March 2023

PAMAM- Cyclodextrin Conjugate Upregulates Brain-Derived Neurotropic Factor in ARPE-19 Cells

Gopika Ashokan
University of South Florida

Follow this and additional works at: https://digitalcommons.usf.edu/etd

Part of the Medicinal Chemistry and Pharmaceutics Commons, Nanoscience and Nanotechnology Commons, and the Ophthalmology Commons

Scholar Commons Citation

This Thesis is brought to you for free and open access by the USF Graduate Theses and Dissertations at Digital Commons @ University of South Florida. It has been accepted for inclusion in USF Tampa Graduate Theses and Dissertations by an authorized administrator of Digital Commons @ University of South Florida. For more information, please contact digitalcommons@usf.edu.
PAMAM- Cyclodextrin Conjugate Upregulates Brain-Derived Neurotropic Factor in ARPE-19 Cells

by

Gopika Ashokan

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science Pharmaceutical Nanotechnology with a concentration in Drug discovery, Delivery, Development and Manufacturing
Department of Pharmaceutical Sciences Taneja College of Pharmacy University of South Florida

Major professor: Subhra Mohapatra, Ph.D Sivakumar Panguluri, Ph.D Manas Biswal, Ph.D

Date of Approval: March 7, 2023

Keywords: PAMAM, TBI, drug delivery, BDNF, nanotechnology

Copyright© 2023, Gopika Ashokan
ACKNOWLEDGMENTS

I thank my PI, Dr. Subhra Mohapatra for helping me throughout this project. I also thank my committee members, Dr. Sivakumar Panguluri and Dr. Manas Biswal for their valuable inputs. I thank Dr. Shyam Mohapatra, Dr. Eleni Markoutsa and Dr. Ryan Green for guiding me. I would like to thank my lab members Karthick Mayilsamy, Andrew McGill, Taylor Martinez for helping me throughout the project. Finally, I thank my dear friend and lab member Kavya Sivakumar for supporting me through all the hard times.
# TABLE OF CONTENTS

List of figures ......................................................................................................................... iv

List of abbreviations ................................................................................................................ v

Abstract .................................................................................................................................... vi

Chapter I: Introduction
  1.1 Background ..................................................................................................................... 1
  1.2 Visual Impairment due to TBI ....................................................................................... 1
  1.3 Role of BDNF in RGC survival ..................................................................................... 3
  1.4 Challenges for ocular drug delivery .............................................................................. 4
  1.5 Advances in nanotechnology for ocular drug delivery .................................................. 5
  1.6 Gene therapy for ocular diseases ................................................................................ 6
  1.7 Dendrimers as gene vectors ......................................................................................... 6

Chapter II: Materials And Methods
  2.1 Materials ....................................................................................................................... 9
  2.2 Visualizing BDNF expression in TBI mouse models .................................................... 9
  2.3 Synthesis of PAMAM- β-CyD Conjugate .................................................................. 10
  2.4 Characterization of PAMAM- β-CyD Conjugate ........................................................... 10
  2.5 Complexation of BDNF expression plasmid with PAMAM- β-CyD ....................... 10
  2.6 Gel electrophoresis ...................................................................................................... 11
  2.7 Assessment of cytotoxicity of nanoparticles on ARPE-19 cells ............................ 11
  2.8 Transfection efficiency of nanoparticles in ARPE-19 cells ........................................ 12
  2.9 Determination of the Bdnf mRNA levels in ARPE-19 cells ...................................... 12
  2.10 Statistical analysis .................................................................................................... 13

Chapter III: Results .................................................................................................................. 14
  3.1 Visualizing BDNF expression in TBI mouse models ................................................... 14
  3.2 Synthesis and Characterization of nanoparticles ....................................................... 15
  3.3 Complexation of BDNF-SEP Plasmid ....................................................................... 17
  3.4 Cytotoxicity Studies .................................................................................................... 19
  3.5 Transfection efficiency ............................................................................................... 20
  3.6 Bdnf mRNA regulation in ARPE-19 cells .................................................................... 21

Chapter IV: Discussion ............................................................................................................ 23

Chapter V: Conclusion .......................................................................................................... 27

References ............................................................................................................................. 28
LIST OF FIGURES

