of -3.18, template modeling score (TM-score) of 0.36 ± 0.12, and root mean square deviation (RMSD) was equal to 14.1 ±8 Å.

**LEDGF<sub>1-326</sub> is Conformationally Stable**

To investigate the conformational stability of LEDGF<sub>1-326</sub> in water, the perturbation in the tertiary structure due to chemical denaturation was measured by the intrinsic fluorescence of tryptophan molecules present in LEDGF<sub>1-326</sub> (Figure 4.3A). Emission spectrum of native LEDGF<sub>1-326</sub> protein, in absence of urea, had a λ<sub>max</sub> at 340 nm and Δλ<sub>1/2</sub> (half width of Δλ) of 56 nm (Figure 4.3Ai). As the concentration of urea
increased from 0 to 5 M, quenching in the fluorescence signal as well as red shift was seen. The signal decreased slowly until 0.9 M urea concentration was reached. Thereafter, there was a sharp decrease in the fluorescence signal until 2.3 M urea concentration was reached. Beyond this concentration, the decrease in the fluorescence signal was minimal. The $\lambda_{\text{max}}$ of LEDGF$_{1-326}$ shifted to 356 nm and $\Delta\lambda_{1/2}$ was 71 nm at 5 M urea. When the ratio of LEDGF$_{1-326}$ fluorescence signal at 340 to 356 nm was plotted as a function of urea concentration, a sigmoidal curve was obtained (Figure 4.3Aii). Using the equations 1 and 2 (described in methods), $\Delta G(H_2O)$ of LEDGF$_{1-326}$ was estimated to be $3.24 \pm 0.48$ kcal mol$^{-1}$, the m-value to be $1.70 \pm 0.22$ kcal mol$^{-1}$M$^{-1}$, and $[\text{urea}]_{1/2}$ to be $1.81 \pm 0.02$ M, indicating that LEDGF$_{1-326}$ is might be stable protein.

Far-UV CD spectroscopy was performed to investigate the perturbation in the secondary structures of LEDGF$_{1-326}$ in presence of urea (Figure 4.3B). The CD signal of the LEDGF$_{1-326}$ was traced against the wavelength at each urea concentration (Figure 4.3Bi). The CD signal continuously became more negative as the concentration of urea was increased. When CD signal at 230 nm was plotted as a function of urea concentration (Figure 4.3Bii), a sigmoidal curve was obtained. Fitting the LEDGF$_{1-326}$ data to equations 1 and 2 indicated a $\Delta G(H_2O)$ of $3.3 \pm 0.4$ kcal mol$^{-1}$, m-value of $1.9 \pm 0.2$ kcal mol$^{-1}$M$^{-1}$, and $[\text{urea}]_{1/2}$ of $1.6 \pm 0.1$ M.

Thermal stability of LEDGF$_{1-326}$ was determined using far-UV CD spectroscopy (Figure 4.3C). The CD signal in the presence of heat as a denaturant was measured from 215 – 250 nm (Figure 3Ci). As the temperature of the LEDGF$_{1-326}$ solution was increased, the negative dip at about 235 nm increased. The CD signal followed the same pattern as chemical denaturation, a pre-transition phase between $\sim30 – 35$ °C, followed by transition
Figure 4.3. LEDGF$_{1-326}$ Conformational Stability Prediction.

A. Fluorescence Spectroscopy (Chemical Denaturation)

B. Circular Dichroism (Chemical Denaturation)

C. Circular Dichroism (Thermal Denaturation)
Figure 4.3. LEDGF<sub>1-326</sub> Conformational Stability Prediction. A) Fluorescence spectroscopy-chemical denaturation: Fluorescence intensity of LEDGF<sub>1-326</sub> decreased as the concentration of urea is increased along with a red shift in the intensity maxima, indicating LEDGF<sub>1-326</sub> is unfolding. C) Circular dichroism-chemical denaturation: The Far-UV CD signal at 230 nm decreased with an increase in urea concentration, indicating perturbation in the secondary structure of LEDGF<sub>1-326</sub>. D) Circular dichroism- thermal denaturation: The Far-UV CD signal at 222 nm decreased with an increase in temperature, indicating unfolding of LEDGF<sub>1-326</sub>. 
phase between ~ 35 – 55 °C, followed by post-transition phase from ~ 55 – 70 °C (Figure 4.3Cii). When this data was fitted using a global fit analysis equation, the \( T_m \) (the melting temperature) of LEDGF\(_{1-326}\) obtained was 44.8 ± 0.2 °C, indicating that LEDGF\(_{1-326}\) will possibly be stable at 25 °C (room temperature).

**LEDGF\(_{1-326}\) Rescues ARPE-19 Cells from Aggregation Mediated Stress**

LEDGF\(_{1-326}\) efficacy to rescue ARPE-19 cells from protein aggregation mediated stress was measured by MTT assay (Figure 4.4 A). Initially, the ability of LEDGF\(_{1-326}\) to increase the viability of ARPE-19 cells in absence of any stress was investigated (Figure 4.4A, no aggregation stress). There was no significant difference in the cell viability in untreated and 0.001 to 50 µg/ml LEDGF\(_{1-326}\) treated cells following 48 hours treatment. At the highest dose of LEDGF\(_{1-326}\) (50 µg/ml), the cell viability was 108.14 ± 5.63 % (right most bar) as compared to 100 ± 13.19 % for untreated cells (left most bar), which was not significantly different. However, in pP23H-Rho transfected ARPE-19 cells having aggregation stress, LEDGF\(_{1-326}\) behaved differently (Figure 4.4A, aggregation stress). Cells expressing P23H mutant rhodopsin showed a decline in cell viability to 48.25 ± 5.62 % (Bar 2). This loss in cell viability could be attributed to toxic effect of expression and accumulation of aggregation prone P23H mutant rhodopsin protein within the cells. When cells expressing P23H mutant rhodopsin (Bar 3– 9) were treated with increasing amounts of LEDGF\(_{1-326}\), an increase in the cell viability was seen. Even at a concentration as low as 0.001 µg/ml, LEDGF\(_{1-326}\) increased the cell viability of ARPE-19 cells from 48.3 ± 5.6 to 77.0 ± 10.2 %. At and beyond this point, the cell viability remained significantly higher as compared to the pP23H-Rho transfected group.
Figure 4.4. LEDGF1-326 Rescues ARPE-19 Cells from Aggregation Mediated Stress.
Figure 4.4. LEDGF<sub>1-326</sub> Rescues ARPE-19 Cells from Aggregation Mediated Stress.

A) MTT assay-LEDGF<sub>1-326</sub> had no effect on the viability of ARPE-19 cells in the absence of aggregation stress at given concentrations. In the presence of aggregation stress, the cell viability of ARPE-19 cells was increased significantly by LEDGF<sub>1-326</sub> at concentrations as low as 0.01 µg/ml. B) Fluorescence Imaging- Representative image of ARPE-19 cells treated with LEDGF<sub>1-326</sub>. Number of live cell nuclei increased with increasing dose of LEDGF<sub>1-326</sub>. C) Cell counting- ARPE-19 cells treated with LEDGF<sub>1-326</sub> in the presence of pP23H-Rho mediated aggregation stress indicated significant increase in number of live cell nuclei in a dose- and time- dependent manner. Data is represented as mean ± S.D. for N=4. *, p<0.05 compared to pP23H-Rho transfected group.
To confirm the results of MTT assay, fluorescence imaging (Figure 4.4B) and cell counting (Figure 4.4C) were also performed. Based on fluorescence images, the cells were counted automatically by high throughput analysis software. Groups treated with LEDGF<sub>1-326</sub> in the presence of P23H-Rho aggregation stress indicated more number of cells per frame as compared to untreated group, indicating the ability of LEDGF<sub>1-326</sub> to prevent the loss of cells due to aggregation stress (Figure 4.4B). Under P23H-Rho aggregation stress, the live cell count decreased to 38 ± 6 % (Figure 4.4C). LEDGF<sub>1-326</sub> increased the live cell count significantly from 38 ± 6 to 118 ± 16 % in a dose-dependent manner in 24 hours. Further, a single treatment of LEDGF<sub>1-326</sub> was effective until day 7 for all doses ranging from 0.005 to 5 µg. P23H-Rho expressing cells treated with LEDGF<sub>1-326</sub> (blue lines) indicated significantly higher number of live cells than untreated group (red line) on day 7.

**LEDGF<sub>1-326</sub> Increases Phagocytic Activity of ARPE-19 Cells**

ARPE-19 cells were transfected with MERTK siRNA and then treated with LEDGF<sub>1-326</sub>. Uptake of 2 µm FluoSpheres was measured to assess the phagocytic activity of ARPE-19 cells (Figure 4.5). In the absence of MERTK siRNA transfection, addition of LEDGF<sub>1-326</sub> increased FluoSphere uptake to 140 ± 10 % compared to the control group (100 %). MERTK siRNA transfection alone did not reduce FluoSphere uptake (113 ± 13 %). In the MERTK siRNA transfected groups, LEDGF<sub>1-326</sub> increased FluoSpheres uptake in a dose-dependent manner, with the uptake being 190.17 ± 21.47 % at 5 µg/ml of LEDGF<sub>1-326</sub>. 
Figure 4.5. LEDGF<sub>1-326</sub> Increases Phagocytic Activity of Retinal Cells. ARPE-19 cells were transiently transfected with 20 pM/ml of MERTK siRNA. Uptake of 2 µm FluoSpheres (100 µg/ml) was monitored to measure the phagocytic activity. LEDGF<sub>1-326</sub> increased the uptake of Fluospheres significantly in a dose-dependent manner, indicating an increase in phagocytic activity of ARPE-19 cells. Data is represented as mean ± S.D. for N=4. p<0.05 compared to MERTK siRNA transfected group.

**LEDGF<sub>1-326</sub> Reduces the Functional and Morphological Loss of Photoreceptors**

LEDGF<sub>1-326</sub> efficacy to reduce the loss of visual function of photoreceptors was investigated in RCS rats by monitoring the electroretinograms (ERGs) (Figure 4.6A). In dark adapted (scotopic) ERG, the b-wave amplitude of 4 weeks old untreated and treated rats ranged from 180.17 ± 27.42 to 216.60 ± 35.30 µV (base ERG), before intravitreal injection was administered (Figure 4.6Ai). At two weeks, after intravitreal injection, the b-wave amplitude ranged from 65.80 ± 15.44 to 91.13 ± 13.94 µV. There was no significant difference in the untreated and LEDGF<sub>1-326</sub> treated groups. However, beyond two weeks, the b-wave amplitude reduction was less in LEDGF<sub>1-326</sub> treated groups.
eight weeks after the intravitreal injection, the b-wave amplitudes of untreated, 0.5, 1.0, and 5 μg of LEDGF<sub>1-326</sub> treated groups were 9.40 ± 4.57, 32.43 ± 10.34, 37.93 ± 0.60, and 57.63 ± 8.81 μV, respectively. The b-wave amplitude of LEDGF<sub>1-326</sub> treated groups was significantly (p<0.05) higher than the untreated group. A dose dependent delay in the decline of b-wave amplitude was seen for the LEDGF<sub>1-326</sub> treated groups. With increasing dose of LEDGF<sub>1-326</sub>, the loss of b-wave amplitude was reduced.

In light adapted (photopic) ERG, the base b-wave amplitude, before intravitreal injection, at 4 weeks ranged from 69.83 ± 16.49 to 80.97 ± 8.60 μV, with no significant difference between the untreated and LEDGF<sub>1-326</sub> treated groups (Figure 4.6Aii). By eight weeks after intravitreal injection, the b-wave amplitude of untreated group reduced from 80.97 ± 8.60 to 10.90 ± 5.64 μV, whereas the decrease was from 79.63 ± 20.30 to 41.33 ± 9.20, 69.83 ± 16.49 to 28.00 ± 7.23, and 68.75 ± 15.93 to 45.78 ± 15.18 μV for 0.5, 1.0, and 5.0 μg of LEDGF<sub>1-326</sub> treated groups, respectively. The b-wave amplitude of LEDGF<sub>1-326</sub> treated groups were significantly (p<0.05) higher than the untreated group after eight weeks of single intravitreal injection of LEDGF<sub>1-326</sub> similar to scotopic ERG.

Morphological analysis of retinal layer was done (Figure 4.6B) for groups treated with 5 μg of LEDGF<sub>1-326</sub> to determine the thickness of the various layers of retinal cells. Representative H&E stained images (Figure 4.6Bii) of wild type healthy SD rat, the untreated RCS rat, and LEDGF<sub>1-326</sub> treated RCS rat indicated ~10-11, 0-1, 3-4 layers thick outer nuclear layer (ONL), respectively. Outer nuclear layer contains the nuclear bodies of the photoreceptors. For both control and LEDGF<sub>1-326</sub> treated groups the ONL was thinnest at the optic nerve head (ONH) region and thickened as it reached the periphery (Figure 4.6Biii). The LEDGF<sub>1-326</sub> treated group consistently indicated thicker
Figure 4.6. LEDGF<sub>1-326</sub> Reduces the Functional and Morphological Loss of Photoreceptors.
Figure 4.6. LEDGF<sub>1-326</sub> Reduces the Functional and Morphological Loss of Photoreceptors. Single intravitreal injection of vehicle (control) or LEDGF<sub>1-326</sub> was given on week 4 to RCS rats. A) Electroretinography: i) For scotopic ERG, rats were dark adapted for 30 min and then ERG was recorded using 0.4 log cd-s/m<sup>2</sup> flashes, ii) For photopic ERG, rats were light adapted using 30 cd/m<sup>2</sup> background light for 3 mins. LEDGF<sub>1-326</sub> significantly reduced the loss in b-wave amplitude in a dose dependent manner, indicating delay in functional loss of photoreceptors. B) Histology at 12 week: i) Vertical section of the eye. ii) Representative H and E stained section of retina. Morphological analysis of iii) Outer nuclear layer (ONL) and iv) Inner nuclear layer (INL). LEDGF<sub>1-326</sub> (5 µg) treated group indicated significantly thicker ONL and INL. Data is presented as mean ± S.D. for N=3. *, p<0.05 compared to corresponding untreated group. Abbreviations: RPE-retinal pigment epithelium, OS- outer segment, ONL-Outer nuclear layer, INL- Inner nuclear layer, IPL- Inner plexiform layer, GCL-ganglion cell layer.
Figure 4.7. LEDGF$_{1-326}$ Reduces Loss of Rod Photoreceptors. Representative fluorescence image of RCS rat eye section. Single intravitreal injection of LEDGF$_{1-326}$ was given to week 4 RCS rats. Immunofluorescence was done at week 12 of eye sections using mouse anti-rhodopsin (1D4) followed by Alexa Fluor® 594 conjugated donkey anti-mouse IgG. Bright red fluorescence (Anti-Rho) was seen in cells treated with LEDGF$_{1-326}$ (1st row, right panel), indicating preservation of rods photoreceptors. The blue fluorescence (DAPI) indicated more nuclear bodies in outer nuclear layer of rats treated with LEDGF$_{1-326}$ (2nd row, right panel).
ONL compared to corresponding control group at every data point with significantly thicker ONL at 0.5, and 2 mm away from ONH at the superior side and at 1.0 and 2.0 mm away from ONH at the inferior side. Inner nuclear layer (INL) was thicker near ONH and thinner near periphery (Figure 4.6Biv). The INL was significantly thicker at 1.0 and 2 mm away from ONH at the superior side and at 1.0 mm away from the ONH on the inferior side in the LEDGF$_{1-326}$ treated group.

Ability of LEDGF$_{1-326}$ to reduce the loss of rod photoreceptors was determined by immunofluorescence (Figure 4.7). Immunolabelling of eye tissue sections with anti-rhodopsin antibody stained rod photoreceptors as red. The ONL and INL were stained blue with DAPI. A thick red band visualized in LEDGF$_{1-326}$ treated group (right panel) indicated presence of large numbers of rod photoreceptors. In the untreated group, this band was almost undetectable. Further, similar to H & E staining (Figure 4.6Bii), the ONL was thicker (blue fluorescence) in LEDGF$_{1-326}$ treated group as compared to untreated group.

**Discussion**

In this study, we successfully cloned and expressed LEDGF$_{1-326}$ in large quantities and characterized its biophysical properties, determined its in vitro ability to enhance phagocytotic activity and ameliorate protein aggregation mediated cellular stress, and in vivo efficacy in rescuing photoreceptors from degeneration. As elaborated below, our in vitro studies indicated that LEDGF$_{1-326}$ improves phagocytic activity and effective in rescuing retinal pigment epithelial cells from death due to aggregation mediated stress. Further, our in vivo studies indicated that intravitreally injected LEDGF$_{1-326}$ can reduce retinal degeneration for at least eight weeks in RCS rats.
In our previous study, we identified *LEDGF* \(_{1-326}\) gene as a potential candidate for rescuing retinal pigment epithelial cells from P23H mutant rhodopsin aggregation induced stress (Baid et al., 2011a). The objective of this study was to develop a protein therapy based on *LEDGF* \(_{1-326}\). To develop *LEDGF* \(_{1-326}\) as a therapeutic agent, it should be produced in tens of milligrams, which in itself is a daunting task. We designed a cloning strategy to express *LEDGF* \(_{1-326}\) as a cytoplasmic protein in pET-28a (+), a bacterial cell based system and confirmed the successful cloning by sequence analysis for correct reading frame, right orientation of the gene, and no mutagenesis during the cloning procedure (Figure 4.1A). Utilizing two-step purification, we were able to produce ~ 95 % pure protein (Figure 4.1B). Since *LEDGF* \(_{1-326}\) is not highly likely to undergo glycosylation based on molecular modeling, and based on our observed efficacy, bacterial biosynthesis of *LEDGF* \(_{1-326}\) is adequate for therapeutic activity. However, the role of other post translational modifications and an alternation in activity of our *LEDGF* \(_{1-326}\) compared to native protein cannot be ruled out. SEC-HPLC of purified *LEDGF* \(_{1-326}\) indicated 5 % higher molecular weight species of *LEDGF* \(_{1-326}\) (Figure 4.2A). Since it has been known that the presence of large aggregates can trigger immunogenic reactions in vivo (Frokjaer and Otzen, 2005), we also investigated the possible formation of high molecular weight aggregates during purification of *LEDGF* \(_{1-326}\) protein, using DLS (Figure 4.2B). Interestingly, the number mean size measurements gave a single narrow size distribution of *LEDGF* \(_{1-326}\), indicating absence of higher molecular weight aggregates. Since MALDI-TOF analysis indicated that *LEGF* \(_{1-326}\) may exists as a monomer and/or dimer (Figure 4.2C), non-reducing SDS-PAGE was utilized to confirm the existence of dimers (Figure 4.2D). Higher molecular weight bands were evident in the
gel, indicating possible existence of dimeric form of LEDGF\textsubscript{1-326}. It appeared that possibly a dynamic equilibrium exists between the monomeric and the dimeric forms of LEDGF\textsubscript{1-326}. However, the ratio of dimer to monomer is currently not established. The far-UV CD of LEDGF\textsubscript{1-326} (Figure 4.2E) indicated that LEDGF\textsubscript{1-326} is predominantly a random coiled structure. Deconvolution of the CD signal further strengthened this view.

To establish a protein to be of therapeutic value it is very important to understand its biophysical nature, which includes protein’s conformational stability. Protein conformational stability is contributed by various environmental factors including pH, ionic strength and temperature. Such information is useful in developing a stable formulation of the protein (Pace, 1990). In this study, fluorescence and CD were used to determine LEDGF\textsubscript{1-326} conformational stability (Figure 4.3). The results obtained from fluorescence spectroscopy for LEDGF\textsubscript{1-326} (Figure 4.3A) indicated that tryptophan residues of native LEDGF\textsubscript{1-326} are partially exposed to aqueous environment (Burstein et al., 1973). LEDGF\textsubscript{1-326} free energy of unfolding, $\Delta G(\text{H}_2\text{O})$, was positive, indicating that the unfolding of LEDGF\textsubscript{1-326} is unfavorable in the absence of any denaturant. The melting temperature of LEDGF\textsubscript{1-326} was predicted to be about 44$^\circ$C indicating that LEDGF\textsubscript{1-326} is stable at room temperature. $\Delta G(\text{H}_2\text{O})$, m-value, $[\text{urea}]_{1/2}$, and $T_m$ together defined the conformational and thermal stability of LEDGF\textsubscript{1-326}.

RPE cells are known to accumulate rod outer segments containing rhodopsin with aging in human eyes (Wyszynski et al., 1989). P23H rhodopsin containing rod outer segment accumulation has also been implicated in vivo in neovascularization of RPE (Nishikawa and LaVail, 1998). Thus, our study utilizing mutant rhodopsin in ARPE-19 cells is pertinent to the in vitro simulation of retinal degeneration models. The in vitro
efficacy data indicated significant decrease in the cell viability of ARPE-19 cells in presence of P23H mutant rhodopsin protein (Figure 4.4). This decrease in the cell viability was previously related to aggregation mediated stress caused by the expression of P23H mutant rhodopsin and the associated aggregates in the cellular environment (Illing et al., 2002). Interestingly, upon treatment with LEDGF\textsubscript{1-326}, ARPE-19 cell viability increased significantly. LEDGF\textsubscript{1-326} protein did not alter the viability of the ARPE-19 cells in the absence of stress, indicating that LEDGF\textsubscript{1-326} is more active in stressed conditions. Since MTT assay is indicative of mitochondrial respiratory state, but not a direct measure of cell viability, we performed high throughput cell counting using Operetta High Content Analysis System. We did not see many dead cells in P23H-Rho group. However, the cell count in P23H-Rho group was significantly lower than the cells not expressing P23H-Rho, suggesting that rhodopsin aggregation arrested cell proliferation. In the presence of LEDGF\textsubscript{1-326}, the number of cells per frame increased compared to controls, indicating that LEDGF\textsubscript{1-326} acts as a proliferating agent in the presence of protein aggregation stress. We previously reported that LEDGF\textsubscript{1-326} reduces oligomers of P23H rhodopsin as well as wild type rhodopsin, while increasing their monomers in a dose-dependent manner (Baid et al., 2011a). LEDGF\textsubscript{1-326} in this earlier study did not affect the total rhodopsin protein expression at low doses. However, at high doses, there was a decrease in the total rhodopsin protein content with no changes in mRNA levels, suggesting that LEDGF\textsubscript{1-326} may be targeting rhodopsin aggregates to degradation pathways, in addition to preventing/disrupting rhodopsin aggregates.