Figure 1: Visual impairment due to TBI ................................................................. 2
Figure 2: BDNF signaling pathway (created using Biorender) ................................ 3
Figure 3: Structure of eye and retina layers .......................................................... 5
Figure 4: Dendrimers as gene vectors ................................................................. 7
Figure 5: Visualizing BDNF expression in TBI mouse model ................................. 14
Figure 6: Synthesis of PAMAM-β-CyD Nanoparticles ........................................... 15
Figure 7: NMR spectra ....................................................................................... 16
Figure 8: Morphology of PAMAM-β-CyD Nanoparticles ....................................... 17
Figure 9: Size characterization .......................................................................... 18
Figure 10: Complexation with BDNF-SEP plasmid ............................................... 18
Figure 11: Cell viability studies ......................................................................... 19
Figure 12: Transfection efficiency of PAMAM and PAMAM-β-CyD complex in ARPE-19 cells .... 20
Figure 13: Bdnf mRNA regulation in ARPE-19 cells ............................................ 21
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARPE</td>
<td>Arising Retinal Pigment Epithelia</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain Derived Neurotropic Factor</td>
</tr>
<tr>
<td>CDC</td>
<td>Centre for Disease Control and Prevention</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial-cell Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro RNA</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotropin-3</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Polyamidoamine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>RFP</td>
<td>Red Fluorescent Protein</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal Ganglion Cells</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPE-65</td>
<td>Retinal Pigment Epithelium specific 65</td>
</tr>
<tr>
<td>SEP</td>
<td>Super Ecliptic Protein</td>
</tr>
<tr>
<td>shRNA</td>
<td>short-hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Silencing RNA</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic Brain Injury</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tropomyosin Receptor Kinase B</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
ABSTRACT

Background: Traumatic Brain Injury (TBI) is a major contributor to death and disability due to motor vehicle accidents, sports, physical abuse, and battlefield injuries. The primary insult to the brain leads to inflammation, vascular dysfunction, and oxidative stress in the brain as well as in the eye. This leads to loss of Retinal Ganglion Cells (RGCs) and downregulation of Brain derived Neurotropic Factor (BDNF). BDNF is a neurotrophic factor that binds to Tropomyosin Receptor Kinase B (TrkB) receptor to promote cell growth, survival, and differentiation. Current treatment strategies do not promote neuronal regeneration. Therefore, novel treatments are needed to restore vision following TBI. Dendrimers are nanoscale, branched polymers that have been widely used to deliver drugs due to their ability to cross the blood-brain barrier (BBB) and blood-retina barrier (BRB). β-cyclodextrin (β-CyD) has a hydrophobic cavity that can encapsulate drugs. We aim to induce BDNF expression in ARPE-19 cells by complexing BDNF plasmid to PAMAM-β-cyclodextrin conjugate.

Methods: In this study, we conjugated β-CyD to Polyamidoamine (PAMAM) dendrimers and used the PAMAM-β-cyclodextrin nanoparticles to deliver BDNF plasmid in retinal pigmented epithelial cell line, ARPE-19. Two different NP (N): plasmid (P) ratios – 2:1 and 5:1 were tested.

Results: BDNF levels were downregulated in TBI mouse retina. Conjugation of β- CyD to PAMAM increased transfection efficiency compared to PAMAM alone. BDNF plasmid was complexed with PAMAM-β-CyD conjugated in different N/P ratios. Higher N/P ratios increase cytotoxicity due to positive charges of PAMAM disrupting the negatively charged cell membrane in the PAMAM-BDNF treatment group. Vice-versa was observed in BDNF
complexed PAMAM-β-CyD treatment groups which might be because β-CyD can interact with phospholipids in the cell membrane. N/P ratio 5:1 increased transfection efficiency and Bdnf mRNA levels compared to 2:1 due to complete condensation of DNA around the PAMAM as a result of higher positive surface charges.