Since RPE cells are specialized to phagocytose photoreceptor outer segments and other cellular debris (Feng et al., 2002), the phagocytic activity of ARPE-19 cells was
determined in the presence of LEDGF<sub>1-326</sub>. Depletion of MERTK receptors in the retinal pigment epithelium is known to inhibit phagocytosis in RPE (Feng et al., 2002). However, MERTK siRNA transfection did not reduce the phagocytosis or uptake of FluoSpheres by ARPE-19 cell in our experimental conditions. It could be possible that under the conditions of the experiment, MERTK was not sufficiently depleted in ARPE-19 cells. Interestingly, LEDGF<sub>1-326</sub> significantly increased ARPE-19 phagocytic activity irrespective of MERTK siRNA transfection. This result is of high interest as this is the first time that LEDGF<sub>1-326</sub> has been shown to enhance phagocytosis.

In the absence of any exact replicate animal model for dry AMD, RCS rat is a useful model in understanding retinal degenerations. RCS rats exhibit primary genetic defect in the RPE due to deletion of MERTK gene, leading to loss of phagocytic activity and toxic accumulation of cell debris. The ultimate pathology in these animals is photoreceptor degeneration (LaVail et al., 1975). Histopathology and ERG are established methods to monitor photoreceptor degeneration and retinal functional loss, respectively. B-waves of ERG specifically indicate photoreceptor health. Therefore, we used histopathology and ERG to monitor the ability of LEDGF<sub>1-326</sub> to reduce retinal degeneration. The decline in the b-wave amplitude in scotopic ERG represents loss or degeneration of rod and cone photoreceptors. In photopic ERG, the rods are bleached, so the response is mostly from the cone photoreceptors. The amplitude of the b-wave reflects the number of functionally active photoreceptors. Single intravitreal injection of LEDGF<sub>1-326</sub> significantly reduced the loss of b-wave amplitude, indicating reduction in the functional loss of photoreceptors for a period of at least two months from the day of
treatment (Figure 4.6A). Further, the functional protection of photoreceptors indicated by ERG correlated with the morphological protection of photoreceptors indicated by histology (Figure 4.6B) and immunofluorescence (Figure 4.7). LEDGF<sub>1-326</sub> rescued photoreceptors, as evidenced by the thicker ONL and thicker band of rhodopsin stained photoreceptors. It also protected INL, which is made up of amacrine and bipolar cells, from degeneration as evident in histological analysis. n-terminal LEDGF<sub>1-326</sub> has stress related element binding domain (Baid et al., 2011a), which is capable of activating stress related proteins (Singh et al., 2006).

Despite some efforts in the current manuscript, the mechanism as to how exactly LEDGF<sub>1-326</sub> works has yet to be investigated at molecular level and is beyond the scope of this manuscript. However, based on existing knowledge, the putative mechanisms of action of LEDGF<sub>1-326</sub> are summarized in Figure 4.8.

We speculate that LEDGF<sub>1-326</sub> may reduce both oxidative as well as endoplasmic reticulum stress (generated by protein aggregation), by upregulating stress response proteins. It may also improve the phagocytic activity of RPE, thereby improving the survival of photoreceptors. Since a close association has been suggested (Salminen et al., 2010) between proteins aggregation stress (RP) and oxidative stress (dry-AMD), a molecule like LEDGF<sub>1-326</sub> can be a universal therapeutic protein to treat multiple retinal degenerative diseases. Further, since many neurodegenerative diseases including Alzheimer’s, Parkinson’s, and Huntington’s have been linked to protein aggregation, a scope exists where LEDGF<sub>1-326</sub> could also possibly be tested in some of these diseases as a therapeutic intervention.
**Figure 4.8. Putative mechanism of action of LEDGF_{1-326}.** A speculative molecular level mechanism of LEDGF_{1-326} to prevent or treat retinal degenerative diseases including dry-AMD and RP.

**Conclusions**

In conclusion, in this study we were able to biosynthesize and purify large quantities of LEDG_{1-326} in aggregate free, highly pure form. LEDGF_{1-326} was found to be conformationally and thermally stable protein at 25 °C. LEDGF_{1-326} was able to prevent the loss of cell viability due to aggregation mediated stress at concentrations ranging from 0.001 to 50 µg/ml. Single intravitreal dose of LEDGF_{1-326} was effective in reducing RCS rats retinal degeneration for over 2 months. Thus, LEDGF_{1-326} is a potential therapeutic agent for retinal degenerative disorders.

**Acknowledgments**

This work was supported, in whole or in part, by National Institutes of Health Grants EY018940, RC1 EY020361, and R21EY17360.
CHAPTER V

DEVELOPMENT OF STABLE AQUEOUS IMMEDIATE RELEASE FORMULATION OF LEDGF_{1-326}

Abstract

Purpose

To develop a stable aqueous formulation of His-tag free lens epithelium derived growth factor fragment (LEDGF_{1-326}).

Methods

His-tag free LEDGF_{1-326} protein was biosynthesized in *Escherichia coli* Bl21 and purified using cation chromatography. LEDGF_{1-326} was, thereafter, formulated in citrate-phosphate buffer (pH 6 to 7.5), with or without an additive mixture (Tween 20, EDTA, and sucrose) and incubated at 25 °C for 60 days. Changes in LEDGF_{1-326} tertiary and secondary structure were monitored by intrinsic tryptophan fluorescence spectra (FL), and far-UV circular dichroism (CD), respectively. LEDGF_{1-326} fragmentation was visualized by SDS-PAGE and insoluble aggregates were quantitated by protein estimation. Formation of subvisible particles was monitored by dynamic light scattering (DLS). Enzyme linked immunosorbent assay (ELISA) was used to quantify immunoreactive LEDGF_{1-326}.

Results

The fluorescence intensity at 342 nm and CD signal at 208 nm of LEDGF_{1-326} significantly decreased by day 3 in citrate-phosphate buffer (pH 6 -7.5) in the absence of
additives. DLS indicated significant increase in the particle size from 7 nm to 200-700 nm. Insoluble aggregates of LEDGF_{1-326} were seen as a visible white pellet. LEDGF_{1-326} also underwent chemical degradation and fragmented to smaller molecular weight proteins as early as day 1 followed by complete loss of its original monomer (40 kDa) by day 7. On the other hand, LEDGF_{1-326} formulated with 0.1 % Tween 20, 1 mM EDTA, and 10 % sucrose in citrate-phosphate buffer pH 6 - 7.5 remained structurally unaltered and aggregate free until day 60. SDS-PAGE indicated no change until day 30, but by day 60 there was appearance of fragmented LEDGF_{1-326} along with LEDGF_{1-326} monomer. The immunoreactivity of LEDGF_{1-326} in the absence of additives decreased to 3.1 ± 2.4 % by day 14 and was undetectable by day 60. In the presence of additives, LEDGF_{1-326} immunoreactivity was 30 ± 4, 78 ± 15, 78 ± 45, 76 ± 9, 102 ± 13, and 58 ± 1 % at pH 6, 6.5, 6.75, 7.0, 7.25, and 7.5, respectively on day 60. At pH 7.0, LEDGF_{1-326} degradation was inhibited better by the mixture of additives when compared to individual additives.

**Conclusions**

LEDGF_{1-326} is highly susceptible to chemical and physical degradation in aqueous formulation during storage at 25 °C leading to formation of fragments and aggregates. Additives Tween 20, EDTA, and sucrose reduced LEDGF_{1-326} degradation. LEDGF_{1-326} remained most immunoreactive in the pH range of 6.75 - 7.25 in the presence of formulation additives.

**Introduction**

In our previous study, we identified lens epithelium derived growth factor fragment (LEDGF_{1-326}) as a protein aggregation reducing agent (Baid et al., 2011a). LEDGF_{1-326} reduced aggregates of wild type as well as mutant P23H rhodopsin in retinal
cells. It also protected ARPE-19 cells from death as a consequence of mutant P23H rhodopsin aggregation stress. We biosynthesized and purified LEDGF\textsubscript{1-326} protein and evaluated its efficacy in a retinal degenerative rat model (Baid et al., 2013). Single intravitreal injection of LEDGF\textsubscript{1-326} reduced functional as well as structural loss of photoreceptors in Royal College of Surgeon (RCS) rats for at least 8 weeks. It also significantly increased the phagocytic efficiency of retinal pigmented cells to 2.4 times. Thus, LEDGF\textsubscript{1-326} is of potential value as a therapeutic protein to treat retinal degenerations such as retinitis pigmentosa (RP) and dry age related macular degeneration (dry AMD). However, the previously synthesized LEDGF\textsubscript{1-326} had histidine (His) tag as a part of the pET-28a(+) plasmid vector in which LEDGF\textsubscript{1-326} was cloned. Fusion of His-tag with a protein is required for purification but His-tag carrying protein is not suitable for clinical use as the His- tag may alter the biological activity (Fonda et al., 2002) as well the structure and biophysical properties (Pedersen et al., 1999; Abdullah and Chase, 2005). The His- tag is generally removed to obtain the native protein. Thus, one objective of this study was to prepare a His-tag-free LEDGF\textsubscript{1-326} as a therapeutic protein for eye diseases.

Protein therapeutics have evolved into a major class of drugs for treatment of various complex disorders including malignant lymphoma (Spiekermann and Hiddemann, 2005), colorectal cancer (Scott et al., 2005), ulcerative colitis (Feagan et al., 2005), heart disease (Hershberger et al., 2005), wet-AMD (Heier et al., 2012), and diabetes (Rekha and Sharma, 2012). There were about 130 protein and peptide products approved by the US FDA by 2008, with the majority being therapeutic proteins and a small number of products being vaccines and diagnostic proteins (Leader et al., 2008).
Based on advances in recombinant DNA technologies, protein biosynthesis and production in large scale is now feasible. However, proteins may undergo spontaneous physical and chemical degradation under normal physiological conditions causing loss in its the biological activity (Stephenson and Clarke, 1989). The biological function of a protein depends on its three dimensional conformation, which is determined by the primary amino acid sequence and higher order secondary, tertiary, and quaternary structure. The three dimensional conformation is maintained by covalent and non-covalent interactions and any disruption in these interactions may lead protein degradation and loss of protein function. Further, during purification, processing, and storage proteins undergo various stresses, which lead to further degradation (Aune and Tanford, 1969; Pace, 1990; Wang, 1999; Arakawa et al., 2001). Since no efforts were previously made to stabilize LEDGF\textsubscript{1-326} formulations and because proteins are notorious for their instability, the second objective of this study was to develop a stable formulation of LEDGF\textsubscript{1-326}, through a rational selection of formulation additives.

Proteins degradation can be broadly classified into two categories; chemical and physical degradation. Chemical instability or degradation involves formation and breakage of covalent bonds resulting in generation of new species; it includes processes such as asparagine isomerization, proteolysis, oxidation, hydrolysis, and beta elimination (Chang and Hershenson, 2002). On the other hand, physical instability or degradation relates to changes in the physical state of protein, with or without changes in the chemical composition, such as adsorption, denaturation, aggregation, and precipitation. In protein formulation, while it is difficult to monitor and control chemical degradation, the physical degradation can be readily monitored and controlled efficiently using proper choice of additives.
formulation excipients (Carpenter et al., 1997). Denaturation is a common phenomenon unique to proteins, resulting from disruption of the native quaternary, tertiary, and/or secondary structures. Denaturation often leads to unfolding, which in turn may lead to formation of soluble or insoluble aggregates. To develop a protein therapeutic, there is a need to optimize protein formulation so as to reduce aggregation and maintain the stability and hence, efficacy and safety, of the protein.

Based on mechanisms of protein degradation, they can be stabilized from days to months by careful consideration of formulation variables including choice of additives (Chang and Hershenson, 2002). There are more than 60 available excipients which can be screened for increasing protein stability in formulation (Jeong, 2012). Among the important factors which affect protein stability and aggregation in aqueous formulation are - pH, buffer species, ionic strength, and stabilizers. pH affects most chemical reactions (e.g., deamidation, hydrolysis, oxidation, cyclic imide formation, and disulfide scrambling) (Khossravi and Borchardt, 2000; Liu et al., 2008). When pH of the formulation is at the isoelectric point of the protein, the protein is most insoluble. This leads to protein precipitation and aggregation. As protein formulation moves away from the pI, proteins are either negatively or positively charged leading to the increase in the solubility of the protein molecule. Insulin for example is insoluble at pH 5.4, which is its isoelectric point (Bolli et al., 1999; Rosskamp and Park, 1999). Adjusting the pH above and below this point increases the solubility. pH far removed from the pI of the protein may increase solubility, but it may also initiate conformational changes (Dill, 1990). pH of the formulation also influences the chemical degradation of proteins. For example, deamidation of insulin occurs at a faster rate at pH less than 4 and more than 7, leading to
polymerization and aggregation (Brange, 1992; Brange et al., 1992a; Brange et al., 1992b; Brange and Langkjaer, 1992; Brange et al., 1992c). Thus, a formulation scientist has to make a careful choice of pH to have acceptable protein solubility and stability. Based on these studies, the insulin formulations (Isophane NPH, Lente, and Ultralente) are made in the range of pH 7-7.8. However, the acceptable pH range varies for each protein depending on the net charge of protein molecule (Townsend and DeLuca, 1990; Kamerzell et al., 2011).

Physical instability and susceptibility of protein to aggregation is related to surface exposure. Proteins, being amphiphilic in nature, tend to migrate to air-water interfaces, leading to protein unfolding and aggregation. Nonionic surfactants such as polysorbate 20 (Tween 20) and 80 (Tween 80) are most widely used to protect the protein, due to their ability to 1) compete with protein molecules for interfaces, 2) increase thermodynamic stability of protein, and 3) foster refolding of proteins (Nema et al., 1997; Kreilgaard et al., 1998; Webb et al., 2002; Kerwin, 2008).

In addition to nonionic surfactants, a non-reducing disaccharide such as sucrose or trehalose is also added to the formulation as a stabilizing agent to minimize protein aggregation (Tiwari and Bhat, 2006; Ohtake and Wang, 2011). Sugars are known to increase the $T_m$ (melting temperature) of proteins in solution and inhibit protein unfolding, thereby increasing protein stability and shelf-life (Carpenter et al., 1997). Unfolding of proteins may lead to exposure of hydrophobic residues at the surface. Thus, a combination of surfactant and a stabilizing agent may have an additive effect. Typically non reducing sugars such as sucrose and trehalose are preferred as a stabilizing agent; however, sucrose is susceptible to acid hydrolysis at low pH (e.g., pH 4.8) and
forms reducing sugars, glucose and fructose (Banks et al., 2009). Therefore, when formulations are made at low pH, trehalose is used instead of sucrose.

Proteins may undergo oxidative degradation when exposed to oxygen, high temperature, metal ions, and other free radical initiators (often present as contamination in many pharmaceutical raw materials) (Akers, 2000; Wasylaschuk et al., 2007). Chelating agents inhibit free radical formation and subsequent protein oxidation by sequestering trace metal ions such as copper, zinc, calcium, etc. Chelating agents like ethylene diamine tetra acetic acid (EDTA) have been used to inhibit oxidation of growth factors.

Based on the above observations, in this study we optimized buffer pH to stabilize LEDGF\textsubscript{1-326} and further included Tween 20 as a nonionic surfactant, sucrose as a stabilizing sugar, and EDTA as a chelating, antioxidant additive to reduce LEDGF\textsubscript{1-326} degradation over a period of 60 days at 25 °C. Further, an enzyme linked immunosorbent assay (ELISA) method was also developed to detect active LEDGF\textsubscript{1-326}.

Materials and Methods

Materials

Isopropyl-β-D-thio-galactoside (IPTG), citric acid, dibasic sodium hydrogen phosphate, ethylene diamine tetra acetic acid (EDTA), Tween 20, sucrose, and sodium azide were purchased from Sigma-Aldrich. AKTAFLPC (GE Healthcare Biosciences, Pittsburgh, PA) was used for protein purification. All chromatograms were analyzed using UNICORN software (GE Healthcare Biosciences, Pittsburgh, PA). All chemicals unless specified were obtained from Sigma-Aldrich and were of reagent or higher grade.
His-Tag Removal

*LEDGF*\(_{1-326}\) gene was amplified from pLEDGF\(_{1-326}\) plasmid (Baid et al., 2013) using 5’AGCAAGCCATGGGCATGACTCGCGATTCAAACCTGGA3’ and 5’AGCAAGAAGCTTCTACTGCTCAGTTTCCATTGGTC3’ primers containing NcoI and HindIII sites, respectively. Thereafter, the amplified LEDGF\(_{1-326}\) gene was ligated into pET-28a (+) after digesting with NcoI and HindIII enzymes. The ligated product was transformed in competent *Escherichia coli* DH5\(\alpha\) cells (Life Technologies, Grand Island, NY) as per user’s manual. The insertion of the gene was confirmed by PCR, restriction digestion and sequencing methods.

*LEDGF\(_{1-326}\)* Biosynthesis and Purification

LEDGF\(_{1-326}\) (His-tag free) was biosynthesized and purified as described previously except for one modification. One step cation exchange chromatography alone was used to purify LEDGF\(_{1-326}\) instead of two step chromatography; i.e., cation exchange followed by gel filtration used for His-LEDGF\(_{1-326}\) (Baid et al., 2013). The purified LEDGF\(_{1-326}\) was extensively dialyzed in citrate-phosphate buffer pH 7.0, and stored at -80 °C until further use.

Formulation Preparation

Citrate-phosphate buffers of pH 6.0, 6.5, 6.75, 7.0, 7.25, and 7.5 were made by mixing 0.1 M citric acid and 0.2 M sodium hydrogen phosphate (Na\(_2\)HPO\(_4\)). For additive containing formulations, Tween 20, EDTA-disodium salt, and sucrose were added at the final concentrations of 0.1 % w/v, 1 mM, 10 % w/v, respectively. LEDGF\(_{1-326}\) was added to obtain a final concentration of 1 or 0.5 mg/ml. Finally the volume was adjusted with deionized water to make the final ionic strength of the citrate-phosphate buffer as per
Table 5.1. Formulation containing 0.02 % sodium azide was tested for any degradation that might happen due to microbial growth. All solutions were sterile filtered through 0.22 µm Millex® syringe filter (EMD Millipore, Billerica, MA). Formulations were stored at 25 °C in a temperature controlled incubator.

Table 5.1. Ionic Strength of Citrate-Phosphate Buffer at Different pH.

<table>
<thead>
<tr>
<th>S.N0.</th>
<th>Formulation pH</th>
<th>Ionic Strength (mM)</th>
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<tbody>
<tr>
<td>1</td>
<td>6.0</td>
<td>137</td>
</tr>
<tr>
<td>2</td>
<td>6.5</td>
<td>139</td>
</tr>
<tr>
<td>3</td>
<td>6.75</td>
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<tr>
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<td>7.25</td>
<td>143</td>
</tr>
<tr>
<td>6</td>
<td>7.5</td>
<td>144</td>
</tr>
</tbody>
</table>

_Fluorescence Spectroscopy_

The steady state fluorescence spectroscopy was done to determine changes in LEDGF$_{1-326}$ tertiary structure. The intrinsic tryptophan (Trp) fluorescence spectrum of formulations were recorded at 280 nm excitation and emission from 300 to 400 nm, with 1 nm increments using Spectramax M5 (Molecular Devices, Downingtown, PA). Fluorescence intensity at 342 nm was plotted for each pH as a function of time. Buffer and inner filter effects were corrected in all samples.

_Circular Dichroism (CD)_

Secondary structural changes of LEDGF$_{1-326}$ were determined by far-UV CD spectrum. Briefly, the formulation was placed in 1 mm quartz cuvette and the spectrum
was recorded from 200 to 280 nm using Chirascan® CD instrument (Applied Photophysics Ltd, UK) at a scan speed of 0.5 sec per data point, step size of 1 nm and bandwidth of 4 nm.

**Dynamic Light Scattering (DLS)**

LEDGF<sub>1-326</sub> protein size was monitored using Nano ZS (Malvern, Westborough, MA). Briefly 100 µl of formulation was placed in a low volume glass cuvette. Using dynamic light scattering, LEDGF<sub>1-326</sub> particle scattering data was collected at a back scattering angle of 173º. An average of 13 scans was performed for each measurement.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

LEDGF<sub>1-326</sub> formulation sample (10 µl) was boiled for 10 min at 75 ºC along with 10 µl of 2X loading buffer. Proteins were size separated on 4 - 15 % mini-PROTEAN TGX gels (BioRad) and visualized using Coomassie Blue staining as per user’s protocol.

**Protein Estimation**

For protein estimation, LEDGF<sub>1-326</sub> formulation was spun down at 10,000 g for 5 min and the supernatant was collected. Protein estimation of supernatant was done using bicinchoninic acid (BCA) assay kit as per user’s manual. For insoluble aggregate estimation, the soluble protein measured at each time point was subtracted from the day 0 protein levels of the corresponding formulation.

**ELISA**

An indirect ELISA method was developed to detect immunoreactivity of LEDGF<sub>1-326</sub>. Briefly, in 96-well plate, 100 µl of either standard LEDGF<sub>1-326</sub> (freshly purified) or formulation sample were coated and incubated overnight at 4 ºC. Wells were washed three times with wash buffer (0.1 % w/v Tween 20 in PBS, pH 7.0) after each
step. Nonspecific binding sites were blocked with blocking solution (0.5 % bovine serum albumin and 0.1 % Tween 20 in PBS pH 7.0) for 4 hours. LEDGF\textsubscript{1-326} was detected by mouse anti-LEDGF antibody (BD Biosciences, San Diego, CA), which was cross detected by a HRP conjugated anti-mouse secondary antibody (BD Biosciences, San Diego, CA). After washing the plate, 3,3',5,5'-Tetramethylbenzidine (TMB) was added. Immunoreactive LEDGF\textsubscript{1-326} was quantified by colorimetric absorbance at 650 nm upon development of blue color.

**Statistical Analysis**

All data are presented as mean ± SD. Data obtained for fluorescence, CD, and DLS across the pH range (6.0- 7.5) was averaged to compare buffer and additive containing buffer formulations on multiple days. Statistics were done by one-way ANOVA followed by Tukey’s post hoc analysis (SPSS, ver.11.5; SPSS, Chicago, IL). p < 0.05 was considered to be statistically significant.

**Results**

*His-Tag Free LEDGF\textsubscript{1-326} Cloning and Purification*

PCR amplification of LEDGF\textsubscript{1-326} gene resulted in a 1000 bp band of LEDGF\textsubscript{1-326} (Figure 5.1). Restriction digestion, ligation and subsequent PCR amplification from ligated product indicated a positive band of LEDGF\textsubscript{1-326}. LEDGF\textsubscript{1-326} protein was purified as a monomer of 36.7 kDa along with faint lower molecular weight proteins indicating that LEDGF\textsubscript{1-326} might have undergone some degradation during purification.

*Additives Preserved LEDGF\textsubscript{1-326} Native Structure and Size*

Effect of the additives Tween 20, EDTA, and sucrose, on LEDGF\textsubscript{1-326} stability was monitored in citrate-phosphate buffer pH 6.0 - 7.5 over a period of 60 days at 25 °C.
The tertiary structure perturbation in LEDGF₁₋₃₂₆ was monitored by measuring the fluorescence of tryptophan (Trp) in LEDGF₁₋₃₂₆ (Figure 5.2A). LEDGF₁₋₃₂₆ fluorescence intensity at 342 nm was comparable across all the pH values (Figure 5.2A) and the average intensity at the six pH conditions was 5163 ± 302 R.F.U. on day 0 for plain buffer formulations. Fluorescence intensity increased to 6198 ± 102 R.F.U. on day 1 and then decreased significantly to 3518 ± 305 R.F.U. by day 3 and 781 ±181 R.F.U. by day 14. There was ~ 43 and 87 % intensity loss by day 3, and 14, respectively, compared to day 1 intensity. The fluorescence spectra (Figure 5.3, left panel) indicated red shift in the fluorescence maxima on day 3 at all pH conditions. By day 7 and beyond, the fluorescence spectra had no evident peak. Comparing the formulations, with and without additives, there was no significant difference in the LEDGF₁₋₃₂₆ fluorescence signal intensity on day 0 and day 1 (Figure 5.2A). In the presence of additives, LEDGF₁₋₃₂₆ fluorescence was significantly preserved and did not indicate any red shift (Figure 5.3 right panel) until day 60, unlike the plain buffer formulations.
Figure 5.2. Additives Preserved LEDGF<sub>1-326</sub> Structural Integrity.
Figure 5.2. Additives Preserved LEDGF1-326 Structural Integrity. A) Fluorescence intensity- LEDGF1-326 tertiary structure perturbation measured as changes in fluorescence intensity at 342 nm following 280 nm excitation. B) Circular dichroism (CD)- LEDGF1-326 secondary structure perturbation measured as changes in ellipticity at 208 nm. C) Dynamic light scattering- LEDGF1-326 hydrodynamic size was measured using 173° backscattering. Data is expressed as mean ± S.D. of N=3. p<0.05 is considered to be significant as compared to day 1 data.
Figure 5.3. Fluorescence Scans of LEDGF<sub>1-326</sub> Formulations at Different Time Points. Fluorescence scans of LEDGF<sub>1-326</sub> formulations were taken using 280 nm excitation wavelength. Each scan represents average of N=3 batches.
Figure 5.4. CD Scans of LEDGF$_{1-326}$ Formulations at Different Time Points. LEDGF$_{1-326}$ secondray structural changes were monitored by far UV CD scans. Each scan represent average of N=3 batches.
Table 5.2. Fluorescence Intensity of LEDGF\textsubscript{1-326} Formulations without Additives.

<table>
<thead>
<tr>
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<th>LEDGF\textsubscript{1-326}</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Days</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>6.0</td>
<td>5251 ± 75</td>
<td>6028 ± 228</td>
<td>3830 ± 458</td>
<td>1421 ± 508</td>
<td>754 ± 162</td>
<td>647 ± 70</td>
</tr>
<tr>
<td>6.5</td>
<td>5205 ± 46</td>
<td>6284 ± 110</td>
<td>3878 ± 224</td>
<td>1289 ± 57</td>
<td>952 ± 223</td>
<td>942 ± 142</td>
</tr>
<tr>
<td>6.75</td>
<td>4559 ± 767</td>
<td>6310 ± 51</td>
<td>3653 ± 393</td>
<td>1092 ± 448</td>
<td>1048 ± 114</td>
<td>809 ± 248</td>
</tr>
<tr>
<td>7.0</td>
<td>5394 ± 94</td>
<td>6169 ± 304</td>
<td>3254 ± 527</td>
<td>1610 ± 29</td>
<td>661 ± 131</td>
<td>536 ± 26</td>
</tr>
<tr>
<td>7.25</td>
<td>5280 ± 79</td>
<td>6239 ± 80</td>
<td>3190 ± 207</td>
<td>1422 ± 76</td>
<td>699 ± 70</td>
<td>674 ± 97</td>
</tr>
<tr>
<td>7.5</td>
<td>5293 ± 54</td>
<td>6163 ± 59</td>
<td>3307 ± 108</td>
<td>1336 ± 121</td>
<td>575 ± 73</td>
<td>396 ± 84</td>
</tr>
<tr>
<td>Average</td>
<td>5163 ± 302</td>
<td>6198 ± 102</td>
<td>3518 ± 305</td>
<td>1361 ± 171</td>
<td>781 ± 181</td>
<td>667 ± 193</td>
</tr>
</tbody>
</table>

The fluorescence emission intensity of LEDGF\textsubscript{1-326} was measured at 342 nm using 280 nm excitation wavelength. Average fluorescence intensity is the mean fluorescence intensity at any particular time point of all pH together. Data represent mean ± S.D. for N=3 batch.
Table 5.3. Fluorescence Intensity of LEDGF$_{1-326}$ in Formulations with Additives.

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>4849 ± 176</td>
<td>6071 ± 66</td>
<td>6047 ± 96</td>
<td>5973 ± 94</td>
<td>5901 ± 145</td>
<td>5174 ± 1383</td>
<td>6281 ± 64</td>
</tr>
<tr>
<td>6.5</td>
<td>5162 ± 33</td>
<td>6197 ± 12</td>
<td>5997 ± 76</td>
<td>5948 ± 85</td>
<td>6016 ± 88</td>
<td>5984 ± 176</td>
<td>6526 ± 181</td>
</tr>
<tr>
<td>6.75</td>
<td>5257 ± 55</td>
<td>6267 ± 117</td>
<td>5693 ± 375</td>
<td>6081 ± 82</td>
<td>5470 ± 781</td>
<td>5864 ± 108</td>
<td>5142 ± 2165</td>
</tr>
<tr>
<td>7.0</td>
<td>4968 ± 92</td>
<td>6270 ± 65</td>
<td>6052 ± 168</td>
<td>5928 ± 98</td>
<td>6027 ± 41</td>
<td>5894 ± 94</td>
<td>5485 ± 311</td>
</tr>
<tr>
<td>7.25</td>
<td>4963 ± 128</td>
<td>6201 ± 182</td>
<td>5396 ± 1032</td>
<td>5898 ± 95</td>
<td>5666 ± 244</td>
<td>5683 ± 253</td>
<td>5913 ± 77</td>
</tr>
<tr>
<td>7.5</td>
<td>3522 ± 1379</td>
<td>6184 ± 96</td>
<td>6066 ± 105</td>
<td>6124 ± 160</td>
<td>6111 ± 92</td>
<td>5984 ± 144</td>
<td>6316 ± 16</td>
</tr>
<tr>
<td>Average</td>
<td>4794 ± 638</td>
<td>6198 ± 72</td>
<td>5875 ± 273</td>
<td>5992 ± 90</td>
<td>5865 ± 247</td>
<td>5764 ± 309</td>
<td>5944 ± 537</td>
</tr>
</tbody>
</table>

The fluorescence emission intensity of LEDGF$_{1-326}$ was measured at 342 nm using 280 nm excitation wavelength. Average fluorescence intensity is the mean fluorescence intensity at any particular time point of all pH together. Data represent mean ± S.D. for N=3 batch.
Table 5.4. Ellipticity of LEDGF$_{1-326}$ in Formulations without Additives.

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>-17.5 ± 0.2</td>
<td>-16.5 ± 0.7</td>
<td>-14.8 ± 0.6</td>
<td>-5.72 ± 3.3</td>
<td>-1.4 ± 0.5</td>
<td>-2.4 ± 0.7</td>
<td>-4.0 ± 0.7</td>
</tr>
<tr>
<td>6.5</td>
<td>-17.4 ± 0.4</td>
<td>-17.2 ± 0.6</td>
<td>-14.6 ± 0.4</td>
<td>-6.6 ± 0.9</td>
<td>-2.0 ± 0.7</td>
<td>-3.1 ± 0.4</td>
<td>-3.8 ± 0.8</td>
</tr>
<tr>
<td>6.75</td>
<td>-17.6 ± 0.0</td>
<td>-16.5 ± 0.3</td>
<td>-13.2 ± 0.9</td>
<td>-1.3 ± 0.8</td>
<td>-1.3 ± 0.2</td>
<td>-2.1 ± 0.5</td>
<td>-2.9 ± 0.5</td>
</tr>
<tr>
<td>7.0</td>
<td>-17.5 ± 0.3</td>
<td>-16.7 ± 0.2</td>
<td>-14.1 ± 1.6</td>
<td>-2.3 ± 1.4</td>
<td>-1.3 ± 0.4</td>
<td>-1.7 ± 0.3</td>
<td>-2.8 ± 0.6</td>
</tr>
<tr>
<td>7.25</td>
<td>-17.5 ± 0.5</td>
<td>-16.2 ± 0.6</td>
<td>-13.0 ± 0.9</td>
<td>-3.2 ± 1.2</td>
<td>-1.6 ± 0.2</td>
<td>-2.0 ± 0.2</td>
<td>-3.1 ± 0.1</td>
</tr>
<tr>
<td>7.5</td>
<td>-17.2 ± 0.3</td>
<td>-16.6 ± 0.5</td>
<td>-13.3 ± 0.1</td>
<td>-1.4 ± 0.6</td>
<td>-0.9 ± 0.3</td>
<td>-1.7 ± 0.1</td>
<td>-1.8 ± 0.2</td>
</tr>
<tr>
<td>Average</td>
<td>-17.4 ± 0.1</td>
<td>-16.6 ± 0.3</td>
<td>-13.8 ± 0.8</td>
<td>-3.4 ± 2.3</td>
<td>-1.4 ± 0.3</td>
<td>-2.2 ± 0.5</td>
<td>-3.1 ± 0.8</td>
</tr>
</tbody>
</table>

The ellipticity of LEDGF$_{1-326}$ was measured using circular dichorism at 208 nm. Average ellipticity is the mean ellipticity at any particular time point of all pH together. Data represent mean ± S.D. for N=3 batch.
Table 5.5. Ellipticity of LEDGF<sub>1-326</sub> in Formulations with Additives.

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>-18.3 ± 0.1</td>
<td>-17.3 ± 0.4</td>
<td>-17.8 ± 0.6</td>
<td>-17.3 ± 0.4</td>
<td>-18.3 ± 0.3</td>
<td>-18.1 ± 0.6</td>
<td>-18.7 ± 0.3</td>
</tr>
<tr>
<td>6.5</td>
<td>-17.9 ± 0.4</td>
<td>-17.5 ± 0.3</td>
<td>-17.7 ± 0.7</td>
<td>-17.2 ± 0.6</td>
<td>-17.9 ± 0.2</td>
<td>-18.0 ± 0.7</td>
<td>-21.0 ± 1.5</td>
</tr>
<tr>
<td>6.75</td>
<td>-17.7 ± 0.1</td>
<td>-17.0 ± 0.9</td>
<td>-17.4 ± 0.6</td>
<td>-16.7 ± 0.3</td>
<td>-17.3 ± 0.9</td>
<td>-17.6 ± 0.6</td>
<td>-16.8 ± 6.5</td>
</tr>
<tr>
<td>7.0</td>
<td>-18.3 ± 0.4</td>
<td>-14.8 ± 0.2</td>
<td>-17.1 ± 0.4</td>
<td>-16.9 ± 0.9</td>
<td>-17.6 ± 0.3</td>
<td>-17.8 ± 0.2</td>
<td>-18.8 ± 0.6</td>
</tr>
<tr>
<td>7.25</td>
<td>-17.6 ± 0.3</td>
<td>-15.8 ± 0.3</td>
<td>-16.9 ± 0.3</td>
<td>-17.0 ± 0.3</td>
<td>-17.4 ± 0.7</td>
<td>-17.9 ± 0.5</td>
<td>-20.2 ± 0.1</td>
</tr>
<tr>
<td>7.5</td>
<td>-17.9 ± 0.6</td>
<td>-16.1 ± 0.4</td>
<td>-17.0 ± 0.3</td>
<td>-16.7 ± 0.1</td>
<td>-17.7 ± 0.5</td>
<td>-17.4 ± 0.1</td>
<td>-19.0 ± 0.1</td>
</tr>
<tr>
<td>Average</td>
<td>-17.7 ± 0.3</td>
<td>-16.4 ± 1.0</td>
<td>-17.3 ± 0.4</td>
<td>-17.0 ± 0.2</td>
<td>-17.6 ± 0.4</td>
<td>-17.8 ± 0.3</td>
<td>-19.1 ± 1.4</td>
</tr>
</tbody>
</table>

The ellipticity of LEDGF<sub>1-326</sub> was measured using circular dichroism at 208 nm. Average ellipticity is the mean ellipticity at any particular time point of all pH together. Data represent mean ± S.D. for N=3 batch.
Table 5.6. Hydrodynamic Size LEDGF\textsubscript{1-326} in Formulations without Additives.

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>6.3 ± 0.1</td>
<td>4.9 ± 1.0</td>
<td>248.8 ± 204.6</td>
<td>609.4 ± 71.2</td>
<td>673.2 ± 40.0</td>
<td>398.2 ± 317.5</td>
<td>60.6 ± 35.0</td>
</tr>
<tr>
<td>6.5</td>
<td>6.2 ± 0.9</td>
<td>6.1 ± 1.0</td>
<td>509.8 ± 23.7</td>
<td>637.9 ± 59.2</td>
<td>605.0 ± 19.9</td>
<td>584.4 ± 54.6</td>
<td>52.3 ± 36.2</td>
</tr>
<tr>
<td>6.75</td>
<td>5.6 ± 0.6</td>
<td>6.4 ± 0.4</td>
<td>665.8 ± 91.2</td>
<td>520.7 ± 265.5</td>
<td>732.7 ± 103.8</td>
<td>530.8 ± 428.7</td>
<td>481.6 ± 276.0</td>
</tr>
<tr>
<td>7</td>
<td>6.7 ± 0.2</td>
<td>5.1 ± 2.1</td>
<td>674.7 ± 77.1</td>
<td>748.0 ± 104.5</td>
<td>54.8 ± 9.5</td>
<td>108.7 ± 59.8</td>
<td>569.8 ± 424.4</td>
</tr>
<tr>
<td>7.25</td>
<td>6.0 ± 1.1</td>
<td>5.1 ± 2.5</td>
<td>689.0 ± 10.6</td>
<td>654.4 ± 63.9</td>
<td>54.1 ± 8.3</td>
<td>62.5 ± 4.4</td>
<td>144.6 ± 144.2</td>
</tr>
<tr>
<td>7.5</td>
<td>6.4 ± 0.4</td>
<td>6.4 ± 0.3</td>
<td>722.8 ± 75.1</td>
<td>734.0 ± 58.9</td>
<td>683.0 ± 47.4</td>
<td>66.7 ± 22.5</td>
<td>308.1 ± 350.7</td>
</tr>
<tr>
<td>Average</td>
<td>-17.4 ± 0.1</td>
<td>6.2 ± 0.4</td>
<td>5.7 ± 0.7</td>
<td>585.1 ± 180.6</td>
<td>650.7 ± 83.9</td>
<td>291.9 ± 241.2</td>
<td>269.5 ± 220.5</td>
</tr>
</tbody>
</table>

The hydrodynamic size of LEDGF\textsubscript{1-326} was measured using dynamic light scattering. Average is the mean size at any particular time point of all pH together. Data represent mean ± S.D. for N=3 batch.
Table 5.7. Hydrodynamic Size LEDGF\textsubscript{1-326} in Formulations with Additives.

<table>
<thead>
<tr>
<th>pH</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>6.5</td>
<td>1.2 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>6.75</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>23.2 ± 38.5</td>
</tr>
<tr>
<td>7</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>7.25</td>
<td>1.2 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>7.5</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Average</td>
<td>1.2 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.0</td>
<td>4.8 ± 9.0</td>
</tr>
</tbody>
</table>

The hydrodynamic size of LEDGF\textsubscript{1-326} was measured using dynamic light scattering. Average is the mean size at any particular time point of all pH together. Data represent mean ± S.D. for N=3 batch.
LEDGF$_{1-326}$ secondary structure perturbation was monitored by far-UV CD. Figure 5.2B shows ellipticity of LEDGF$_{1-326}$ at 208 nm for pH 6.0 to 7.5 formulations as a function of time. CD spectra indicated that LEDGF$_{1-326}$ is primarily a random coiled protein (Figure 5.4). LEDGF$_{1-326}$ had an average ellipticity of -17.5 ± 0.1 mdeg at 208 nm on day 0 on average when all pH values were considered together. This value reduced significantly to -13.9 ± 0.8 and -3.4 ± 2.3 mdeg by day 3 and day 7, respectively in plain buffer formulations (Figure 5.2B). LEDGF$_{1-326}$ ellipticity was comparable on day 0 in groups with or without additives (Figure 5.2B). Unlike plain buffer formulations, additive containing formulations preserved LEDGF$_{1-326}$ ellipticity up to day 60.

The average hydrodynamic (particle) size of LEDGF$_{1-326}$ for the entire pH range was 7 ± 1 nm on day 0 in plain buffer formulations (Figure 5.2C) as indicated by DLS. By day 3, LEDGF$_{1-326}$ particle size increased to 200-700 nm in plain buffer formulations. In the presence of additives, LEDGF$_{1-326}$ had a size of ~1 nm on day 0 and no change in size was observed up to day 60 at all pH values (Figure 5.2C).

**Additives Reduced LEDGF$_{1-326}$ Fragmentation**

SDS-PAGE indicated LEDGF$_{1-326}$ was a 36.7 kDa protein (Figure 5.5). On day 0 there were faint bands of small molecular weight fragments in all formulations. In plain buffer formulations, the lower molecular weight fragments intensified as early as day 1. By day 7, the monomeric LEDGF$_{1-326}$ bands completely disappeared above pH 6. Additives delayed the intensification of lower molecular weight bands until day 60. On day 60, significant amount of LEDGF$_{1-326}$ monomeric bands were visible in additive containing formulation, although with intensified lower molecular weight band.
**Figure 5.5. Additives Reduced LEDGF<sub>1-326</sub> Fragmentation.** Equal amount of sample (10 μl) of sample was loaded in each well. SDS-PAGE (reducing) indicated LEDGF<sub>1-326</sub> monomer to be a 36.7 kDa protein. In the absence of additives, LEDGF<sub>1-326</sub> possibly underwent chemical degradation and fragmentation. Representative image from N=3 batches.

LEDGF<sub>1-326</sub> (10 μg)

Additives= 0.1 % Tween 20 + 1 mM EDTA + 10 % Sucrose
Additives Reduced LEDGF<sub>1-326</sub> Aggregate Formation

The mean soluble LEDGF<sub>1-326</sub> protein content on day 0 in plain buffer formulation pH 6.0 - 7.5 was 417 ± 21 µg/ml which decreased to 142 ± 60 µg/ml by day 7 (Figure 5.6A). Thereafter, there was high variation in the protein content in plain buffer formulations depending on pH. However, there was no clear trend. By day 60, the total soluble protein content ranged from 172 to 456 µg/ml in plain buffer formulations. On the other hand, soluble LEDGF<sub>1-326</sub> protein content was more consistent in the presence of additives and was in range of 440- 470 µg/ml on day 60.

Percentage of aggregates was calculated from the soluble protein content (Figure 5.6B). Insoluble aggregates of LEDGF<sub>1-326</sub> were formed as early as day 3 in plain buffer formulations and by day 7, on an average for all pH conditions, 64.6 ± 14.0 % of LEDGF<sub>1-326</sub> aggregated (Figure 5.6B). LEDGF<sub>1-326</sub> aggregation was highly variable across the pH range beyond day 7. In the presence of additives, LEDGF<sub>1-326</sub> aggregates remained below the quantification limit until day 60. An exception was seen on day 30 when the aggregation was on an average for all pH values was 22.3 ± 9 %. (Figure 5.6B). Visible particles were seen in plain buffer formulations, while the additive containing formulations were clear until day 60 (Figure 5.7).

Additives Preserved LEDGF<sub>1-326</sub> Immunoreactivity

LEDGF<sub>1-326</sub> immunoreactivity was quantified using an indirect ELISA (Figure 5.8). On day 0, mean LEDGF<sub>1-326</sub> immunoreactivity was 76.9 ± 4.8 % in plain buffer formulations at pH 6.0 - 7.5 (Figure 5.8). On day 14, when ELISA was repeated, the mean LEDGF<sub>1-326</sub> average immunoreactivity for all pH values was 3.1 ± 2.4 %. By day 60, immunoreactive LEDGF<sub>1-326</sub> was undetectable. On the other hand, in the presence of
Figure 5.6. Additives Reduced Aggregate Formation. For soluble protein content, formulation was centrifuged at 10,000 g for 5 mins and supernatant protein estimation was done using BCA assay. A) Soluble LEDGF$_{1-326}$ protein. B) LEDGF$_{1-326}$ aggregates determined indirectly from soluble protein content. Data is expressed as mean ± S.D. for N=3 batches. p<0.05 indicates statistically significant differences as compared to day 1 data.
Figure 5.7. Additives Reduced LEDGF<sub>1-326</sub> Precipitation. On Day 30, LEDGF<sub>1-326</sub> formulation images were taken using a standard camera. Representative images from 3 batches.
Figure 5.8. Additives Preserved LEDGF\textsubscript{1-326} Immunoreactivity. LEDGF\textsubscript{1-326} immunoreactivity against anti-LEDGF antibody was tested on day 0, 14, and 60 using ELISA. Data represents means ± S.D. for N=3 batches. No immunoreactive LEDGF\textsubscript{1-326} was detected in LEDGF\textsubscript{1-326} formulations without additives (left panel) at all pH values on day 60. * indicates p<0.05 as compared to corresponding pH data on day 0, 14, or 60.