**Conclusion:** BDNF complexed PAMAM-β-CyD conjugate are able to transfec ARPE-19 cells and upregulate Bdnf mRNA levels. This study using ARPE-19 cells can be further validated for ocular drug delivery to the posterior segment in-vivo animal models of TBI. For future studies, the hydrophobic cavity of β-CyD can be utilized to encapsulate drugs to decrease inflammation, oxidative stress, and intraocular pressure in the eye. The nanoparticles-mediated gene therapy may be a promising approach for gene therapy for retinal degeneration post-TBI, in the future.
CHAPTER I: INTRODUCTION

1.1 Background

Traumatic brain injury (TBI) is a major cause of death and disability in the United States. According to the Centre for disease Control and Prevention (CDC), TBI is defined as a bump, blow or jolt to the head that affects the functions of the brain. TBI is caused due to falls, motor vehicle accidents, sports, war injuries, domestic violence, assaults, etc [1]. TBI can be classified into three categories based on the total of motor, verbal, and eye-opening scores, also known as the Glasgow coma scale (3-15), as follows- Mild (13-15), Moderate (9-12) and severe (3-8) [2]. Reports indicate that there were approximately 64,632 TBI related deaths in 2020 [3]. TBI can also be classified into open or closed headed injuries. Open headed injuries are caused when there is a penetrating force to the skull and close headed injuries are usually non penetrating [4]. TBI can lead to primary or secondary injuries. The primary injuries usually refer to a direct mechanical injury to the brain where there is shear stress and compressions involved due to acceleration or deceleration. The secondary injuries include increased intracranial pressure, hypoxia, axonal injury leading to inflammation, cell damage and cell death. Patients with TBI suffer from cognitive, behavioural, and motor deficits. TBI also is an associated risk factor in Alzheimer’s disease and dementia [5].

1.2 Visual impairment due to TBI

Visual impairment caused by TBI ranges from 9-38% based on the severity and the mechanism of injury [6]. TBI can impact vision due to retinal degeneration, lesions in retina, choroidal rupture, damage to the afferent and efferent optic pathways [7]. Optic nerve damages due to TBI often
results in the loss of retinal ganglion cells (RGCs) \[8\]. Das et al showed RGC loss in the retina seven days post repeated TBI (rTBI) induction in mice. In this study, rTBI led to retinal damage by microglia and muller-cell activation \[9\]. While the rods and cones, transmit the signals from light stimulation, the RGCs connect the retinal output to different sites of the visual processing centre in the central nervous system \[10\]. Post TBI, several pathophysiological changes

\textit{Figure 1: Visual impairment due to TBI}
occur in the brain that lead to secondary damage. These mechanisms include excitotoxicity, mitochondrial dysfunction, oxidative stress, neuroinflammation, axonal degeneration, and apoptosis \cite{11}. This results in visual dysfunction including photosensitivity, blurred vision, diplopia, photophobia, reading problems and oculomotor deficits \cite{12}.

Patients with vision deficits after TBI are often treated with vision therapy. Vision therapy is defined as a sequence of neuromuscular activities to develop, rehabilitate, and enhance visual skills and processing \cite{13}. These include orthoptic therapy, oculomotor vision rehabilitation, prisms and occluders and pharmacological interventions \cite{14}. Even though, these are the most commonly practiced methods, there is no scope for regeneration of RGCs after retinal degeneration.

1.3 Role of BDNF in RGC survival

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{BDNF_signal.png}
\caption{BDNF signaling pathway (created using Biorender)}
\end{figure}
Neurotrophic factors are effective in slowing retinal neurodegeneration. Brain derived Neurotropic Factor (BDNF) has increased RGC survival in chronically hypertensive rats. BDNF gene delivery to the retina has improved RGC survival in glaucoma models. BDNF is a neurotrophic factor that binds to TrkB (Tropomyosin Receptor Kinase B) receptor to promote cell growth, survival, and differentiation. Cheng et al showed that after transection of optic nerve, the TrkB mRNA levels in the RGCs reduced indicating trauma induced downregulation of neurotrophic factor. When treated with TrkB inducing gene and exogenous BDNF, the RGCs showed 76% survival after two weeks [15].

1.4 Challenges for ocular drug delivery

The anterior section of the eye consists of the cornea, conjunctiva, sclera and anterior uvea. The sclera is an avascularized structure [16]. Topical formulations are the most commonly used treatment method. Due to lacrimal fluid, the drug is instantly removed from the eye surface. With these formulations, the bioavailability is less than 5%. Systemic absorption occurs rapidly leading to low ocular bioavailability [17]. The cornea is made up of tight junctions which do not allow paracellular transport of drugs to the posterior segment. These formulations do not reach the posterior segment of the eye, that consists of retina, vitreous, and choroid and is highly vascular. Intravitreal and subretinal routes of administration are invasive. The blood aqueous barrier in the anterior segment consists of epithelial cells limiting the availability of drugs into the aqueous humour [18]. The blood retinal barrier in the posterior segment of the eye is comprised of retinal pigment epithelium and blood capillaries. Even though the drugs can access the choroid all extra vascular space through leaky walls, the retinal pigment epithelium and retinal endothelium limits the distribution of drugs [17]. The therapeutics are also not
sustained in the ocular tissues for a long time. These challenges need to be overcome by using a novel approach that can increase the bioavailability of the drug administered.