Individual Additives are Less Effective as Compared to Combination

To understand the effect of individual additives on LEDGF\textsubscript{1-326} stability, 1 mg/ml LEDGF\textsubscript{1-326} formulations were prepared with individual additives at pH 7.0 (Figure 5.9 and 5.10). LEDGF\textsubscript{1-326} fluorescence intensity at 342 nm decreased significantly from 8410 ± 116 to 2178 ± 22 R.F.U in plain citrate-phosphate buffer formulation by day 30.
Figure 5.9. Individual Additives Could Not Prevent LEDGF<sub>1-326</sub> Structural Integrity. A and B) Fluorescence intensity of LEDGF<sub>1-326</sub> at an emission wavelength of 342 nm following excitation at 280 nm. C and D) Circular dichroism (CD) - Ellipticity of LEDGF<sub>1-326</sub> at 208 nm. Ellipticity for day 30 could not be plotted (Figure C) due to significant noise. E and F) Hydrodynamic size of LEDGF as measured by dynamic light scattering. Data represent mean ± SD for N=3 batches. *, p<0.05 indicates significant difference as compared to day 0 data.
LEDGF<sub>1-326</sub> fluorescence intensity in the presence of 0.02 % azide, 0.1 % Tween 20, 1 mM EDTA, and 10 % sucrose was not significantly different from the plain citrate-phosphate buffer formulation on day 0. On day 30, the fluorescence intensity was 9136 ± 241, 4925 ± 1249, 4056 ± 979, and 6370 ± 592, R.F.U. for 0.02 % azide, 0.1 % Tween 20, 1 mM EDTA, and 10 % sucrose, respectively, indicating some loss of fluorescence as compared to day 0 but it was significantly higher as compared to plain buffer formulation on day 60. LEDGF<sub>1-326</sub> fluorescence was 25, 59, 48, and 76 % on day 30 in plain buffer, Tween 20, EDTA, and sucrose formulation, respectively. On the other hand, combination of additives preserved the fluorescence signal till day 30 (Figure 5.9B). LEDGF<sub>1-326</sub> had no significant changes in the CD signal until day 14 for all formulations; however, on day 30, there was significant background noise at 208 nm and the data was unreliable for day 30 (Figure 5.9C). The combination of additives significantly preserved the CD signal until day 30 (Figure 5.9D).

LEDGF<sub>1-326</sub> had a mean hydrodynamic size of 7 ± 1 nm on day 0 in all formulations except sucrose containing formulation (Figure 5.9E). In the presence of sucrose, LEDGF<sub>1-326</sub> size decreased to 1 nm. By day 30, LEDGF<sub>1-326</sub> particle size increased significantly to 578 ± 366, 726 ± 444, 490 ± 423, and 1052 ± 125 nm, in plain buffer, and Tween 20, EDTA, and sucrose containing formulations, respectively. For sodium azide group, instead of increase in size, there was a decrease in size from 7 to 4 nm.

LEDGF<sub>1-326</sub> monomer band intensity in SDS-PAGE was comparable in all formulations from day 0 to day 14 (Figure 5.10) except for the blank group. By day 7,
Figure 5.10. Individual Additives Could Not Prevent LEDGF<sub>1-326</sub> Fragmentation. The monomer band of LEDGF<sub>1-326</sub> reduced over a period of 14 days due to LEDGF<sub>1-326</sub> degradation and fragmentation as indicated by reducing SDS-PAGE. Representative image from N=3.

there was thinning of 36.7 kDa band indicating loss of LEDGF<sub>1-326</sub> monomers. On day 14, appearance of lower molecular weight bands in all individual additive groups were more pronounced as compared to day 0. Combination of additives had less fragmentation on day 14. The plain buffer formulation had the lowest amount of 36.7 kDa protein band on day 14.
Discussion

In the absence of 3-D structure of LEDGF\textsubscript{1-326}, it is very difficult to model/predict domains that are highly susceptible to degradation. However, based on its amino acid back bone, LEDGF\textsubscript{1-326} contains 54 lysine residues (15.5 \%) susceptible to glycation, 20 aspartate residue (6.1 \%) susceptible to isomerization, 12 asparagine amino acids (3.7 \%) that may undergo deamidation and peptide bond hydrolysis, and 5 methionines (1.5 \%), 1 cysteine residue (0.3 \%), 5 histidines (1.5 \%), 2 tryptophans (0.6 \%), and 3 tyrosines (0.9 \%) susceptible to oxidation. His-LEDGF\textsubscript{1-326}, being a random coiled structure with low melting temperature and low free energy of unfolding (Baid et al., 2013) may easily be unfolded with changes in buffer pH and other excipients including surfactants, stabilizers and temperature (Figure 5.11). The partially or fully unfolded LEDGF\textsubscript{1-326} may expose the hydrophobic amino acids to surrounding environment, making it more susceptible to chemical degradation leading to fragmentation. Once degraded, fragmented LEDGF\textsubscript{1-326} may aggregate to form subvisible or visible particles leading to LEDGF\textsubscript{1-326} precipitation. On the other hand, unfolded LEDGF\textsubscript{1-326} may also undergo physical aggregation, forming high molecular weight aggregates. Thus, unfolding of LEDGF\textsubscript{1-326} could be a precursor of LEDGF\textsubscript{1-326} degradation. Based on the above hypothesis, we carefully designed a formulation of LEDGF\textsubscript{1-326} that retained > 90 \% monomeric form for 60 days at 25 °C.

A pH range of 6.0 - 7.5 was selected as it falls under the normal physiological pH (7.0 - 7.4) of vitreous humor (Foster, 2008). Since the theoretical pI of LEDGF\textsubscript{1-326} was 9.23, LEDGF\textsubscript{1-326} remains in complete ionic form in this pH range with minimal chances of precipitation. To maintain stable pH, citrate-phosphate buffer was chosen. Citrate buffers are known to reduce oxidative degradation by (Avanti et al., 2011). However,
since the pH range of citrate buffer is 3.0 - 6.2 and that of phosphate buffer is 6-8, citrate-phosphate buffer was made so as to increase the buffering range from 6.2 to 8.0. A low ionic (~130 mM) strength was selected (Table 5.1) to minimize LEDGF<sub>1-326</sub> salting out effect (Simpson, 2010). Salting out of proteins vary from proteins to proteins, it also depends upon the pH of the buffer. For example, fibrinogen is salted out at 0.8 M ammonium sulphate (Berg JM, 2002). Typically the buffer concentration of 20-400 mM is used for protein formulations (Simpson, 2010). Tween 20 was used to reduce surface adsorption and unfolding of LEDGF<sub>1-326</sub> (Webb et al., 2002). EDTA was used to reduce metal ion catalyzed oxidation of proteins (Fransson and Hagman, 1996). Sucrose was used to decrease unfolding of LEGF<sub>1-326</sub> and thus reduce subsequent chemical
degradation. Since the formulation was made within the range of pH 6.0 - 7.5, sucrose is stable in this pH range and does not form reducing sugars (Banks et al., 2009); since glycation occurs at pH < 4, there were minimal chances of glycation of the lysine residues in LEDGF<sub>1-326</sub>.

Loss in LEDGF<sub>1-326</sub> fluorescence signal within 3 days indicated loss of soluble protein. The red shift in fluorescence maxima indicated unfolding of LEDGF<sub>1-326</sub>, while loss in ellipticity in CD indicated secondary structural perturbation (Figures 5.2A and 5.2B). Additives significantly (~100%) preserved LEDGF<sub>1-326</sub> tertiary and secondary structure until day 60 (Figures 5.2A and 5.2B). These results demonstrate that LEDGF<sub>1-326</sub> is primarily degraded due to unfolding, and exposure of its hydrophobic residues to surrounding water environment.

Following unfolding, LEDGF<sub>1-326</sub> might have undergone aggregation, leading to appearance of subvisible particles in plain buffer formulations by day 3 (Figure 5.2C). LEDGF<sub>1-326</sub> particle size did not increase in additive containing formulations until day 60 (Figure 5.2C). LEDGF<sub>1-326</sub> hydrodynamic size was interestingly 1 nm as opposed to 7 nm in the presence of additives. It is known that sucrose is prefentially excluded from the protein surface causing hydration of the protein molecule and shifting the thermodynamic equilibrium towards the more compact conformation; however, sucrose is not known to change the secondary sturtures of a protein (Kendrick et al., 1997). LEDGF<sub>1-326</sub> is primarily a random coiled protein; there is a possibility that it might have been compacted by sucrose without any changes in the secondary structure content.

Additives not only increased LEDGF<sub>1-326</sub> physical and chemical stability, they also maintained LEDGF<sub>1-326</sub> immunoreactivity (Figure 5.8). The ELISA method
developed in this current study demonstrated immunoreactivity of LEDGF<sub>1-326</sub> towards anti-LEDGF antibody that detects LEDGF<sub>1-326</sub> domain aa85-188, which is just 23 amino acid away from the PWWP domain (aa5 to aa62) and contains nuclear localization signal (aa148 to aa156), an important domain for LEDGF<sub>1-326</sub> activity (Singh et al., 2006). Thus, ELISA is based on active segments of LEDGF<sub>1</sub>.

We wanted to investigate if individual excipient alone can stabilize LEDGF<sub>1-326</sub>. This would have allowed us to remove other additives and reduce the burden of complex formulation. Although sucrose was most effective in reducing LEDGF<sub>1-326</sub> structural loss (Figure 5.9a), it was not as effective as the combination of additives in reducing LEDGF<sub>1</sub><sub>326</sub> instability (Figure 5.9 and 5.10).

In the presence of sodium azide, LEDGF<sub>1-326</sub> maintained the fluorescence signal and ellipticity (Figure 5.9A and 5.9B) but indicated loss in size (Figure 5.9C) and fragmentation (Figure 5.10). Azide was used in the formulation to investigate the possibility of LEDGF<sub>1-326</sub> degradation due to bacterial contamination; however, azide is known to react with carbonyl group to form amines in the presence of acid. Thus, there is a possibly that sodium azide binds to LEDGF<sub>1-326</sub> protein, leading to its chemical degradation.

LEDGF<sub>1-326</sub> degradation was also dependent on LEDGF<sub>1-326</sub> concentration. LEDGF<sub>1-326</sub> underwent physical and chemical degradation much faster in 0.5 mg/ml formulation as compared to 1 mg/ml LEDGF<sub>1-326</sub> formulation (day 3 vs day 30) (Figures 5.9 and 5.10). Proteins are indicated to be less stable at concentration < 50 µg/ml and tend to aggregate due to surface adsorption (Simpson, 2010). Therefore, proteins are typically stored at high concentration >1 mg/ml. Our data also demonstrated that
LEDGF$_{1-326}$ was less stable at 0.5 mg/ml as compared to 1 mg/ml possibly due to surface adsorption.

Because of low melting temperature of LEDGF$_{1-326}$, accelerated studies at higher temperatures (> 40 °C) to predict the shelf life at low temperatures was not feasible for LEDGF$_{1-326}$. Thus, using the $Q_{10}$ approach, first introduced by Simonelli & Dresback, an estimation of shelf life was done for 4 °C (Simonelli AP, 1972). According to this approach, $Q_{10}$ is the factor by which the reaction rate constant increases for a 10 °C temperature increase. The equation to calculate the shelf life is

$$t_{90}(T_2) = \frac{t_{90}(T_1)}{(\Delta T/10)^{Q_{10}}}$$

where $t_{90}(T_2)$ is the estimated shelf life at $T_2$ °C, $t_{90}(T_1)$ is the shelf life at given temp (T$_1$), and $\Delta T$ is the difference in the temperature T$_1$ and T$_2$. The $Q_{10}$ value was estimated to be 3 (Simonelli AP, 1972). The shelf life of LEDGF$_{1-326}$ additive containing formulation at 4 °C was calculated to be 19.8 months from above equation.

**Conclusions**

The data in this study demonstrated that LEDGF$_{1-326}$ protein was susceptible to conformational instability and loss of immunoreactivity upon storage. In aqueous formulation, combination of Tween 20, EDTA and sucrose not only provided structural stability but also prevented fragmentation and retained LEDGF$_{1-326}$ immunoreactivity for 2 months. Although no significant differences were found in the physical and chemical stability of LEDGF$_{1-326}$ in pH range of 6-7.5, LEDGF$_{1-326}$ immunoreactivity was significantly higher in the pH range of 6.5-7.25 as compared to pH 6 and 7.5 in the presence of additives.
CHAPTER VI

LEDGF<sub>1-326</sub> NANOASSEMBLIES ENHANCE CELLULAR UPTAKE AND IN VIVO PERSISTENCE AND EFFICACY

Abstract

Purpose

To design a stable self-assembled nano delivery system of LEDGF<sub>1-326</sub> to enhance its cellular uptake, in vivo persistence, and efficacy.

Methods

LEDGF<sub>1-326</sub> nanoassemblies were formed by Zn(II) addition and characterized using dynamic light scattering, intrinsic tryptophan fluorescence, far-UV circular dichroism, and UV-Vis spectroscopy. Alexa-LEDGF<sub>1-326</sub> nanoassembly uptake in retinal cells was determined by fluorometry. Persistence and ocular tissue distribution of intravitreally injected Alexa-LEDGF<sub>1-326</sub> nanoassemblies was studied using noninvasive fluorophotometry followed by ELISA in Sprague Dawley (SD) rats. The ability of intravitreally injected LEDGF<sub>1-326</sub> nanoassemblies to protect photoreceptor function was assessed by electoretinography (ERG) in Royal College of Surgeon (RCS) rats.

Results

Zn(II) induced secondary and tertiary structural changes in LEDGF<sub>1-326</sub> that were reversible in the presence of EDTA. LEDGF<sub>1-326</sub> self-assembled in the presence of Zn(II) in a dose dependent manner. At 10 mM Zn(II), LEDGF<sub>1-326</sub> nanoassemblies had a diameter of 28 ± 5 nm. Nanoassemblies delayed LEDGF<sub>1-326</sub> structural changes and the
initiation of fragmentation from 3 to 14 days at 25 °C. LEDGF<sub>1-326</sub> nanoassembly uptake was 2.5 times that of control LEDGF<sub>1-326</sub> in retinal cells. After one single intravitreal injection, the mean residence time was 22-296 fold higher for nanoassemblies, with the values being 6.4, 3.7, and 5.7 days for nanoassembly and 0.3, 4.1, and 6.5 hrs for control LEDGF<sub>1-326</sub> in aqueous humor, vitreous humor, and choroid-retina, respectively of SD rats. ELISA based activity of nanoassemblies was 6.5-fold higher as compared to LEDGF<sub>1-326</sub> in the retina on day 14. RCS rats treated with nanoassemblies had higher b-wave amplitude in scotopic ERG and photopic ERG compared to LEDGF<sub>1-326</sub> at 10 weeks after intravitreal injection.

**Conclusion**

Zn(II) induced structural changes in LEDGF<sub>1-326</sub> allowing formation of nano sized self-assembly. Nanoassemblies increased cellular uptake, and enhanced *in vivo* persistence and efficacy of LEDGF<sub>1-326</sub>.

**Introduction**

We previously identified lens epithelium derived growth factor fragment (LEDGF<sub>1-326</sub>) as a potential therapeutic agent for treatment of retinal degeneration including dry AMD and retinitis pigmentosa. Retinal pigmented epithelial (RPE) cell that suffered protein aggregation stress due to mutant P23H rhodopsin expression were rescued by LEDGF<sub>1-326</sub> (Baid et al., 2011a). LEDGF<sub>1-326</sub> also reduced P23H/WT rhodopsin aggregates in RPE cells. Intravitreal injection of LEDGF<sub>1-326</sub> protein was seen to preserve the b-wave amplitude of rod and cone photoreceptors in retinal degenerative Royal College of Surgeon (RCS) rats (Baid et al., 2013). LEDGF<sub>1-326</sub> also improved the morphology of photoreceptor in RCS rats as compared to untreated group.
The unique anatomy and physiology of the eye impedes drug delivery, especially delivery of protein drugs, to the back of the eye disorders including retinal degenerative diseases (Kompella et al., 2010a). Topical route of administration is inefficient in delivering drugs to the back of the eye because of the presence of various static (cornea, conjunctiva, and lens), and dynamic (tear drainage, vascular clearance in several tissues including conjunctiva, episclera, and choroid, lymphatic clearance, aqueous fluid circulation, and vitreous fluid circulation) barriers (Gaudana et al., 2010; Thrimawithana et al., 2011). On the other hand, requirement of high doses, systemic side effects along with systemic degradation and delivery of low concentrations at target site due to blood-tissue barriers are major challenges for the intravenous route (Baid et al., 2011b). Local delivery, such as an intravitreal injection, places the drug in the proximity of the retina (the target tissue for retinal diseases) and provides effective drug levels in the retina, especially for permeable molecules and those that act in the extracellular space. Macromolecules such as LEDGF_{1-326} are most suited for intravitreal injections as their half-life in vitreous is relatively longer than small molecule drugs. Vitreous half-life of a small molecule such as diclofenac is 2.9 hr (Durairaj et al., 2009), while bevacizumab, a 150 kDa macromolecule, has a vitreous half-life of 4.32 days (Bakri et al., 2007b) in rabbits. Although bevacizumab’s longer half-life allows dosing once a month, monthly intravitreal injections may lead to various complications such as retinal detachment, retinal and vitreous hemorrhage, endophthalmitis, increased intraocular pressure, floaters, transient blurry vision, retinal tears, patient non-compliance and infections (Jager et al., 2004; Peyman et al., 2009; Wu and Chen, 2009). Several studies indicated the inability of
proteins to effectively penetrate cells due to poor plasma membrane permeability (Schmidt-Erfurth et al., 1998; Gros et al., 2006). While bevacizumab acts by sequestering extracellular VEGF (Presta et al., 1997; Ferrara et al., 2004), activity of LEDGF$_{1-326}$ requires cellular entry since it is a transcriptional factor (Singh et al., 2000). Thus, there is a need for a LEDGF$_{1-326}$ delivery system that can enhance cellular uptake and prolong protein residence in the eye.

Biodegradable micro- or nanoparticles can potentially enhance and sustain drug delivery for weeks to months (Shelke et al., 2011). However, the traditional methods of preparation of these particles involve use of organic solvents such as dichloromethane, which can denature proteins (Estey et al., 2006). Incomplete and erratic release, immunogenicity, and loss of efficacy are few consequences of protein denaturation by organic solvents or polymer matrix. Further low encapsulation efficiency and challenges in particle sterilization without damaging the protein are other hurdles associated with the preparation of conventional polymeric slow release systems. Therefore, the objective of this study was to develop non-polymeric systems for enhanced and sustained delivery of LEDGF$_{1-326}$.

We hypothesized that metal ion addition to LEDGF$_{1-326}$ can form nanoassemblies which enhance and sustain LEDGF$_{1-326}$ delivery. Metal ions are integral to several proteins and control folding and stability of proteins and hence their physiological functions (Zhou et al., 2005; Andreini et al., 2006). Approximately one-third of structurally characterized proteins contain metals, and it is estimated that about the same proportion of all proteins are metalloproteins. Many transcription factors are bound to
zinc (Andreini et al., 2006; Ebert and Altman, 2008). Among the metals that bind to proteins naturally, zinc is the most commonly found metal. Zinc does not undergo oxidation/reduction reactions as its d-shell is filled and thus provides stability to proteins (Vallee and Auld, 1990). It allows proteins to modify their binding site affinity and function due to its ability to take different coordination numbers and forms several types of ligating residues. Previously, a subcutaneous sustained delivery system of BMS-686117, a GLP-1 receptor peptide (11 amino acid) agonist, was prepared using zinc (Qian et al., 2009). The zinc modified BMS-686117 had prolonged terminal $t_1/2$ of 8.5 hr, a mean residence time (MRT) of 16 hr, and a $C_{max}$ value 6–8 times lower than the drug solution. Zinc was also used previously to form stable suspensions of insulin (51 kDa) in the form of hexamers, in order to sustain insulin monomer release over short durations (Heinemann et al., 2000). Zinc is part of age-related eye disease study (AREDS) formulation, as a dietary supplement for dry AMD (2001; Jiang et al., 2007). Despite zinc being known as therapeutically effective and efficient to form coordinate bonds with proteins, it has never been explored as a delivery platform for increasing protein cellular uptake and persistence in eye, especially for several days.

In this study, we determined the effect of Zn(II) on LEDGF$_{1-326}$ structure formulated Zn(II) induced nano-sized self-assemblies of LEDGF$_{1-326}$ and investigated their cellular uptake. Further, we tested the ability of nanoassemblies to prolong LEDGF$_{1-326}$ delivery and enhance its activity in delaying retinal degeneration in RCS rats.

**Abbreviations**

Zn(II)- zinc divalent ion

Nano (xx) assembly- LEDGF$_{1-326}$ nanoassembly prepared with xx mM Zn(II)
Materials and Methods

ARPE-19 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cell culture reagents and Alexa Fluor® 488 dye were obtained from Invitrogen Corporation (Carlsbad, CA). Chromic acid, HCl, and NaOH were obtained from Fisher Scientific (Pittsburgh, PA). Tris base, zinc chloride, and ethylene di-amine-terta-acetic acid (EDTA) were obtained from Sigma-Aldrich (St. Louis, MO). LEDGF<sub>1-326</sub> was synthesized and purified as per the methods described previously (Baid et al., 2013).

Nanoassembly Preparation

Lyophilized LEDGF<sub>1-326</sub> was dialyzed overnight in pH 7.4 Tris buffer (25 mM Tris-HCl and 100 mM NaCl) at 4 ºC. Zn(II) stock solution (100 mM) was prepared by dissolving zinc chloride in Tris buffer. For nanoassembly preparation, Zn(II) solution was diluted to a final concentration of 0.1, 1, or 10 mM using Tris buffer containing LEDGF<sub>1-326</sub> (final concentration 1 mg/ml) and incubated at 37 ºC for 24 hr to form nano[0.1], nano[1], and nano[10] assemblies, respectively (Table 6.1). LEDGF<sub>1-326</sub> (1 mg/ml) with no Zn(II) under similar conditions in Tris buffer served as a control. All solutions were filtered with 0.2 µm filter before nanoassembly preparation.

Dynamic Light Scattering (DLS)

Nanoassembly size distribution and homogeneity was measured using zeta sizer Nano ZS (Malvern, Westborough, MA) and DLS technique. Briefly, protein sample was placed in 150 µl quartz cuvette and data was collected at 173º backscatter angle with eleven scans averaged for final size distribution plot. The time dependent changes in size and stability of nanoassemblies were monitored by measuring the number size distribution profile at different time points.
Transmission Electron Microscopy

An aliquot of LEDGF$_{1-326}$ control or nano[10] assembly was coated on 400 mesh copper grids with carbon coated formvar support for 3 min. The sample was negatively stained with 2 % w/v uranyl acetate solution for 1 min and washed once with filtered MilliQ water. The grid was blotted dry and the coated sample was analyzed on Philips Technei TEM operating at 80 kV.

Fluorescence Spectroscopy

Changes in the tertiary structure of LEDGF$_{1-326}$ were determined by measuring steady state intrinsic fluorescence of tryptophan. Briefly, 150 µl of protein sample (1 mg/ml) was placed in a quartz cuvette and emission spectra were recorded from in 1 nm increments from 300 to 400 nm, following excitation at 280 nm, using Spectramax M5 (Molecular Devices, Downingtown, PA). Six scans per data point were averaged.

Circular Dichroism (CD)

Far-UV CD spectra were recorded to determine the secondary structural changes in LEDGF$_{1-326}$. Briefly, 150µl of the protein sample (1 mg/ml) was placed in 1 mm quartz cuvette and the CD spectrum was recorded at a step size of 1 nm and the bandwidth of 4 nm from 200 to 280 nm using Chirascan® CD instrument and at a scan speed of 0.5 secs per data point (Applied Photophysics Ltd, UK). Buffer background was subtracted from all scans. All scans were done in triplicate. To determine the secondary structure percentage, CDNN 2.1 software (Dr. Gerald Bohm, Martin-Luther-University at Halle, Wittenberg, Germany, UK) was used to deconvolute the CD spectrum.
**Chelation**

For investigating whether nanoassemblies formed are reversible in their structural and size properties, effect of EDTA on nanoassembly properties was investigated. Briefly, LEDGF\(_{1-326}\) nano[10] assembly was prepared as stated above. A 500 mM stock solution of EDTA was made in Tris buffer. EDTA solution was added to final concentration of 50 mM to LEDGF\(_{1-326}\) control and nano[10] assembly. The formulations were incubated for 4 hr at room temperature. Thereafter, DLS, fluorescence, CD, and UV-vis measurements were done as described above.

**Alexa Fluor 488® Conjugation**

Lyophilized LEDGF\(_{1-326}\) stored at -80°C was solubilized and dialyzed (5,000 Da cutoffs) overnight using freshly made 100 mM NaHCO\(_3\) buffer, pH 8.4, with three buffer exchanges. Absorbance of the dialyzed protein was accurately measured using BCA assay (Thermo Fisher Scientific, Rockford, IL) as per user’s manual. LEDGF\(_{1-326}\) and Alexa Fluor® 488 was mixed in 1:4 molar ratios and stirred for 1 hour at room temperature. The unreacted dye was removed from the conjugate by dialysis (5,000 Da cutoffs) using tris-HCl buffer with three buffer exchanges at 4 °C.

**Cell Uptake**

For cell uptake study, Alexa conjugated LEDGF\(_{1-326}\) was used to prepare the nano[10] assembly. ARPE-19 (50,000) cells (Passage #29) were seeded in 24-well plates in serum containing DMEM-F12 medium. After 24 hr, the medium was aspirated out and cells were washed with 100 µl of serum-free medium. Thereafter, cells were treated with 200 µl of either Alexa-LEDGF\(_{1-326}\) control or nano[10] assemblies at three different concentrations (2, 10, 25 µg/ml) for 2 and 6 hr in serum free medium at 37 °C. Cells just
with buffer, and 10 mM Zn(II) were maintained as additional controls. After 2 or 6 hr, cells were washed with cold PBS (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) twice, followed by 2 washes with acid PBS (pH 5.0; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH adjusted with HCl). Thereafter, cells were lysed with 1% v/v Triton X-100 for 20 min at room temperature, scraped and collected. The fluorescence of the cell lysate was measured at 488 excitation and 519 emission using Spectramax M5. Each data point was an average of six scans. LEDGF₁-₃₂₆ control or nano[10] assembly cell uptake was calculated from the standard curve of serially diluted Alexa-LEDGF₁-₃₂₆ control or nano[10] assemblies, respectively. Data represent mean ± S.D. for N=3.

**Animal Handling**

Adult male Sprague Dawley (SD) rats (150-180 g) were purchased from Harlan Laboratories for conducting pharmacokinetic studies (Indianapolis, IN). For the purpose of efficacy studies, homozygous RCS rats were maintained and bred in the animal housing facility of University of Colorado, Aurora, CO, as per an Institutional Animal Care and Use Committee approved protocol. Animals were treated as per the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

**Ocular Fluorophotometry**

The pharmacokinetic of Alexa 488 labeled LEDGF₁-₃₂₆ control and nano[10] assemblies were studied using an ocular fluorophotometer (Fluorotron Master®, OcuMetrics Inc., Mountain View, CA) in non-diseased SD rats. Fluorotron measures the fluorescence signal in terms of sodium fluorescein (NaF) concentration in different parts
of eye along an optical axis from the back of the eye to the front of the ye. The instrument reports 150 data points for fluorescence measurement at every 0.25 mm interval along this axis. Ocular tissue layers are assigned specific data point based on autofluorescence of choroid-retina, lens, and cornea.

A drop of 0.5 % w/v tropicanamide (Akorn, Lake Forest, IL) was used to dilate the pupil before scanning. A baseline scan was performed to measure the intrinsic fluorescence of various ocular tissues. Thereafter, a single intravitreal injection of 5 µl of Alexa-LEDGF1-326 control or nano[10] assembly formulation was administered in the right eye of the SD rats, leaving the contralateral eye as a blank. Subsequent scans were taken at specified times beginning at 2 min until day 14 after injection. A standard curve was established by measuring the fluorescence signal of Alexa 488 LEDGF1-326 control or nano[10] assemblies in a cuvette using Fluorotron Master®. The standard curves were used to convert NaF values obtained from the tissue scans to actual concentration of Alexa 488 LEDGF1-326 control and nano[10] assemblies in the ocular tissues. Scans were performed in 4 rats for each group.

**Pharmacokinetic Analysis**

Non-compartmental pharmacokinetic analysis was performed for Alexa 488 conjugated LEDGF1-326 control and nano[10] assembly using WinNonlin software (version 1.5, Scientific consulting, Inc.). Initial protein concentration of the dye conjugate was estimated using absorbance at 280 nm. The dye conjugate samples were then diluted and analyzed using Fluorotron Master®. The standard curve thus obtained was used in converting tissue fluorescence signals in NaF concentration into LEDGF1-326 concentrations. The reported concentrations and units for pharmacokinetic parameters are
to be interpreted as those based on LEDGF$_{1-326}$ concentrations. The concentration-time data obtained from the fluorescence scans was used to determine the C$_{\text{max}}$ (the maximum concentration reached by drug in any given tissue), the mean residence time, and the area under curve (AUC) after intravitreal injection.

**ELISA**

After fluorophotometry in SD rats on day 14, eyes were enucleated and stored at -80 °C. Cornea, aqueous humor, lens, vitreous humor, retina, choroid-RPE, and sclera were isolated under frozen conditions and placed in preweighed tubes. Tissues were homogenized in 500 µl of PBS buffer containing protease inhibitor (cOmplete, Mini, Roche Applied Sciences, Branchburg, NJ) tissue disruptor under ice cold conditions. The supernatant was collected by centrifugation at 10,000 g for 5 mins and used for ELISA. For ELISA, 100 µl of either standard LEDGF$_{1-326}$ (freshly purified) or tissue lysates were coated overnight at 4 °C in triplicates in 96-well plates. Wells were washed three times with wash buffer (0.1 % w/v Tween 20 in PBS pH 7.0) after each step. The nonspecific binding sites were blocked with blocking solution (0.5 % w/v bovine serum albumin, and 0.1 % Tween 20 in PBS pH 7.0) for 4 hours. LEDGF$_{1-326}$ was detected by mouse anti-LEDGF antibody (1:1,000) (BD Biosciences, San Diego, CA) which was cross detected with HRP conjugated anti-mouse secondary antibody (1:10,000) (BD Biosciences, San Diego, CA). After through washing of the plate with water, 3,3',5,5'-Tetramethylbenzidine (TMB) was added. Immunoreactive LEDGF$_{1-326}$ control and nano[10] assemblies were quantified by colorimetric absorbance at 650 nm upon development of blue color at 20 min. Solutions volume used was 200 µl and all incubations were done at room temperature.
**Cell Viability Assay**

ARPE-19 cells were used to determine the cell survival function of LEDGF$_{1-326}$ in the presence of aggregation mediated stress. Briefly, ARPE-19 cells were grown as described earlier (Baid et al., 2013). For cell viability assay, 10,000 cells were seeded in 96-well plates in serum containing DMEM-F12 medium. After 24 hr, the medium was aspirated out and cells were washed with 100 µl of serum free medium. Thereafter, cells were treated with 200 µl of either LEDGF$_{1-326}$ control or nano[10] assembly formulation for 24 hr at 37 ºC. No cells (just the medium), cells with 25 mM Tris buffer, cells with 25 mM Tris + 10 mM Zn(II) were maintained as additional controls. After 24 hr, the medium was aspirated out and 200 µl of fresh serum free medium was added to each well. Diluted 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) reagent (20 µl of 5 mg/ml in PBS) was added to each well and the plate was incubated for 3 hr at 37 ºC. The MTT containing medium was aspirated out and the formazan crystals formed were dissolved in 200 µl of DMSO. The absorbance of the color developed was measured at 570 nm using Spectramax M5. The percentage viability of APRE-19 cells was calculated as compared to the control group. Date represent mean ± S.D. of N=3 samples.

**Electroretinography (ERG)**

ERG was done to determine the functional activity of photoreceptors in retinal degenerative RCS rat. Briefly, 4 week old RCS rats were dark adapted for at least 30 min and thereafter all procedures were done under dim red light. A mixture of 80 mg/kg of ketamine and 12 mg/kg of xylazine was injected intra-peritoneally to anesthetize rats. A
topical drop of 0.5% tropicanamide (Akorn, Lake Forest, IL) was used to dilate the pupil, and 2.5% hypromellose (Akorn, Lake Forest, IL) was thereafter used to keep the eye moist and cataract free. The animal was placed on a 37 °C heated water jacket. Needle reference electrodes (LKC Technologies Inc., Gaithersburg, MD) were inserted into animal’s tail and right cheek. DTL plus silver electrodes (LKC Technologies Inc., Gaithersburg, MD) were positioned on both eyes touching the cornea. Full field electroretinograms (ERG) were collected as per the International Society for Clinical Electrophysiology of Vision (ISCEV) (Marmor et al., 2009). Brief flashes of 0.9 log cd–s/m² with 10 secs interval were given and scotopic ERG was recorded. Dark-adapted oscillatory potentials (OP) were obtained from the dark-adapted eye, using 0.4 log cd–s/m² flash stimulus. Thereafter, the animal was light adapted for 3 min with a background light of 30 cd/m². Photopic ERG was recorded at 0.9 log cd–s/m² with background light on. Flicker ERG was obtained with 0.4 log cd–s/m² stimuli, with 30 stimuli per second (30 Hz) flashes. For each animal at least three ERGs were averaged.

After the base ERGs were taken, rats were injected intravitreally with drug formulations using a 34 gauge needle (1/2 inch long with 45º taper) connected to 10 µl Hamilton glass syringe (Hamilton Co., NV). Intravitreal injection of 2 µl of 2.5 mg/ml sterile filtered LEDGF₁₋₃₂₆ control or nano[10] assemblies was administered in one eye. Equal volume of buffer (25 mM Tris-HCl, 100 mM NaCl, pH 7.4) or Zn(II) (25 mM Tris-HCl, 100 mM NaCl, 10 mM Zn(II), pH 7.4) was injected in the contralateral eye. ERGs were recorded every alternate week till 14th week. Thereafter rats were euthanized. Data is presented for mean ± SD of N=3 or 4 animals.
Results

**LEDGF\textsubscript{1-326} Self-Assembled to Form Nanostructures in the Presence of Zn(II)**

Formation of LEDGF\textsubscript{1-326} nanoassemblies in the presence of Zn(II) was monitored by DLS (Figure 6.1A). The hydrodynamic diameter of LEDGF\textsubscript{1-326} was 9 ± 1 nm, which remained unaltered in the absence of Zn(II) during 24 hr incubation at 37 ºC (Figure 6.1A, and Table 6.1). On the other hand LEDGF\textsubscript{1-326} incubated with different concentrations of Zn(II) underwent an increase in size, indicating formation of nanoassemblies. In the presence of 0.1 mM Zn(II), there was a delay in size increase until 24 hr. However, 1 and 10 mM Zn(II) increased LEDGF\textsubscript{1-326} size within 30 min of incubation. At 24 hr incubation, LEDGF\textsubscript{1-326} formed nano[0.1], nano[1], nano[10] assemblies of 22 ± 5, 26 ± 5, and 28 ± 5 nm size with 0.1, 1.0, 10 mM Zn(II), respectively.

TEM images of nano[10] assemblies indicated the presence of nanostructures (Figure 6.2, right most panel). Some nanoassemblies clung to each other, while others were present as individual particles. In the absence of Zn(II), LEDGF\textsubscript{1-326} (Figure 6.2, middle panel) had no such structures.

**Nanoassembly Formation Induced LEDGF\textsubscript{1-326} Structural Changes**

Changes in the tertiary structure of LEDGF\textsubscript{1-326}, due to nanoassembly formation, were measured by intrinsic tryptophan fluorescence (Figure 6.2A). The fluorescence emission maximum (E\textsubscript{max}) of LEDGF\textsubscript{1-326} (1mg/ml) was at 342 nm with an intensity of 7643 R.F.U. (Figure 6.1C) in the absence of Zn(II). Formation of nanoassembly induced a red shift of ~2 nm in E\textsubscript{max} and a Zn(II) concentration dependent decrease in fluorescence intensity. Interestingly, the fluorescence signal of nano[10] assembly
Table 6.1. Particle Size of Nanoassemblies.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>LEDGF&lt;sub&gt;1-326&lt;/sub&gt;</th>
<th>Zn(II)</th>
<th>Number Mean Diameter (nm)</th>
<th>Polydispersity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/ml)</td>
<td>(mM)</td>
<td>0.5 hr</td>
<td>12 hr</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>0</td>
<td>0.69</td>
<td>0.68</td>
</tr>
<tr>
<td>LEDGF&lt;sub&gt;1-326&lt;/sub&gt;</td>
<td>1</td>
<td>0</td>
<td>9 ± 1</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Nano[0.1] assembly</td>
<td>1</td>
<td>0.1</td>
<td>9 ± 1</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Nano[1] assembly</td>
<td>1</td>
<td>1</td>
<td>24 ± 2</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>Nano[10] assembly</td>
<td>1</td>
<td>10</td>
<td>22 ± 5</td>
<td>17 ± 3</td>
</tr>
</tbody>
</table>

Nanoassemblies were prepared by adding LEDGF<sub>1-326</sub> to the final concentration mentioned above to Zn(II) containing Tris buffer (25 mM Tris-HCl, 100 mM NaCl, at pH 7.4) and incubating at 37 °C for 24 hr. Nanoassemblies were named based on Zn(II) content. Thus nano[0.1], nano[1], and nano[10] assembly contained 0.1, 1, 10 mM Zn(II), respectively. Formation of nanoassemblies was monitored at specified time interval using DLS. Data represent mean ± S.D. of N = 3 or more batches. * indicates data represent N=1 batch.
Figure 6.1.  LEDGF<sub>1-326</sub> Nanoassemblies Formed in the Presence of Zn(II).  A) Dynamic light scattering- LEDGF<sub>1-326</sub> size increased from 7 ± 1 to 28 ± 5 nm due to self assembly. Representative graph from N=3 or more batch.  B) Transmission electron microscopy- Representative image of i) buffer (25 mM Tris-HCl, 100 mM NaCl, at pH 7.4), ii) LEDGF<sub>1-326</sub>, and iii) nano[10] assembly.  While no particles were visible in buffer and LEDGF<sub>1-326</sub>, structures <50 nm were evident in Nano[10] formulation. Representative image from N=3 batch.
Figure 6.2. Zn(II) Induced Structural Changes in LEDGF$_{1-326}$. Structural pertubation in LEDGF$_{1-326}$ due to nanoassembly formation was investigated by, A) Fluorescence spectroscopy- LEDGF$_{1-326}$ fluorescence was quenched in the presence of Zn(II), indicating exposure of hydrophobic residues to polar environment. B) Circular dichorism-A shift of negative peak from 230 to 220 nm and higher negative ellipticity below 210 nm suggests formation of α-helix in LEDGF$_{1-326}$ in the presence of Zn(II). C) UV-Vis spectroscopy- LEDGF$_{1-326}$ UV absorbance was quenched in the presence of Zn(II) at all concentrations assessed except 10 mM. Data represents means ± S.D. for N=3 batches.
was lost significantly after 30 min of incubation at 37 °C (Figure 6.3) and regained within 24 hr of incubation (Figure 6.2A). The fluorescence intensity at 342 nm was 4582, 3884, and 5348 R.F.U. for nano[0.1], nano[1], and nano[10] assemblies after 24 hr incubation at 37 °C, indicating there was loss of fluorescence due to formation of nanoassemblies.

![Graph](image)

**Figure 6.3. Initial Loss of Fluorescence Signal.** Transient change in the fluorescence signal was seen 30 mins after addition of Zn(II). The change is signal was most prominent in the Nano[10] assembly. This loss in fluorescence signal was gained in next 24 hr as seen in Figure 6.2. Data represent mean ± S.D. for N=1 batch.

**Nanoassembly Retained Size upon Dilution**

To investigate the changes in the secondary structure, far-UV CD spectrum of LEDGF$_{1-326}$ was recorded from 205-260 nm (Figure 6.2B). LEDGF$_{1-326}$ CD spectrum consisted of a negative peak at 230 nm and a strong negative peak below 200 nm. Deconvolution of this spectrum indicated LEDGF$_{1-326}$ to be a random coiled protein (Table 6.2). In the presence of Zn(II), significant changes in CD spectrum were observed. The negative peak at 230 nm shifted towards left (to lower wavelength) and the
negative ellipticity below 200 nm increased. Changes in the CD signal was dependent on the Zn(II) concentration, being slow for nano[0.1] assembly and fastest for nano[10] assembly (data not shown). Deconvolution and comparison of CD spectra of nanoassemblies with LEDGF$_{1-326}$ indicated an increase in $\alpha$-helix content with an increase in Zn(II) concentration; however, these differences were not substantial.

Table 6.2. Nanoassembly Induced $\alpha$-helix Formation.

<table>
<thead>
<tr>
<th></th>
<th>LEDGF$_{1-326}$</th>
<th>Nano[0.1]</th>
<th>Nano[1]</th>
<th>Nano[10]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix (%)</td>
<td>14.3</td>
<td>15.1</td>
<td>15.9</td>
<td>16.70</td>
</tr>
<tr>
<td>Antiparallel (%)</td>
<td>15.3</td>
<td>14.7</td>
<td>14.2</td>
<td>13.7</td>
</tr>
<tr>
<td>Parallel (%)</td>
<td>18.9</td>
<td>18.2</td>
<td>17.5</td>
<td>16.8</td>
</tr>
<tr>
<td>Beta-turn (%)</td>
<td>21.1</td>
<td>20.8</td>
<td>20.6</td>
<td>20.3</td>
</tr>
<tr>
<td>Random coil (%)</td>
<td>52.2</td>
<td>51.1</td>
<td>50.1</td>
<td>49.0</td>
</tr>
<tr>
<td>Total sum</td>
<td>121.9</td>
<td>119.9</td>
<td>118.2</td>
<td>116.5</td>
</tr>
</tbody>
</table>

The far-UV CD spectra obtained in Figure 2B were deconvulated using CDNN 2.1 software. Nanoassemblies had more $\alpha$-helix as compared to LEDGF$_{1-326}$. However, the difference was not remarkable.

The absorbance maximum of LEDGF$_{1-326}$ was at 276 nm. In the presence of 0.1 and 1 mM Zn(II), there was a decrease in LEDGF$_{1-326}$ absorbance but no change in the presence of 10 mM Zn(II). The absorbance at 276 nm ($A_{\text{max}}$) was 0.47, 0.33, 0.32, and 0.46 A.U. for LEDGF$_{1-326}$, and nano[0.1], nano[1], and nano[10] assemblies, respectively.

Stability of nanoassembly over dilution was studied using DLS (Figure 6.4 and Table 6.3). Size of nano[10] assembly once formed (~28 nm) did not change when...
Figure 6.4. **Nanoassembly Size is Resistant to Dilutions.** Nanoassembly formulation was diluted with Tris buffer (25 mM Tris-HCl, 100 mM NaCl, at pH 7.4 and 25 °C). Particle size was measured using dynamic light scattering. A) Once formed, nanoassembly size remained at about 28 nm despite dilution. B) The diluted nanoassemblies retained their size even after storage at 4 °C for 24 hr following dilution. Data represent mean ± S.D. of N=1 batch.
diluted 2 and 4 times (Figure 6.4A). Further, when the diluted nano[10] assembly was stored at 4 °C for 24 hr, there was no significant change in size (Figure 6.4B).

### Table 6.3. Particle Size of Nanoassembly.

<table>
<thead>
<tr>
<th></th>
<th>Number Mean Diameter (nm)</th>
<th>PDI</th>
<th>Number Mean Diameter (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undiluted</td>
<td>27.32</td>
<td>0.131</td>
<td>28.51</td>
<td>0.09</td>
</tr>
<tr>
<td>2 fold dilution</td>
<td>28.46</td>
<td>0.118</td>
<td>31.67</td>
<td>0.251</td>
</tr>
<tr>
<td>4 fold dilution</td>
<td>30.61</td>
<td>0.18</td>
<td>28.74</td>
<td>0.119</td>
</tr>
</tbody>
</table>

Nanoassemblies were prepared by adding LEDGF\textsubscript{1-326} to 10 mM Zn(II) containing tris buffer (25 mM Tris-HCl, 100 mM NaCl, at pH 7.4) and incubating at 37 °C for 24 hr. Once prepared, nanoassemblies were diluted with plain buffer and changes in size measured by DLS. Thereafter, the diluted samples were stored at 4 °C for 24 hr and size was measured again. Data represent mean ± S.D. of N = 1 batch.

**EDTA Destabilized Nanoassembly**

To investigate the nature of chemical interaction between LEDGF\textsubscript{1-326} and Zn(II), we evaluated the effect of EDTA on nano[10] assembly properties (Figure 6.5). Addition of EDTA did not alter LEDGF\textsubscript{1-326} size or fluorescence, CD, and UV-Vis spectra. On the other hand, nano[10] assembly’s size decreased from 25 ± 4 to 9 ± 2 nm upon EDTA addition (Figure 6.5A, Table 6.4). The fluorescence signal of nano[10] assembly increased and there was no significant difference to that of LEDGF\textsubscript{1-326} (Figure 6.5B).