1.5 Advances in nanotechnology for ocular drug delivery

Nanomedicine is an emerging technology for gene therapy. Various nano- drug delivery methods have been developed for ocular drug delivery like liposomes, polymeric nanoparticles, dendrimers, and micelles. Nanoparticles help in increased drug solubility, increased bioavailability, sustained and target drug release \[19\]. Their surface properties can be modified to increase target specificity or to conjugate drugs based on their physiochemical properties. There are a few nano medicines in the market for ocular delivery which include liposomes, nano emulsions, micelles and hydrogels.

![Figure 3: Structure of eye and retina layers](image-url)
1.6 Gene therapy for ocular diseases

Gene therapy has become a promising treatment option for tissues that cannot regenerate. A successful gene therapy is largely dependent on the route of administration and the choice of vectors. Viral and non-viral vectors have been used to deliver genes. Luxturna, a viral vector delivering human retinal protein, was approved by Food and Drug Administration (FDA) in 2017 to treat biallelic RPE-65 mutation that leads to total blindness \textsuperscript{20}. Viral gene vectors have the disadvantages of eliciting an immune response and lower loading capacity. Non- viral vectors, on the other hand, are non-immunogenic and have higher loading capacity \textsuperscript{21}. Gene therapy using non-viral vectors are focused on inreasing the transfection efficiency, therapeutic effect, and minimizing cytotoxicity \textsuperscript{22}.

1.7 Dendrimers as gene vectors

Dendrimers are branched, tree-like structures with an increase in the number of functional groups with each generation. Higher the generation of the dendrimers, higher the molecular weight and number of surface functional groups. They are capable of binding to nuclei acids due to the surface charge density \textsuperscript{23}. The dendrimers condense the genetic material like pDNA (plasmid DNA), shRNA (short- hairpin RNA) siRNA (silencing RNA) and miRNA (micro RNA) containing phosphate groups forming a dendriplex due to the amine groups present on their surface. The complexes can now interact with the cell membrane due to presence of negative charge and are endocytosed. Inside the endosomes, the dendrimers act as a proton sponge which leads to endosomal rupture and release of the therapeutics into the cytoplasm. Eventually, the nucleic acids enter the nucleus \textsuperscript{24}. 
Dendriplexes are endocytosed through cell membrane and the proton sponge effect leads to the release of nucleic acids. In this study, the dendriplexes will be used to deliver BDNF expressing plasmid DNA to produce BDNF.

Polyamidoamine (PAMAM) has been the most researched family of dendrimers. It consists of an ethylenediamine core branching out with repeating units of methacrylate with each generation \[23\]. A study by Shakhbazau et al. showed that PAMAM G4 dendrimers had higher transfection efficiency of a plasmid encoding neurotrophic factor-3 (NT-3) compared to PAMAM G3, G5, G6 and G4-OH and saw an increase of BDNF (Brain Derived Neurotrophic Factor), GDNF (Glial-cell Derived Neurotrophic Factor) and NT-3 levels compared to the control group \[25,26\].

This approach of delivering dendrimers complexed with plasmids encoding neurotrophic factors can help in neurodegenerative diseases \[27\].

Figure 4: Dendrimers as gene vectors
The transfection efficiency of dendrimers can be increased by conjugating them with cyclodextrins [28]. Cyclodextrins are cyclic oligosaccharides of α-D-glucopyranose units. They have a central hydrophobic cavity and hydrophilic surface. The most common types of cyclodextrins are α-, β- and γ- cyclodextrins that contain six, seven and eight units of D-glucopyranose units, respectively [29]. Cyclodextrins can disrupt the biological membrane by complexing with phospholipids and cholesterol [30]. Cyclodextrin conjugated with PAMAM, PEI (Polyethyleneimine) has been used to deliver pDNA, shDNA and siRNA [31,32]. Cyclodextrin is an FDA approved drug excipient and can be used for ocular delivery due to their ability to increase aqueous solubility, drug absorption and decrease inflammation.