Similarly, CD and UV-Vis spectra of LEDGF\textsubscript{1-326} nano[10] assemblies were not significantly different to that of control LEDGF\textsubscript{1-326} (Figure 6.5C and 6.5D). High UV absorbance in both LEDGF\textsubscript{1-326} control and nano[10] assembly below 260 nm is due to the presence of EDTA, which has an absorbance maxima at 230 nm. These studies
Figure 6.5. Nanoassemblies were Disrupted in the Presence of EDTA. EDTA was added to nanoassembly and LEDGF<sub>1-326</sub> in 1:5 molar ratio (zinc : EDTA) and incubated for 4 hr at 25 °C. A) Dynamic light scattering- Addition of EDTA reduced nanoassembly size from 25 ± 4 to 9 ± 2 nm, while LEDGF<sub>1-326</sub> size remained at 9 ± 0 nm. B) Fluorescence spectroscopy- The fluorescence signal of LEDGF<sub>1-326</sub>, quenched due to nanoassembly formation, was regained upon EDTA addition and was similar to LEDGF<sub>1-326</sub> with EDTA. C) Circular dichroism- All changes in LEDGF<sub>1-326</sub> CD, which occurred due to nanoassembly formation, reverted back in presence of EDTA. D) UV-Vis spectroscopy- Nanoassembly and LEDGF<sub>1-326</sub> in the presence of EDTA suggested similar absorbance. Data represents mean ± S.D. of N=3 batches. *, p<0.05 was considered to be statistically significant as compared to control LEDGF<sub>1-326</sub> group.
suggest the reversibility between nanoassembly and LEDGF₁₋₃₂₆ that is governed by effective Zn(II) concentration in formulation buffer.

**Table 6.4. Particle Size in the Presence of EDTA.**

<table>
<thead>
<tr>
<th></th>
<th>LEDGF₁₋₃₂₆</th>
<th>Nano[10] assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Size (nm)</td>
<td>PDI</td>
</tr>
<tr>
<td>No EDTA</td>
<td>7.5 ± 1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>EDTA</td>
<td>8.5 ± 0.4</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

Nanoassemblies were prepared by adding LEDGF₁₋₃₂₆ to 10 mM Zn(II) containing Tris buffer (25 mM Tris-HCl, 100 mM NaCl, at pH 7.4) and incubating at 37 °C for 24 hr. Once prepared, nanoassemblies were treated with 50 mM EDTA and changes in size were measured by DLS. Data represent mean ± S.D. of N = 3 batches.

**Nanoassembly Reduced LEDGF₁₋₃₂₆ Degradation**

LEDGF₁₋₃₂₆ structural stability in nanoassembly at 25 °C was monitored for 1 month using DLS, SDS-PAGE, CD, and fluorescence (Figure 6.6). LEDGF₁₋₃₂₆ size decreased from 8 ± 1 to 5 ± 1 nm on day 3 (Figure 6.6A). Nano[1] assembly exhibited a size of 27 ± 1 at day 0, which decreased to 4 ± 3 nm on day 7. On the other hand, nano[10] assembly indicated no change in size until day 14 but by day 30 there was a significant change in size change from 27 ± 1 to 8 ± 2 nm.

Monomer band of 36.7 kDa was seen in SDS-PAGE for LEDGF₁₋₃₂₆, and nano[1], and nano[10] assembly on day 0 (Figure 6.6B). By day 3 there was complete loss of this band in LEDGF₁₋₃₂₆. Nano[1] and nano[10] assemblies delayed this loss until day 7 and 30, respectively.

The fluorescence spectrum of LEDGF₁₋₃₂₆ indicated significant decrease in fluorescence intensity at 342 nm from 4866 on day 0 to 4158 and 2723 R.F.U. on day 7.
Figure 6.6. Nanoassemblies Reduced LEDGF$_{1-326}$ Degradation.
Figure 6.6. Nanoassemblies Reduced LEDGF<sub>1-326</sub> Degradation. LEDGF<sub>1-326</sub> and nanoassemblies were incubated at 25 °C and LEDGF<sub>1-326</sub> stability was monitored for 30 days. A) Dynamic light scattering- Nanoassemblies formed with 10 mM Zn(II) retained their size until day 14 while LEDGF<sub>1-326</sub> size decreased from 8 ± 1 to 5 ± 1 nm by day 3. Data represent mean ± S.D. of N=3 batch. B) SDS-PAGE (reducing)- Nano[10] assemblies retained 36.7 kDa monomeric band of LEDGF<sub>1-326</sub> until day 14. Representative image of N=3 batch. C) Fluorescence spectroscopy- Nano[10] assembly retained the fluorescence signal until day 30 while LEDGF<sub>1-326</sub> fluorescence signal declined significantly from day 7 onwards. Data represent mean ± S.D. of N=3 batch. D)- Circular dichroism- Nano[10] assemblies reduced CD signal loss until day 14 suggesting LEDGF<sub>1-326</sub> secondary structure was preserved in nanoassemblies. Data represents mean ± S.D. of N=3 batches.
and 30, respectively (Figure 6.6C). Fluorescence intensity of nano[1] assembly, on the other hand, remained unchanged until day 7; however, on day 14 onwards significant signal loss were observed. The fluorescence intensity at 342 nm was 2474, 2512, 2184, and 2029 R.F.U. on day 0, 7, 14, and 30, respectively, for nano[1] assembly. Nano[10] assembly being most robust, had no significant difference in fluorescence intensity until day 14, and marginal decrease in fluorescence intensity was observed on day 30. The fluorescence intensity at 342 nm for nano[10] assembly was 2710, 2735, 2698, and 2527 R.F.U. on day 0, 7, 14, and 30, respectively.

The CD spectra of LEDGF1-326 indicated a significant loss in ellipticity from day 1 onwards and by day 30 there was complete loss of ellipticity (Figure 6.6D). The loss in ellipticity was also seen in nano[1] assembly but was less as compared to LEDGF1-326. But for nano[10] assembly, there was no significant loss in CD signal until day 14; by day 30, despite some loss in ellipticity, a significant CD signal persisted.

**Nanoassembly Increased LEDGF1-326 Retinal Cell Uptake**

The cellular uptake of LEDGF1-326 and nano[10] assembly was investigated in ARPE-19 cells using fluorometric measurements of Alexa labeled LEDGF1-326 (Figure 6.7). ARPE-19 cells exhibited dose and time dependent uptake. Although the total uptake (Figure 6A) increased with increasing dose, the percent uptake (Figure 6.7B) decreased. Following 2 hr of incubation with 2, 10, and 25 µg/ml of LEDGF1-326 or nano[10] assembly, the uptake was 0.5 ± 0.1, 1.0 ± 0.3, and 1.4 ± 0.2 µg for LEDGF1-326 as compared to 0.7 ± 0.1, 1.0 ± 0.2, and 2.2 ± 0.5 µg for nanoassembly, respectively (Figure 6.7A). Nano[10] assembly uptake was higher as compared to LEDGF1-326 in each
ARPE-19 cells were incubated with 2, 10, 25 µg/ml of Alexa-LEDGF$_{1-326}$ or nano[10] assemblies at 37 °C for 2 and 6 hrs. Fluorescence of LEDGF$_{1-326}$ and nanoassembly was measured to determine ARPE-19 cell uptake. A) Total retinal cell uptake- ARPE-19 cell had significantly higher level of nanoassembly uptake as compared to LEDGF$_{1-326}$ at 6 hr incubation. B) Percent uptake- The percent uptake decreased with increasing dose for both LEDGF$_{1-326}$ and nano[10] assemblies, suggesting an upper limit for cellular uptake capacity of retinal cells for the macromolecules and their self-assemblies within 6 hr incubation time. Data represent mean ± S.D. of N=4 batch. * indicates p<0.05, ** indicates p<0.005 compared to corresponding LEDGF$_{1-326}$ treated group.

Further, with an increase in incubation time there was an increase in uptake. At 6 hr incubation, uptake was 1.2 ± 0.2, 1.2 ± 0.2, and 1.9 ± 0.2 µg for LEDGF$_{1-326}$ and 2.0 ± 0.1, 2.9 ± 0.5, and 2.9 ± 0.2 µg for nanoassembly. At 6 hr, nanoassembly uptake was significantly higher (p < 0.05) as compared to corresponding LEDGF$_{1-326}$ group.

Following 2 hr of incubation with 2, 10, and 25 µg/ml of LEDGF$_{1-326}$ or nano[10] assembly, the percent uptake was 24.7 ± 7.7, 10.1 ± 3.3, and 5.9 ± 0.23 % for LEDGF$_{1-326}$ as compared to 37.3 ± 3.9, 7 ± 1.5, and 9 ± 2% for nanoassembly, respectively.
(Figure 6.7B). With increase in the incubation dose, there was a decrease in the
percentage uptake for both LEDGF$_{1-326}$ and nano[10] assembly. At 6 hr incubation, the
percent uptake was $60 \pm 11.1$, $12 \pm 1.7$, and $7.7 \pm 0.9 \%$ for LEDGF$_{1-326}$ as compared to
$101.2 \pm 4.6$, $29.5 \pm 5$, and $11.7 \pm 0.8 \%$ for nanoassembly for 2, 10, and 25 µg/ml,
respectively. The percent uptake of nanoassembly was significantly higher ($p < 0.005$)
than corresponding LEDGF$_{1-326}$ group.

**Nanoassembly Persisted in Ocular Tissues for Days**

Nanoassembly persistence in SD rat eye was monitored by the fluorescence
intensity of Alexa labeled LEDGF$_{1-326}$ using ocular fluorophotometry (Figure 6.8). Figure
6.8A is an average of blank scans (N=7 animals) of SD rat eye before intravitreal
injection. Autofluorescence was seen at $24 \pm 0$, $51.1 \pm 0.87$, and $88 \pm 0$ data points, which
corresponded to choroid-retina, lens, and cornea, respectively. Based on this data,
choroid-retina, vitreous humor (value between corid-retina and lens), lens, aqueous
humor (value between lens and cornea), and cornea tissues positions were assigned at 24,
40, 50, 70, and 88 data point, respectively.

Figure 6.8B, and 6.8C are standard curves obtained as sodium fluorescein (NaF)
concentration (ng/ml) equivalent to actual LEDGF$_{1-326}$ concentration (µg/ml) for Alexa
488 LEDGF$_{1-326}$ control and nano[10] assembly. These curves were used to convert the
fluorescence signals into concentrations of Alexa 488 LEDGF$_{1-326}$ control and nano[10]
assembly.

Figure 6.8D, and 6.8E are average fluorescence scans (N=4 animals) from 2 min
to 14 days for LEDGF$_{1-326}$ control and nano[10] assembly after intravitreal l injection as
Figure 6.8. Nanoassemblies Prolonged LEDGF<sub>1-326</sub> *In Vivo* Persistence.
Figure 6.8. Nanoassemblies Prolonged LEDGF\textsubscript{1-326} \textit{In Vivo} Persistence. Single intravitreal injection of 5 µg of Alexa labeled LEDGF\textsubscript{1-326} or nano[10] assembly was dosed in non-disease SD rats. Fluorescence in ocular tissue was measured non-invasively using Fluorotron Master® before intravitreal injection, and from 2 min to 14 days after intravitreal injection. A) Auto fluorescence- Ocular tissue fluorescence in absence of any fluorescent dye. B) LEDGF\textsubscript{1-326} standard curve- Standard curve used to convert the fluorescence signal to actual LEDGF\textsubscript{1-326} concentration. C) Nanoassembly standard curve- Standard curve used to convert the fluorescence signal to actual nanoassembly concentration. D) Mean ocular fluorescence scans of LEDGF\textsubscript{1-326} treated groups for N=4 animals. E) Mean ocular fluorescence scans of nanoassembly treated group for N=4 animals.
Figure 6.9. Distribution and Retention of Nanoassemblies in Ocular Tissues. Single intravitreal injection was given in right eye of either LEDGF$_{1-326}$ or nano[10] assembly in SD rats. The contralateral eye was kept blank. LEDGF$_{1-326}$ and nanoassembly concentrations were determined in A) Vitreous humor, B) Chorid-retina, and C) Aqueous humor using the fluorescence scans obtained from Fluorotron Master®. Nanoassemblies persisted at significantly high levels until day 14 while LEDGF$_{1-326}$ was undetectable at $\leq 3$ days of intravitreal injection. Data represents mean ± S.D. of N=4 animals.
obtained from Flurotron Master®. A high fluorescence signal in vitreous humor after 2 min of injection suggested that injection was done in mid-vitreous (Figure 6.8D, and 6.8E). Fluorescence signal declined rapidly within 24 hr in both Alexa 488 LEDGF₁-326 control (Figure 6.8D) and nanoassembly group (Figure 6.8E). While fluorescence signal of Alexa 488 LEDGF₁-326 control was undetectable by day 3, signal from its nanoassembly persisted until day 14.

**Nanoassembly Improved Pharmacokinetic Profile of LEDGF₁-326**

**Aqueous Humor**

In aqueous humor, the base line calculated from the blank scan (Figure 6.8A) was equivalent to 0 µg/ml of LEDGF₁-326 (Figure 6.9A). At 2 min, after intravitreal injection, 3.4 ± 3 and 4.9 ± 2.4 µg/ml of LEDGF₁-326 control and nano[10] assembly were measured in aqueous humor, respectively (Figure 6.9C). LEDGF₁-326 control level dropped below the baseline within 6 hr, while LEDGF₁-326 nano[10] assembly persisted until day 14 and had a significantly high concentration of 0.6 ± 0.1µg/ml on day 14.

**Vitreous Humor**

A fluorescence signal equivalent to 3 ± 0.5 ng/ml of NaF was observed in the vitreous humor in blank scans (Figure 6.8A), which was equivalent to 0 µg/ml (base line) of LEDGF₁-326 (Figure 6.9B). In LEDGF₁-326 injected rats, 292 ± 106 µg/ml of LEDGF₁-326 was measured in the vitreous after 2 min of intravitreal injection, which declined rapidly to 127 ± 74 µg/ml in 30 min (Figure 6.9B). LEDGF₁-326 vitreous concentration was below the base line (0 µg/ml) by day 3. Nano[10] assembly on the other hand persisted for days in the vitreous. The concentration of nanoassembly after 2 min of
Figure 6.10. Nanoassemblies Enhanced LEDGF<sub>1-326</sub> Retention in Eye Tissues. A) C<sub>max</sub>, B) AUC, and C) Mean residence time were determined using non-compartmental analysis with WinNonlin (version 1.5), where, AUC is the area under the curve and C<sub>max</sub> is the maximum drug concentration in a given tissue after intravitreal injection. Data represent mean ± S.D. of N=4 animals. .*indicates p<0.05, compared to corresponding LEDGF<sub>1-326</sub> treated group.
intravitreal injection was 321 ± 54 µg/ml followed by an initial rapid decline to 100 ± 45 µg/ml in 30 min. Thereafter, nano[10] assembly level declined at a slower rate and significantly higher level (p<0.05) of LEDGF<sub>1-326</sub> nano[10] assembly, as compared to LEDGF<sub>1-326</sub>, persisted until day 14. On day 14, 0.7 ± 0.1 µg/ml of nano[10] assembly was quantified, which was significantly higher (p<0.005) than LEDGF<sub>1-326</sub> , and the base line levels.

**Choroid-Retina**

Nano[10] assembly also persisted in the choroid-retina until day 14. The autofluorescence signal of choroid-retina was 10 ± 4 ng/ml of NaF (Figure 6.8A), equivalent to 0.1 µg/ml of LEDGF<sub>1-326</sub>. Thus 0.1 µg/ml of LEDGF<sub>1-326</sub> was considered as base line for choroid-retina (Figure 6.9C). LEDGF<sub>1-326</sub> concentration in choroid-retina was 13.2 ± 10.8 µg/ml after 2 min of intravitreal injection, which increased to 30.0 ± 22.6 µg/ml at 30 min. Thereafter, there was a rapid decline in LEDGF<sub>1-326</sub> level, and by day 1, LEDGF<sub>1-326</sub> was undetectable. On the other hand, nano[10] assembly level was 14.3 ± 9.8 µg/ml after 2 min of intravitreal injection, which increased to 21.5 ± 12.8 µg/ml at 30 min. By day 1, nano[10] assembly level dropped to 2.0 ± 1.1 µg/ml, and followed a slow decline over period of 14 days. Nano[10] assembly persisted at significantly high (p<0.05) level until day 14 as compared to LEDGF<sub>1-326</sub> and base line. On day 14 nano[10] assembly level was 0.6 ± 0.2 µg/ml in choroid-retina.

The peak concentrations (C<sub>max</sub>) of LEDGF<sub>1-326</sub> control and nanoassemblies were similar (Figure 6.10A). Following intravitreal injection, the AUC was 105.9, 12.7, and 13.0 times higher for nanoassembly as compared to LEDGF<sub>1-326</sub> in aqueous humor, vitreous humor, and choroid-retina, respectively (Figure 6.10B). The mean residence time was 6.4, 3.7,
and 5.7 days for nanoassembly and 0.3, 4.1, and 6.5 hr for LEDGF$_{1-326}$ in aqueous humor, vitreous humor, and choroid-retina, respectively.

**Immunoreactive Nanoassembly Persisted in Retina**

Following noninvasive pharmacokinetic study with fluorophotometry, animals were euthanized and ocular tissues were dissected and immunoreactive LEDGF$_{1-326}$ control and nano[10] assembly levels were measured in each ocular tissue and blood by ELISA (Figure 6.11). Tissues obtained from uninjected blank eyes indicated absence of immunoreactive endogenous LEDGF$_{1-326}$. Nano[10] assembly and LEDGF$_{1-326}$ was undetectable in all other ocular tissues and blood sample except retina. In retina, LEDGF$_{1-326}$ control and nano[10] assembly levels were 1.3 ± 0.4 and 0.2 ± 0.2 µg/g of tissue, respectively, on day 14. Nano[10] assembly group had significantly high drug (p<0.05) levels compared to control group.

**Nanoassembly Improved Retinal Cell Survival**

Efficacy of nanoassembly in improving the ARPE-19 cell viability under serum starvation was determined by MTT assay (Figure 6.12). Compared to untreated cells (100 % viability), LEDGF$_{1-326}$ treated cells had increased viability in a dose-dependent manner. ARPE-19 cell viability increased to 124 ± 11, 148 ± 128, and 159 ± 44 % following treatment with 0.01, 0.1, and 1.0 µg/ml of LEDGF$_{1-326}$, respectively. When treated with equivalent amount of nano[10] assembly, ARPE-19 cell viability was 150 ± 2, 180 ± 22, and 200 ± 8 % following 0.01, 0.1, and 1.0 µg/ml of nano[10] assembly treatment, respectively. Although nano[10] assembly improved ARPE-19 survival as compared to LEDGF$_{1-326}$ there was no statistically significant difference.
Figure 6.11. Nanoassemblies Enhanced Immunoreactive LEDGF<sub>1-326</sub> Retention in Retina. Ocular tissues were dissected following pharmacokinetic study on day 14 and levels of immunoreactive LEDGF<sub>1-326</sub> were estimated using an ELISA. Significantly high levels of immunoreactive LEDGF<sub>1-326</sub> were detected in the retina on day 14 after intravitreal injection, suggesting nanoassembly uptake and persistence in the retina. Data represent mean ± S.D. of N=4 animals. * indicates p<0.05 compared to corresponding LEDGF<sub>1-326</sub> treated group.

Nanoassembly Improved and Prolonged Retinal Degeneration Treatment

Nanoassembly efficacy was tested in RCS rats using electroretinography (ERG) (Figure 6.13).

Scotopic ERG

The b-wave amplitude on week 4 was 313 ± 32 µV for all rats (Figure 6.13A). Beyond 4 week, there was a decline in the b-wave amplitude in all groups’ however the rate of decline was the slowest for nano[10] assembly treated groups. LEDGF<sub>1-326</sub> treated rats had significantly (p<0.05) higher b-wave amplitude as compared to buffer treated rats at week 12. However, by week 14, there was no significant difference in buffer,
Figure 6.12. Nanoassemblies Improved Retinal Cell Survival. ARPE-19 cells were treated with nano[10] assembly or LEDGF1-326 in serum starved condition for 24 hr. Cell viability was estimated by MTT assay. Data represent mean ± S.D. of N=3. No statistical difference was obtained when LEDGF1-326 group was compared with corresponding nano[10] assembly group.

Zn(II), and LEDGF1-326 treated rats. On the other hand, nano[10] assembly treated rats had significantly higher (p<0.05) b-wave amplitude at week 12 and 14 as compared to buffer and Zn(II) treated rats. The b-wave amplitude was 17 ±10, 11 ± 7, 42 ± 15, and 65 ± 15 µV, respectively, for buffer, Zn(II), LEDGF1-326, and nanoassembly treated rats on week 14. Nanoassembly treated group had significantly higher (p<0.05) b-wave amplitude on week 12 and 14 as compared to the buffer, and Zn(II) group.

Photopic ERG

In photopic ERG, the b-wave amplitude was 105 ± 23 µV in all groups (when averaged together) at week 4 (Figure 6.13B). Thereafter, it declined until 14th week. On week 14, the b-wave amplitude was 12 ± 7, 11 ± 7, 22 ± 5, and 40 ± 10 µV, in buffer, Zn(II), LEDGF1-326, and nano[10] assembly treated rats, respectively. While LEDGF1-326
Figure 6.13. Nanoassemblies Enhanced and Prolonged LEDGF\textsubscript{1-326} efficacy. Single intravitreal injection of 5 µg of LEDGF\textsubscript{1-326} or nano[10] assembly was given to 4 weeks old retinal degenerative RCS rats. Electroretinograms (ERG) were recorded on 4\textsuperscript{th} week before injection and thereafter every alternate week until 14\textsuperscript{th} week. A) B-wave amplitude of scotopic ERG, B) B-wave amplitude of photopic ERG, C) Oscillatory potential amplitude, and D) 30 Hz flicker amplitude. Data represent mean ± S.D. of N=3 animals.
delayed photopic ERG loss until week 12, nano[10] assembly prolonged its efficacy to week 14. Nano[10] assembly treated rats had significantly (p<0.05) higher b-wave amplitude as compared to buffer, and Zn(II) treated rats on week 14.

Oscillatory Potential (OP)

The OP amplitude in all groups (when averaged together) was 91.8 ± 11.5 µV at week 4 (Figure 6.13C). There was a decrease in OP amplitude across all groups by week 14. On week 14, the OP amplitudes were 32 ± 5, 33 ± 8, 36 ± 8, and 36 ± 9 µV for buffer, Zn(II), LEDGF_{1-326}, and nano[10] assembly treated groups, respectively. There was no significant difference in oscillatory potential amongst all groups.