In this study, we hypothesize that use of β- cyclodextrin conjugated PAMAM dendrimers complexed with a BDNF expressing plasmid can upregulate BDNF in a retinal pigmented epithelial cell line, ARPE-19. These cells are present in the posterior segment of the eye and thus can serve as a good in-vitro model for evaluating ocular drug delivery strategies.
CHAPTER II: MATERIALS AND METHODS

2.1 Materials
Polyamidoamine (PAMAM) was purchased from Dendritech (Midland, MI, USA). Carboxymethyl-β-cyclodextrin was purchased from Sigma Aldrich (Milwaukee, WI, USA). 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) was purchased from Acros Organics (Geel, Belgium). N-hydroxy succinimide (NHS), Dialysis Membrane (MWCO 3500), PureLink HiPure Plasmid Maxiprep kit, TRIzol reagent, Verso cDNA synthesis kit and Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 was purchased from ThermoFisher Scientific (Waltham, MA, USA). Anti-BDNF antibody [EPR1292] was purchased from Abcam (Waltham, MA, USA). BDNF-SEP was a gift from Ryohei Yasuda (Addgene plasmid #83955; http://n2t.net/addgene:83955; RRID: Addgene_83955). pHAGE-CMV-hIL7-IRES-ZsGreen-W was a gift from David Baltimore (Addgene plasmid #26532; http://n2t.net/addgene:26532; RRID: Addgene_26532).

2.2 Visualizing BDNF expression in TBI mouse models.
Eyes were obtained from mice subjected to rTBI (repeated TBI) that were previously fixed in 4% Paraformaldehyde and were used for immunohistochemical staining [9]. The eyes were embedded in paraffin blocks for sectioning. The eyes were sectioned using a microtome into 20µm thick sections. The sections were deparaffinized using xylene, 100% ethanol, 95% ethanol, 75% ethanol, 50% ethanol, 30% ethanol and water, in the same order. The tissues were incubated in antigen unmasking solution for 30 minutes and washed with PBS three times. Then, they were
permeabilized using 3% H₂O₂ for 30 minutes and washed with PBS three times. Non-specific antigens were blocked using 10% NGS for 45 minutes and washed with PBS three times. Anti-BDNF antibody was diluted in the ratio 1:750 and sections were incubated at 4°C overnight and washed with PBS thrice. Goat Anti-Rabbit IgG secondary antibody was diluted in the ratio 1:1000 and sections were incubated in the dark for 2 hours. Then, DAPI staining was done. Finally, the sections were washed with PBS thrice, and the section were observed under the Keyence microscope.

2.3 Synthesis of PAMAM- β-CyD Conjugate

To conjugate β cyclodextrin (β-CyD) to PAMAM dendrimers, 2ml of (PBS) was used to dissolve carboxymethyl-β-cyclodextrin (57.31 mg, 41.62 µmol) and stirred well with equimolar amounts of EDC (6.46 mg, 41.62 µmol) and NHS (4.79mg, 41.62 µmol) for 4 hours. PAMAM (50 mg, 3.52 µmol) was then added to this reaction mixture and stirred well at room temperature for 24 hours. This reaction mixture was dialyzed against deionized water for 48 hours and lyophilized to get β-CyD-PAMAM conjugate.

2.4 Characterization of PAMAM- β-CyD Conjugate

Nuclear Magnetic Resonance (NMR) gives the number of protons associated to the carbon atoms present in the molecule. About 1mg of the nanoparticle was dissolved in 500 uL of deuterated water for NMR characterization. The conjugation was analyzed by identifying the characterization peaks of PAMAM and β-CyD. The hydrodynamic diameter of the nanoparticle was measured using Malvern ZS-100 ZetaSizer to obtain the data on the size.

2.5 Complexation of BDNF expression plasmid with PAMAM- β-CyD

The BDNF-SEP plasmid was grown on a LB Agar plate containing Ampicillin. A colony of the bacteria was taken for culturing overnight in LB broth with Ampicillin (100 µg/ml) and the plasmid
was isolated as per the instructions in the PureLink HiPure Plasmid Maxiprep kit. The concentration and purity of the isolated plasmid was measured using a nanodrop spectrophotometer. The isolated plasmid was added to the CyD-PAMAM conjugate while vortexing and incubated at room temperature for 15 minutes. Various batches of nanoparticles complexed with plasmid DNA were prepared with two N/P ratios- 1:1, 2:1, 5:1 and 10:1. The N/P ratio was calculated as,

\[
\frac{N}{P} = \frac{\mu g \text{ of vector}}{\frac{\text{molecular weight of repeating monomer}}{\text{number of amines}}} \div \frac{\mu g \text{ of DNA}}{330}
\]

2.6 Gel electrophoresis

1% agarose gel was made using 1X TAE (Tris-Acetate EDTA) Buffer. Free BDNF- plasmid, dendriplex and NP-Plasmid complex were run on the gel for 45 minutes at 80mV. The gel was observed under UV-light to observe DNA bands.