30 Hz Flicker

At week 4, the 30 hz flicker amplitude was 10 ± 2 µV for all groups (when averaged together) (Figure 6.13D). There was a decrease in the flicker amplitude in all groups with time; however, at week 14, the flicker amplitude of nano[10] assembly group was significantly higher than buffer and Zn(II) groups. The flicker amplitude at week 14 was 2 ± 0.4, 3.7 ± 0.4, 2.4 ± 0.6, 5.2 ± 2.2 µV for buffer, Zn(II), LEDGF_{1-326}, and nano[10] assembly treated groups, respectively.

Discussion

In this manuscript we developed a polymeric free metal ion induced nano delivery system which offered several advantages. The nanoassemblies 1) reduced physical and chemical degradation, 2) increased cellular uptake, 3) improved ocular tissue exposure and persistence, and 4) enhanced efficacy of LEDGF_{1-326}.

Zn(II) is known to cause structural changes in protein upon binding (Baudier and Gerard, 1983; Hitomi et al., 2001; Li et al., 2008) and our data is supportive of structural
changes in LEDGF1-326. The decrease in the fluorescence signal at 340 nm and a red shift of E_{max} (Figure 6.2A) indicated that Zn(II) might have induced unfolding of LEDGF1-326. The CD signal indicated secondary structural changes in LEDGF1-326 (Figure 6.2B). Similarly UV-Vis signal also indicated structural perturbation of LEDGF1-326 (Figure 6.2C).

![Figure 6.14. Putative Pathway of Nanoassembly Formation.](image)

**Figure 6.14. Putative Pathway of Nanoassembly Formation.** Zn(II) induced structural shuffling in LEDGF1-326 leading to the formation of nanoassemblies.

We hypothesized that Zn(II) induced temporary unfolding of LEDGF1-326, followed by Zn(II) protein interaction and finally refolding and formation of nanoassemblies (Figure 6.14). LEDGF1-326 in nanoassemblies is conformationally different than native LEDGF1-326 (Table 6.2). The time required to undergo all the above
changes was dependent on Zn(II) concentration. The higher the Zn (II) concentration, the faster was the nanoassembly formation. Our time dependent size data (Table 1), fluorescence data at 30 min (Figure 6.3) and fluorescence, CD and UV-Vis data at 24 hr (Figure 6.2) supports this hypothesis. However, measuring the kinetics of nanoassembly formation was beyond the scope of this manuscript and will be investigated in future.

The nanoassembly size was resistant to dilutions (Figure 6.4) but susceptible to EDTA chelation (Figure 6.5), consistent with chemical coordinate bond formation between Zn(II) and LEDGF_{1-326}. EDTA being a strong chelating agent, it might have sequestered Zn(II), resulting in disruption of nanoassemblies and reversal of all structural changes in LEDGF_{1-326}. The data further suggested that LEDGF_{1-326} structural changes are not permanent and can be reversed back to native form upon Zn(II) removal.

Structural changes in LEDGF_{1-326} due to nanoassembly formation had a positive effect on LEDGF_{1-326} stability. LEDGF_{1-326} in nanoassembly was significantly resistant to tertiary and secondary structure perturbation (Figure 6.6C, and 6.6D). Nanoassembly prevented LEDGF_{1-326} from unfolding, and fragmenting (Figure 6.6B). On the other hand, LEDGF_{1-326} underwent significant unfolding (Figure 6.6C, and 6.6D), and loss of monomeric band (Figure 6.6B) in the absence of Zn(II). LEDGF_{1-326} particle size reduction also supported structural degradation of LEDGF_{1-326} in the absence of Zn(II) (Figure 6.6A).

Uptake of LEDGF_{1-326} in ARPE-19 cells was time- and concentration- dependent (Figure 6.7). At 2 hr, there was no significant difference in LEDGF_{1-326} and nanoassembly uptake. However, upon 6 hr incubation, while LEDGF_{1-326} uptake increased marginally, nanoassembly uptake increased significantly. The data suggests
that either nanoassembly uptake is delayed or once engulfed; nanoassembly is retained in
the retinal cells for longer time as compared to LEDGF\textsubscript{1-326}. The percentage uptake
decreased with increasing concentration for both LEDGF\textsubscript{1-326} and nanoassembly,
suggesting a limit to retinal uptake efficiency (Figure 6.7B).

Significantly high levels of nanoassemblies were present in ocular tissues
including vitreous, choroid-retina, and aqueous humor for 2 weeks, while LEDGF\textsubscript{1-326}
was cleared out in 3 days (Figures 6.8, 6.9, and 6.10). The mean residence time was 22-
296 fold higher for nanoassemblies compared to LEDGF\textsubscript{1-325}. In the past, a direct
correlation has been identified between the molecular weight and persistence or clearance
of a molecule from the vitreous humor (Durairaj et al., 2009). Our data strengthens the
established theory. With an increase in the size of the molecule with the formation of
nanoassembly, the drug persisted for longer time vitreous and subsequently in other
ocular tissues. The pharmacokinetic study clearly suggested increased persistence or
reduced clearance of nanoassembly as compared to LEDGF\textsubscript{1-326}, possibly due to an
increase in particle size.

Interestingly, ELISA (Figure 6.11) detected LEDGF\textsubscript{1-326} in the retina, providing a
first time evidence of LEDGF\textsubscript{1-326} transport to retina after intravitreal injection. Presence
of significantly higher LEDGF\textsubscript{1-326} in the retina at 2 weeks after intravitreal injection with
nanoassemblies when compared to the native protein is consistent with increased
persistence, and reduced degradation of LEDGF\textsubscript{1-326}, when delivered as nanoassembly, in
ocular tissue.

Nanoassemblies improved survival of serum starved ARPE-19 cells as compared
to plain LEDGF\textsubscript{1-326} upon 24 hr treatment (Figure 6.12). However; we did not find a
statistical difference. Different ERG measurements were done to determine the effect of nanoassemblies on various retinal cells (Figure 6.13). The scotopic ERG measures rod photoreceptor functionality, photopic ERG measures cone photoreceptor functionality, b-wave amplitude indicates the electrical activity specifically in retinal cells post-synaptic to the photoreceptors (Miller and Dowling, 1970). Oscillatory potential reflect activity initiated by amacrine cells in the inner plexiform layer of the retina (Wachtmeister and Dowling, 1978; Yonemura and Kawasaki, 1979; Heynen et al., 1985), and the 30 Hz flicker reflects cone functionality. LEDGF$_{1-326}$ delayed functional loss of photoreceptors (rods and cones) in RCS rat until 12$^{th}$ week as seen in scotopic and photopic ERGs (Figure 6.13A and 6.13B). However, beyond week 12, LEDGF$_{1-326}$ efficacy was reduced and there was no significant difference in b-wave amplitude of LEDGF$_{1-326}$, buffer, and Zn(II) treated rats. Nanoassembly treated rats had higher b-wave amplitude as compared to LEDGF$_{1-326}$ throughout the study (Figure 6.13A, 6.13B). Interestingly, despite initial loss of b-wave in scotopic ERG by week 6, there was no further significant loss in b-wave amplitude beyond 6$^{th}$ week and until 14$^{th}$ week in the nanoassembly treated rats. Nanoassembly also prolonged efficacy of LEDGF$_{1-326}$; the 30 Hz flicker amplitude loss was reduced until 14$^{th}$ week (Figure 6.13D). There was no statistically significant difference in OP amplitudes of all groups suggesting LEDGF$_{1-326}$ and its nanoassemblies might not be playing a role in the amacrine cell functionality (Figure 6.13C). In summary, nanoassemblies improved and prolonged LEDGF$_{1-326}$ efficacy in treating retinal degeneration until week 14 after a single dose. It is possible that nanoassemblies may persistently improve the performance of photoreceptors beyond 14$^{th}$ week, while
LEDGF\textsubscript{1-326} may no longer be effective after 12\textsuperscript{th} week. However, we did not test this hypothesis as the animals were euthanized at week 14.

**Conclusions**

In conclusion, Zn(II) induced self-assemblies of LEDGF\textsubscript{1-326} in a concentration-dependent manner. The nanoassemblies induced structural changes in LEDGF\textsubscript{1-326}, which were reversible in the presence of chelating agent like EDTA. Nanoassembly not only increased in vitro cellular uptake and in vitro and *in vivo* stability of LEDGF\textsubscript{1-326}, but also prolonged it’s *in vivo* tissue exposure. It also improved and prolonged LEDGF\textsubscript{1-326} efficacy in a retinal degeneration animal model. Since no organic solvent was used in the preparation of nanoassemblies, the issues related to presence of organic solvent in sustained release drug delivery systems commonly used for protein encapsulation is not a concern with nanoassemblies. Thus, this novel delivery system is a promising alternative for sustaining therapeutic protein delivery in ocular tissues.
CHAPTER VII

DEVELOPMENT OF A POLYMERIC SUSTAINED DELIVERY SYSTEM FOR LEDGF<sub>1-326</sub> USING SUPERCRITICAL CARBON DIOXIDE INFUSION AND PRESSURE QUENCH TECHNOLOGY

Abstract

**Purpose**

The objective of the current study was to prepare a novel polymeric sustained delivery system for LEDGF<sub>1-326</sub>, while avoiding protein denaturing organic solvents commonly used in preparing polymeric particles.

**Methods**

Effect of supercritical carbon dioxide (SC CO<sub>2</sub>) on LEDGF<sub>1-326</sub> protein was investigated by monitoring structural characteristics using fluorescence spectroscopy, circular dichroism, size exclusion chromatography, and SDS-PAGE; size using dynamic light scattering; and presence of nuclear binding domain using ELISA. LEDGF<sub>1-326</sub> coated poly-lactic acid (PLA) nanoparticles (NP) were incorporated into poly-lactic-co-glycolide (PLGA) microparticles (MP) using SC CO<sub>2</sub> infusion and pressure quench technology to form nanoparticles in porous microparticles (NPinPMP). NPinPMP were characterized for morphology and *in vitro* release. *In vivo* LEDGF<sub>1-326</sub> delivery from NPinPMP was monitored using noninvasive ocular fluorophotometry in Sprague Dawley (SD) rats after a single intravitreal injection.
Results

Exposure to SC CO$_2$ did not alter LEDGF$_{1-326}$ size and structure but increased its immunoreactivity to 2.3 fold and monomer content to 2 fold. NPinPMP of ~11.56 µm diameter, encapsulating LEDGF$_{1-326}$ were formed. NPinPMP released LEDGF$_{1-326}$ with an initial ~20% burst release followed by slowly release over a 3 month period in vitro. NPinPMP persisted and sustained LEDGF$_{1-326}$ release in vivo for 2 months in the SD rat vitreous, while LEDGF$_{1-326}$ solution was undetectable by day 3. Immunoreactive LEDGF$_{1-326}$ was present in rat retinas at 2 months after a single intravitreal injection of LEDGF$_{1-326}$ either as NPinPMP suspension or solution, with the levels being higher for NPinPMP.

Conclusion

LEDGF$_{1-326}$ was successfully encapsulated in NPinPMP without being exposed to any organic solvent by the SC CO$_2$ infusion and pressure quench technology. NPinPMP sustained LEDGF$_{1-326}$ release for 3 months in vitro and 2 months in vivo. Thus, NPinPMP is a promising technology that can be used to reduce dosing frequency and sustain the delivery of therapeutic proteins in treating chronic posterior segment eye disorders.

Introduction

Age related macular degeneration (AMD) is a common retinal disease that affects about 8 million people in the USA and the number is expected to rise to 12 million by 2020 (Jager et al., 2008). Many protein drugs including ranibizumab (Lucentis®, Genentech, CA; a monoclonal antibody Fab fragment) (Heier et al., 2006b), bevacizumab (Avastin®, Genentech, CA; a full length monoclonal antibody) (Spaide et al., 2006), and aflibercept (Eylea®, Regeneron Pharmaceuticals, NY; VEGF receptor fusion protein) are
FDA approved for treating the wet form of AMD (Dixon et al., 2009). While no FDA approved drug is available for treating the dry form of AMD, which is more common compared to wet AMD, growth factors including ciliary derived neurotrophic factor (CNTF) (Sieving et al., 2006), brain-derived neurotropic factor (BDNF) (LaVail et al., 1998), and others (Faktorovich et al., 1992; Delyfer et al., 2004; Leveillard et al., 2004a; Leveillard et al., 2004b; Andrieu-Soler et al., 2005; Delyfer et al., 2005) are under investigation. Lens epithelium derived growth factor fragment (LEDGF\textsubscript{1-326}) was identified in our lab as a potential protein drug for the treatment of retinal degenerations. LEDGF\textsubscript{1-326} reduced protein aggregation and rescued retinal cells from aggregation mediated damage (Baid et al., 2011a). It also delayed retinal degeneration for eight weeks in Royal College of Surgeons rat model retinal degeneration after a single intravitreal injection (Baid et al., 2013).

Treatment of wet AMD requires frequent intravitreal injections of the above drugs; frequent injections lead to several complications such as cataracts, endophthalmitis, and retinal detachment, potentially leading to patient noncompliance (Jonas et al., 2003; Sampat and Garg, 2010). Novel drug delivery systems which could sustain and/or control drug release for extended periods of time in the eye have gained major attention during the last two decades (Edelhauser et al., 2008). Biodegradable (e.g., those based on poly lactide-co-glycolide (PLGA) or poly caprolactone (PCL)) and non-biodegradable (e.g., those based on poly vinyl alcohol (PVA) and ethylene vinyl acetate (EVA)) implants provide a platform for sustaining drug release for months to years (Lee et al., 2010).
NT-501 (Neurotech USA), an intraocular nondegradable polymeric implant, is undergoing clinical trials for the delivery of ciliary neurotropic factor (CNTF) for the treatment of dry AMD (Kauper et al., 2012). However, the implant requires surgical placement (Lee et al., 2010). Biodegradable implant manufacturing procedures including melt extrusion may not be suitable for protein drugs. Biodegradable micro- or nanoparticles are alternatives to implants for sustained drug release (Shelke et al., 2011). Micro- or nanoparticles based on PLA/PLGA were extensively investigated for the delivery of therapeutic proteins and peptides (Cohen et al., 1991; Geroski and Edelhauser, 2000; Lam et al., 2000; Takada et al., 2003). Proteins have been encapsulated in the polymeric particles using numerous methods including double emulsion solvent evaporation, spray drying, and ultrasonic atomization methods (Jain, 2000; Berkland et al., 2001; Takada et al., 2003). Emulsion solvent evaporation method is the most commonly used technique for protein encapsulation because of relatively simple steps, convenience of controlling manufacturing parameters, and cost effective production. However, particle preparation by this method and other methods indicated above involves use of organic solvents such as dichloromethane or ethyl acetate, which can cause protein instability (Estey et al., 2006). Further, use of high energy sonication and high temperature during particle preparation have deleterious effect on proteins, resulting in loss of biological activity and/or gain of immunogenicity (van de Weert et al., 2000; Hermeling et al., 2004; Estey et al., 2006; Short, 2008; Ye et al., 2010).

Supercritical fluid carbon dioxide (SC CO$_2$) has been used in the past in pharmaceutical industry as an alternative medium to replace organic solvents, due to its non-toxic, nonflammable, and high availability. At and above a temperature of 31 °C and...
a pressure of 72 bar (1044 psi) (Figure 7.1), CO\textsubscript{2} has both liquid-like as well as gas-like properties and is referred to as supercritical (SC) CO\textsubscript{2}. SC CO\textsubscript{2} has excellent solvent (Nalawade et al., 2006), anti-solvent (Meng et al., 2008), and plasticizing (Hao et al., 2004) properties. SC CO\textsubscript{2} has been successfully used for the development of nano- and micro-particles (Benedetti et al., 1997; Reverchon et al., 2002). Amorphous polymers such as PLGA expand and become porous in the presence of SC CO\textsubscript{2}, while crystalline polymers such as PLA do not exhibit such changes (Kompella and Koushik, 2001). The differential action of SC CO\textsubscript{2} on different polymers have been utilized in the past to form expanded porous and non-porous particles (Moshashae et al., 2000; Koushik and Kompella, 2004; Mayo et al., 2010) and protein powders (Cape et al., 2008). CO\textsubscript{2} plasticized PLGA particles under the supercritical conditions and when the pressure was released or quenched, it enforced expansion of microparticles and formed pores in it (Koushik and Kompella, 2004). During this process, SC CO\textsubscript{2} also removed the residual organic solvent from the microparticles, rendering them almost completely free from the organic solvent.

In this current project, we utilized the above knowledge about SC CO\textsubscript{2} and formulated a novel particulate system named as “nanoparticles in porous microparticles” (NPinPMP). We thereafter investigated the possibility of NPinPMP to encapsulate and sustain the \textit{in vivo} delivery of LEDGF\textsubscript{1-326}.

\textbf{Materials and Methods}

L-PLA (inherent viscosity 1.0 dL/g) and PLGA 50:50 with acid end group (inherent viscosity of 0.67 dL/g) were obtained from Durect Corporation (Birmingham,
Figure 7.1. Supercritical Fluid CO\textsubscript{2} Phase Diagram. CO\textsubscript{2} behave as supercritical fluid above 304 °K and 72 bar/1044 psi. The different colors and shapes in the figure are arbitrarily chosen to represent the compaction of molecules in various states.

AL, USA). Bicinchoninic acid (BCA) kit was obtained from Thermo Scientific (Pierce Biotechnology, IL, USA). HPLC grade dichloromethane (DCM) and other solvents were obtained from Fisher Scientific (Pittsburgh, PA). Liquid carbon dioxide (99.95%) obtained from Airgas Company (Denver, CO). All other materials unless specified were obtained from Sigma Chemical Co. (St. Louis, MO). LEDGF\textsubscript{1-326} was prepared as previously described (Baid et al., 2013).

Particle Preparation

Polymeric particles were prepared using double emulsion solvent evaporation method. First 100 mg of polymer was dissolved in 1 ml of DCM. To this 200 µl of deionized water was emulsified by sonicating (Misonix Inc., USA) for 1 min under ice
cold condition at a power level of 10 W. The primary emulsion was then dispersed in 50 ml of 2 % polyvinyl alcohol solution by sonicating for 5 min at 30 W. The freshly prepared nanoparticles were then hardened along with solvent removal by stirring for 3 hr at room temperature. PLA nanoparticles were collected by centrifugation (Beckman, USA) at 12,000 rpm for 15 min at 4°C, washed and finally suspended in 50 ml of deionized water. Particles were dried by lyophilizing for 24 hrs. For PLGA microparticles homogenization at 10,000 rpm for 1 min (Virtishear Cyclone®, USA) was done instead of sonication.

**Supercritical (SC) CO₂ Treatment**

Briefly, 100 µl of LEDGF₁-₃₂₆ (10 mg/ml) was added to 5 mg of PLA nanoparticles (NP) and incubated at 4°C for 30 min and lyophilized overnight. Thereafter, LEDGF₁-₃₂₆ coated PLA NP were mixed with plain PLGA MP (1:9) and placed in a closed high pressure vessel. The particles were exposed to supercritical (SC) CO₂ at a temperature of 33°C and pressure of 1150-1200 psi for 30 min. After SC CO₂ exposure, the pressure was released over a minute and the NPinPMP formed were collected. NPinPMP were washed for 30 min and drug loading was estimated. Particles were lyophilized overnight and used for further studies. Similarly, LEDGF₁-₃₂₆ solution was exposed to SC CO₂ for investigating effect of SC CO₂ on protein conformation.

**Drug Loading and Encapsulation Efficiency**

Drug loading/encapsulation was measured indirectly by subtracting the protein quantity measured after washing from the total protein loaded in NPinPMP. The total theoretical loading was 2 % w/w and the measured LEDGF₁-₃₂₆ in wash buffer was 23 %
of the total LEDGF\textsubscript{1-326}. This accounts for a loading of 1.54 % w/w, with a loading efficiency of 77%.

**In Vitro Drug Release**

LEDGF\textsubscript{1-326} encapsulated NPinPMP were evaluated for *in vitro* release in PBS pH 7.4. About 10 mg of NPinPMP was weighed and dispersed in 1 ml of PBS pH 7.4 and incubated at 37 °C under shaking at 200 rpm (Max Q shaker incubator, Thermo scientific, Asheville, NC, USA). At pre-determined time points, the suspended particles were centrifuged at 13,000 g for 15 min and the supernatant was collected. The pellet comprising particles was re-suspended in 1 ml of fresh PBS pH 7.4 and incubated. The LEDGF\textsubscript{1-326} content in the samples was estimated using micro BCA assay as per the user’s manual.

**Fluorescence Spectroscopy**

The steady state fluorescence spectroscopy was done to determine the changes in the tertiary structure before and after SC CO\textsubscript{2} treatment of LEDGF\textsubscript{1-326}. The intrinsic tryptophan (Trp) fluorescence spectra of LEDGF\textsubscript{1-326} were recorded from 300 to 400 nm, at 280 nm excitation, with every 1 nm increment using Spectramax M5 (Molecular Devices, Downingtown, PA). Buffer and inner filter effects were corrected for all fluorescence values.

**Circular Dichroism (CD)**

Secondary structural changes of LEDGF\textsubscript{1-326} were determined by far-UV CD. Briefly, LEDGF\textsubscript{1-326} sample was placed in 1 mm quartz cuvette and spectra were recorded at a scan speed of 0.5 sec per data point, step size of 1 nm and the bandwidth of
4 nm from 200 to 280 nm using Chirascan® CD instrument (Applied Photophysics Ltd, UK).

**Dynamic Light Scattering (DLS)**

LEDGF$_{1-326}$ protein hydrodynamic size was monitored using Nano ZS (Malvern, Westborough, MA). Briefly 100 µl of sample was placed in low volume glass cuvette. Using dynamic light scattering technique, LEDGF$_{1-326}$ particle scattering data was collected at 173° backscatter angle. An average of 13 scans was done for each measurement.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

LEDGF$_{1-326}$ samples (10 µg) were boiled for 10 min at 75 °C in 10 µl of 2x loading buffer. Protein samples were loaded on 4-15 % mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA) and size separated by electrophoresis. Separated proteins were visualized using Coomassie Blue staining as per user’s protocol.

**Size Exclusion Chromatography (SEC)**

LEDGF$_{1-326}$ samples were size separated using size exclusion chromatography (Waters, Milford, MA). Briefly, Agilent Bio SEC-3 size exclusion column was equilibrated with PBS buffer pH 7.4 at flow rate of 1 ml/min. Injection volume of 10 µl of sample was given and LEDGF$_{1-326}$ size separation was monitored by UV absorbance.

**Ocular Fluorophotometry**

Adult male Sprague Dawley (SD) rats (150-180 g) were purchased from Harlan Laboratories (Indianapolis, IN). Animals were treated as per the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic
and Vision Research. Animal protocols followed during this study were approved by the
Institutional Animal Care and Use Committee of the University of Colorado Anschutz
Medical Campus, Aurora, CO, USA.

In vivo delivery of LEDGF$_{1-326}$ from NPinPMP was evaluated following
intravitreal administration of Alexa Fluor® 488 conjugated LEDGF$_{1-326}$ in NPinPMP in
non-diseased SD rats. Briefly, LEDGF$_{1-326}$ was conjugated with Alexa Flour® 488 dye
according to the user’s manual (Alexa Fluor® 488 Protein Labeling Kit, Invitrogen,
USA). Alexa-LEDGF$_{1-326}$, was thereafter encapsulated into NPinPMP similar to the
procedures described earlier. Rats were anesthetized by intraperitoneal injection of
ketamine (35 mg/kg)/xylazine (5 mg/kg). Betadine solution was applied on the eye
surface to avoid any infection post intravitreal injection. Thereafter, 5 µl of 1 mg/ml
suspension of Alexa-LEDGF$_{1-326}$ encapsulated NPinPMP in PBS pH 7.4 was injected
intravitreally in the right eye using a 30-G needle. Left eyes were injected with 5 µl of
mixture of Alexa-LEDGF$_{1-326}$ and LEDGF$_{1-326}$ solution. The mixture was made such that
it had initial fluorescence equivalent to NPinPMP, while the total dose of LEDGF$_{1-326}$
remains same. Fluorescence of Alexa-LEDGF$_{1-326}$ was monitored periodically using
ocular fluorophotometry (Fluorotron Master™, Ocumetrics, CA, USA) until the
fluorescence reached the baseline. Baseline fluorescence values of eyes were monitored
before injecting the formulations. At each time point, three fluorophotometric scans were
taken per animal and averaged. Since Fluorotron Master™ provides fluorescein (NaF)
concentration for each fluorescence value, a standard curve for Alexa-LEDGF$_{1-326}$ was
obtained using a cuvette adapter in Fluorotron Master™. The standard curve was used to
convert fluorescein equivalent concentrations provided by fluorophotometer to corresponding LEDGF<sub>1-326</sub> concentration in each study group. At the end of study, animals were sacrificed, eyes were enucleated and ocular tissues were separated. LEDGF<sub>1-326</sub> was extracted and estimated using ELISA.

**ELISA**

An indirect ELISA method was used to determine immunoreactive LEDGF<sub>1-326</sub> in samples. Briefly, in 96-well plate, 100 µl of either LEDGF<sub>1-326</sub> standards (freshly purified) or LEDGF<sub>1-326</sub> samples were coated and incubated overnight at 4 °C in triplicates. Wells were washed three times with wash buffer (0.1 % w/v Tween 20 in PBS pH 7.0) after each step. The nonspecific binding sites were blocked with blocking solution (0.5 % bovine serum albumin, and 0.1 % Tween 20 in PBS pH 7.0) for 4 hours. LEDGF<sub>1-326</sub> was detected by mouse anti-LEDGF antibody (1:1,000) (BD Biosciences, San Diego, CA) which was cross detected with HRP conjugated anti-mouse secondary antibody (1:10,000) (BD Biosciences, San Diego, CA). After thorough washing of the plate finally 3,3’,5,5’-Tetramethylbenzidine (TMB) was added. All solutions added were 200 µl and unless specified, all incubation were done at room temperature. Immunoreactive LEDGF<sub>1-326</sub> was quantitated by colorimetric absorbance at 650 nm upon development of blue color after 20 mins.

**Statistical Analysis**

Data represented as the mean ± SD. Statistical analysis was done by one-way ANOVA followed by Tukey’s post hoc analysis (SPSS, ver.11.5; SPSS, Chicago, IL). p ≤ 0.05 was considered to be statistically significant.
Results

Effect of SC CO\(_2\) Exposure on LEDGF\(_{1-326}\)

Effect of SC CO\(_2\) exposure on LEDGF\(_{1-326}\) was investigated by intrinsic fluorescence spectroscopy, far-UV CD, DLS, reducing SDS-PAGE, SEC, and ELISA (Figure 7.2). The fluorescence and CD spectrum of LEDGF\(_{1-326}\) before and after exposure to SC CO\(_2\) had no difference (Figure 7.2A and 7.2B). The hydrodynamic size of LEDGF\(_{1-326}\) as measured by DLS was ~ 8 nm before and after SC CO\(_2\) exposure (Figure 7.2C). Reducing SDS-PAGE indicated no changes in the monomer band of LEDGF\(_{1-326}\) before and after SC CO\(_2\) exposure (Figure 7.2D). SEC was done to determine the monomer content in LEDGF\(_{1-326}\) protein (Figure 7.2E). SEC indicated that LEDGF\(_{1-326}\) monomer retention time was 10 min. Before SC CO\(_2\) treatment the relative area under the curve (AUC) for LEDGF\(_{1-326}\) monomer was 25 % which increased to 49 % when exposed to SC CO\(_2\). LEDGF\(_{1-326}\) immunoreactivity increased 2 fold with SC CO\(_2\) exposure (Figure 7.2F).

NPinPMP Preparation and Characterization

Aqueous coated LEDGF\(_{1-326}\) nanoparticles were infused in porous microparticles during SC CO\(_2\) exposure (Figure 7.3). The mean particle size of PLA and PLGA was ~200 nm and, ~2 \(\mu\)m, respectively. Upon exposure to SC CO\(_2\), PLGA particle size increased to 11.5 \(\mu\)m. The surface morphology of microparticle (MP) changed from non-porous to highly porous (PMP) particles with NP infused inside the PMP forming NPinPMP (Figure 7.4). LEDGF\(_{1-326}\) loading was estimated to be 1.54 % with 77 % encapsulation efficiency.
Figure 7.2. SC CO₂ effect on LEDGF₁-326. LEDGF₁-326 was exposed to SC CO₂ for 30 min at 1150-1200 psi and 33 °C. Changes in LEDGF₁-326 conformation, size, monomer content, and immunogenicity was investigated by A) Fluorescence spectroscopy, B) Far-UV CD, C) dynamic light scattering, D) SDS-PAGE (Reducing), E) Size exclusion chromatography, and F) ELISA. Data represent mean ± S.D. of N=1. For ELISA data represent mean ± S.D. of N=3 samples (same batch). * indicates p<0.05 is statistically significant.
Figure 7.3. **Illustration of NPinPMP Preparation.** Poly lactic-co-glycolic acid (PLGA) microparticles (MP) expand and porosify in presence of SC CO$_2$, while poly lactic acid (PLA) nanoparticles (NP) coated with LEDGF$_{1-326}$ infuse inside the porous microparticles (PMP) to form NPinPMP.

**NPinPMP Sustained In Vitro Release of LEDGF$_{1-326}$**

*In vitro* release of LEDGF$_{1-326}$ from NPinPMP was monitored in PBS buffer at 37 °C. LEDGF$_{1-326}$ was released from NPinPMP in by phasic profile (Figure 7.5). An initial fast burst release within 24 hr was seen followed by slow sustained release for 3 months. NPinPMP had initial release of 20 ± 2 % within 24 hr with a complete release of 95 ± 6 % of LEDGF$_{1-326}$ in 3 month period.

**NPinPMP Persisted and Sustained LEDGF$_{1-326}$ Delivery in Ocular Tissues**

Persistence of LEDGF$_{1-326}$ encapsulated NPinPMP in SD rat was measured using the fluorescence signal of Alexa-LEDGF$_{1-326}$ (Figure 7.6). Figure 7.6A and 7.6B are fluorescence scans of LEDGF$_{1-326}$ solution and NPinPMP, respectively, from 5 min to 2 month after single intravitreal injection. Soon after intravitreal injection,
a peak fluorescence of LEDGF₁-326 was observed in vitreous which rapidly declined within 24 hr for solution and was undetectable by day 3 (Figure 7.6A). In NPinPMP injected rats, there was initial peak fluorescence with a rapid decline in 24 hr followed by emergence of a peak on day 25 which declined slowly over a period of 2 months (Figure 7.6B).

![Figure 7.4. Change in Surface Morphology after SC CO₂ Exposure.](image)

Nanoparticle  Microparticle
X50000  X1500

NPinPMP  NPinPMP
X1000  X3000

Figure 7.4. Change in Surface Morphology after SC CO₂ Exposure. PLGA microparticles expanded due to exposure to SC CO₂ and morphology changes to porous surface. The PLA nanoparticles did not expand but infused inside the porous PLGA microparticles and partly blocked the pores.
The fluorescence signal in vitreous obtained from the scans (Figure 7.6A and 7.6B) was converted to actual LEDGF$_{1-326}$ concentration (Figure 7.7A). After 5 min of intravitreal injection, a peak value of 21 and 18 µg/ml of LEDGF$_{1-326}$ were observed in solution and NPinPMP respectively. LEDGF$_{1-326}$ solution peak concentration quickly declined to 2 µg/ml within 24 hr, and was below the baseline by day 3. On the other hand NPinPMP maintained 4 µg/ml of LEDGF until day 7. Thereafter, LEDGF$_{1-326}$ concentration in vitreous increased to 41 µg/ml by day 25 followed by slow decline to 2 µg/ml by day 50 in NPinPMP.

Following fluorophotometry, LEDGF$_{1-326}$ immunoreactivity was investigated in various ocular tissues and in blood after 2 months by indirect ELISA (Figure 7.7B). LEDGF$_{1-326}$ was undetectable in blank eye tissues indicating absence of any detectable quantity of endogenous LEDGF$_{1-326}$. Interestingly, LEDGF$_{1-326}$ was detectable for both
Figure 7.6. NPinPMP had Prolonged Persistence in Ocular Tissues. Persistence of NPinPMP in rat vitreous was investigated by measuring the fluorescence of Alexa-LEDGF$_{1-326}$ encapsulated NPinPMP by Fluorotron Master®. Single intravitreal injection of 5 µl of 1 mg/ml of Alexa-LEDGF$_{1-326}$ NPinPMP or solution was administered in SD rat eye. Fluorotron scans were taken noninvasively from 5 min to 2 months. Data represents average of N=4 independent animals eye scans.
Figure 7.7. NPinPMP Sustained LEDGF<sub>1-326</sub> In Vivo Release. A) Amount of Alexa-LEDGF<sub>1-326</sub> released in SD rat vitreous was calculated from the fluorescence scans obtained in Figure 7.6. B) ELISA- Following pharmacokinetic study, LEDGF<sub>1-326</sub> ELISA was done to detect immunoreactive LEDGF<sub>1-326</sub> in various ocular tissues. AH= Aqueous Humour, VH=Vitreous Humour Significant high levels of LEDGF<sub>1-326</sub> were present in retina for both NPinPMP and solution injected rats. LEDGF<sub>1-326</sub> was also detected in choroid in solution injected group. Data represent average of N=4 independent animals ± SD. *, p<0.05 as compared to corresponding blank group.
solution and NPinPMP group in retina, being 25 ± 7 and 20 ± 7 ng/ml, respectively. LEDGF<sub>1-326</sub> solution was also detected in choroid at the level of 5 ± 2 ng/ml.

**Discussion**

In the current study we developed a novel NPinPMP for sustained delivery of LEDGF<sub>1-326</sub>. SC CO<sub>2</sub> exposure was mild and had no deleterious effect on LEDGF<sub>1-326</sub>. There was neither tertiary and secondary structure perturbation nor any chemical degradation or fragmentation of LEDGF<sub>1-326</sub> as indicated by fluorescence spectroscopy, CD, DLS, and SDS-PAGE (Figure 7.2). Interestingly SC CO<sub>2</sub> almost doubled the monomeric content of LEDGF<sub>1-326</sub> as indicated by SEC (Figure 2E), that might have led to increase in immunoreactivity in ELISA (Figure 7.2F).

LEDGF<sub>1-326</sub> encapsulated NPinPMP (Figure 7.3) were prepared using the SC CO<sub>2</sub> infusion and pressure quench technology. Initially under high pressure, SC CO<sub>2</sub> diffused in amorphous PLGA microparticles, decreased the glass transition temperature and plasticized the microparticles (Koushik and Kompella, 2004). On the other hand SC CO<sub>2</sub> had no effect on crystallin PLA nanoparticles. During pressure quenching, SC CO<sub>2</sub> expanded rapidly forming porous enlarged microparticles, in situ infused with LEDGF<sub>1-326</sub> coated nanoparticles. SEM images (Figure 7.4) clearly indicated changes in the surface morphology, particle size, and presence of nanoparticle inside microparticle. This technology completely eradicated LEDGF<sub>1-326</sub> exposure to denaturing organic solvents, harsh sonication, and hours of continuous stirring; typically unavoidable in traditional emulsion solvent evaporation method of particle preparation.
Complete release of LEDGF$_{1-326}$ from NPinPMP was observed over a period of 3 months \( \textit{in vitro} \) (Figure 7.4). Non-invasive ocular fluorophotometry indicated a sustain release of LEDGF$_{1-326}$ from NPinPMP over a period of 2 months. While LEDGF$_{1-326}$ solution got cleared away within 3 days, NPinPMP demonstrated continuous release until 2 months. A second peak of high fluorescence at about 25 days after initial lag phase in NPinPMP group indicated possible degradation of polymer and release of high level of LEDGF$_{1-326}$.

ELISA demonstrated LEDGF$_{1-326}$ uptake and persistence in retina after 2 months of intravitreal injection. Immunoreactive LEDGF$_{1-326}$ retinal level was similar in NPinPMP and solution, with solution indicating slightly higher level. One possible explanation could be that NPinPMP may be persisting in vitreous and cannot diffuse in the retina. LEDGF$_{1-326}$ released from the NPinPMP diffused inside the retina and ELISA detected only free LEDGF$_{1-326}$ (released LEDGF$_{1-326}$ from NPinPMP). Unreleased LEDGF$_{1-326}$, bound to NPinPMP, might be undetectable. Another interesting finding from ELISA was the presence of immunoreactive LEDGF$_{1-326}$ in choroid-retina in solution injected rats; this suggested the mobility of LEDGF$_{1-326}$ across retinal layers after intravitreal injection.

**Conclusion**

SC CO$_2$ infusion and pressure quench technology successfully formed novel NPinPMP, which sustained LEDGF$_{1-326}$ \( \textit{in vitro} \) and \( \textit{in vivo} \) release for 3 and at least 2 months, respectively. NPinPMP is a novel sustained delivery system for protein drugs.
CHAPTER VIII

SUMMARY AND FUTURE DIRECTIONS

Dry age related macular degeneration (dry AMD) and retinitis pigmentosa (RP) are two major forms of retinal degeneration. Because of the multifactorial nature of the above two diseases, it is very challenging to develop a therapeutic agent. Currently there is no FDA approved drug therapy for dry AMD or RP.

Proteins have gained much success in the treatment of other eye diseases such as wet AMD. However, due to their complex structure, they have to be biosynthesized, unlike small molecules which can be chemically synthesized. Further, because of their large molecular size, they are highly susceptible to chemical and physical degradation. Thus, protein production and stabilization is a daunting task and needs to be optimized for each individual therapeutic protein.

Retinal degeneration is a posterior eye disorder which requires prolonged treatment. A major hurdle in the treatment of retinal degeneration or any other posterior eye segment disease is the need of repeated intravitreal injections, which increase the risk of cataract, endophthalmitis, and retinal detachment. A sustained delivery system may minimize these risks. However, current methods of protein encapsulation in polymeric particles expose proteins to harsh conditions such as organic solvents and sonication, leading to degradation, aggregation, and functional loss of proteins. Additionally, cellular entry of proteins acting within the cell is another barrier in developing some
protein therapeutics. For the purpose of this project we identified LEDGF, a transcriptional factor, acting intracellularly as a therapeutic agent. However, LEDGF has HIV integrase binding domain and may promote HIV infection. Thus, there is a need to engineer a new protein fragment of LEDGF.

To solve the above problems, the overall objectives of this project were to 1) identify a novel protein fragment of LEDGF for effective therapy of retinal degenerative diseases, 2) biosynthesize, purify, and characterize the novel protein, 3) develop stable aqueous formulations, and 4) develop innovative sustained delivery systems to reduce frequent intravitreal injections of the therapeutic protein.

**Specific Aim 1**

To determine the efficacy of LEDGF\textsubscript{1-326} gene to reduce protein aggregation in retinal pigmented epithelial cells.

**Summary**

We hypothesized that lens epithelium derived growth factor (1-326), a novel fragment of LEDGF, has the ability to reduce protein aggregation and aggregation mediated cellular toxicity. To test this hypothesis, we developed an *in vitro* protein aggregation model by expressing mutant P23H rhodopsin in retinal pigmented epithelial cells. We, thereafter, tested LEDGF\textsubscript{1-326} efficacy to reduce mutant P23H rhodopsin aggregates and aggregation mediated retinal cell damage. Our study demonstrated that LEDGF\textsubscript{1-326} improved retinal cell survival that was compromised due to expression and aggregation of mutant P23H rhodopsin. LEDGF\textsubscript{1-326} also reduced oligomers of mutant P23H and wild type rhodopsin in these cells.
**Future Directions**

Based on the above results, future investigations include

1) Understand the molecular mechanism of LEDGF₁-₃₂₆ involved in reducing P23H rhodopsin aggregation.

2) Test LEGF₁-₃₂₆ efficacy in animal models of retinitis pigmentosa such as transgenic P23H rats. Early intervention vs late intervention would be particularly useful to compare in this regard.

3) Test LEDGF₁-₃₂₆ efficacy in reducing other protein aggregates commonly involved in neurodegenerative diseases.

**Specific Aim 2**

To biosynthesize, purify, and characterize LEDGF₁-₃₂₆ protein and determine its efficacy in inhibiting retinal degeneration.

**Summary**

We hypothesized that LEDGF₁-₃₂₆ will be effective in rescuing photoreceptors of retinal degenerative Royal College of surgeons (RCS) rats. To test this hypothesis, we biosynthesized LEDGF₁-₃₂₆ in a bacterial expression system and purified and characterized its biophysical properties. Thereafter, we tested its efficacy to reduce retinal degeneration in RCS rats. Our study led to high scale production of LEDGF₁-₃₂₆ in pure aggregate free form. LEDGF₁-₃₂₆ was a stable, randomly coiled protein. Single intravitreal injection of LEDGF₁-₃₂₆ reduced the functional and morphological loss of photoreceptors in RCS rats, indicating its potential value in treating retinal degenerative diseases.
Future Directions

Based on the above results, future investigations include

1) Test LEDGF$_{1-326}$ efficacy in other retinal degenerative animal models including those associated with oxidative stress such as Sod1(-/-) and Sod2(-/-) mice.

2) Test LEDGF$_{1-326}$ immunogenicity and toxicity.

3) Test LEDGF$_{1-326}$ multidose and/or combination therapy.

4) Investigate molecular mechanism of LEDGF$_{1-326}$ efficacy.

Specific Aim 3

To develop a stable aqueous formulation of LEDGF$_{1-326}$.

Summary

We hypothesized that LEDGF$_{1-326}$ can be stabilized by using pharmaceutical additives. We used Tween 20, EDTA, and sucrose to formulate LEDGF$_{1-326}$ in citrate-phosphate buffer in the pH range of 6-7.5 and monitored changes in LEDGF$_{1-326}$ conformation and activity over 2 months at 25 °C. Our study demonstrated that additives preserved LEDGF$_{1-326}$ tertiary structure, reduced its propensity to fragment and/or aggregate and preserved its immunoreactivity.

Future Directions

Based on the above results, future investigations include

1) Formulate stable LEDGF$_{1-326}$ lyophilized product.

2) Understand the degradation pathway of LEDGF$_{1-326}$ and formulate a better formulation utilizing this knowledge.

3) Engineer more stable mutants of LEDGF$_{1-326}$ without losing its efficacy.
Specific Aim 4

To formulate a metal ion induced nanoassembly of LEDGF\textsubscript{1-326} to increase cellular uptake and \textit{in vivo} persistence and efficacy.

**Summary**

We hypothesized that LEDGF\textsubscript{1-326} nanoassembly will have increased uptake, and prolonged \textit{in vivo} persistence and efficacy as compared to LEDGF\textsubscript{1-326} solution. To test this hypothesis, we engineered LEDGF\textsubscript{1-326} nanoassemblies using Zn(II) and studied its pharmacokinetics and efficacy in rat models. Our study demonstrated that Zn(II) induces nanoassembly formation in LEDGF\textsubscript{1-326}. Nanoassemblies reduced LEDGF\textsubscript{1-326} structural instability, and increased its \textit{in vitro} cell uptake. Nanoassemblies also increased LEDGF\textsubscript{1-326} persistence in the eye, and improved and prolonged its efficacy in a rat model.

**Future Directions**

Based on the above results, future investigations include

1) Utilize nanoassemblies to formulate other therapeutic proteins and peptides.

2) Investigate the safety, toxicity, and immunogenicity of the nanoassemblies.

3) Improve nanoassembly stability to increase the shelf life by optimizing the formulation components.

Specific Aim 5

To develop an organic solvent free polymeric sustained release system for LEDGF\textsubscript{1-326} using supercritical (SC) CO\textsubscript{2} technology.

**Summary**

We hypothesized SC CO\textsubscript{2} technology will enable LEDGF\textsubscript{1-326} encapsulation in the polymeric particles without the need of LEDGF\textsubscript{1-326} being exposed to organic solvents.
To test this hypothesis, we first investigated the effect of SC CO\textsubscript{2} on LEDGF\textsubscript{1-326} structure and immunogenicity. Thereafter, we formulated a novel nanoparticles in porous microparticles (NPinPMP) encapsulated with LEDGF\textsubscript{1-326} using SC CO\textsubscript{2} infusion and pressure quench technology and monitored its \textit{in vitro} and \textit{in vivo} release profile. Our data demonstrated that SC CO\textsubscript{2} does not denature LEDGF\textsubscript{1-326} and NPinPMP sustain LEDGF\textsubscript{1-326} release for a few months.

\textit{Future Directions}

Based on these results the future investigations include

1) Determine efficacy of LEDGF\textsubscript{1-326} loaded NPinPMP to reduce retinal degeneration.

2) Determine LEDGF\textsubscript{1-326} stability in NPinPMP.

3) Optimize the method to scale-up particle production.
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APPENDIX A

PUBLICATIONS