2.7 Assessment of cytotoxicity of nanoparticles on ARPE-19 cells

Around $4 \times 10^3$ ARPE-19 cells were plated per well in a 96 well plate using DMEM: F12K (1:1) medium and incubate at 37° C with 5% CO$_2$. When the cells reached 80% confluency, the media was removed and washed with PBS. The cells were treated using different batches of nanoparticles and incubated with serum-free media for 24, 48 and 72 hours. Control group was treated with PBS. The cytotoxicity was measured using Cell TiterGlo luminescent cell viability assay. The CTG reagent was prepared as per manufacturer’s instructions, and it was mixed with complete media in 1:1 ratio. 100µl of this mixture was added to the wells and the placed on the shaker for 15 minutes. Then the supernatant was added to another plate and luminescence was measured under using Synergy H4 hybrid reader (BioTek).
2.8 Transfection efficiency of nanoparticles in ARPE-19 cells

Around $4 \times 10^3$ ARPE-19 cells were plated per well in a 96 well plate using DMEM: F12K (1:1) medium and incubate at 37° C with 5% CO₂. When the cells reached 80% confluency, the media was removed and washed with PBS. Control group was treated with PBS. The other groups were treated using different batches of PAMAM and nanoparticles complexed plasmids. TdTomeo plasmid was used to check the transfection efficiency. The cells incubated with serum-free media for 24, and 48 hours. The cells were observed for red fluorescence under Keyence microscope.

2.9 Determination of the Bdnf mRNA levels in ARPE-19 cells

Around $1.5 \times 10^5$ ARPE-19 cells were plated on a 6 well plate using DMEM: F12K (1:1) medium and incubate at 37° C with 5% CO₂. When the cells reached 80% confluency the media was removed and washed with PBS. The cells were treated using different batches of nanoparticles complexed with BDNF-SEP and incubated with serum-free media for 24, 48 and 72 hours. Control group was treated with PBS. 250ul of Trizol was added to each well and the cells were collected by gently scraping. To 1ml of the sample, 200ul of chloroform was added and shaken vigorously for 15 seconds. It was incubated at room temperature for 15 minutes and centrifuged at 10,000× g for 15 minutes. The supernatant containing the nucleic acids was collected and 500ul of Isopropyl alcohol was added to it. It was then incubated for 10 minutes and centrifuged at 12,000 × g for 10 mins. The supernatant was discarded and 500ul of 70% Ethanol was added. It was centrifuged at 12,000 × g for 5 minutes. The pellet was air dried, and the extracted RNA was resuspended in water. The concentration and purity of the RNA was measured using a nanodrop spectrophotometer. 2000 ng of RNA from each treatment group was taken and cDNA was prepared using Verso cDNA Synthesis kit for Polymerase Chain Reaction (PCR) in ProFlex™ PCR System (Applied Biosystems). Then, the cDNA was diluted using water in the ratio 1:10. A primer mix
was prepared using EvaGreen forget me not mix, *Bdnf* primer and water in the ratio 2:0.5:1.5. 1ul of cDNA and 4ul of primer mix was added to a 384 well plate and quantitated under CFX384 Real-Time 384-well qPCR Detection System (Biorad).

### 2.10 Statistical analysis

All data are presented as mean ± Standard Error of Mean (SEM). Statistical significance was determined by one-way ANOVA and two-way ANOVA with a Dunnett’s and Tukey’s test, respectively. A p value of less than 0.05 was considered statistically significant for all comparisons.
CHAPTER III: RESULTS

3.1 Visualizing BDNF expression in TBI mouse

BDNF expression in the retina of rTBI mice was compared to Sham mice \(^9\). The retinal tissues were sectioned into 20µm thick sections using a microtome and immunostained for BDNF. The expression of BDNF (green) and the nuclei (blue) is shown in Figure 5. It can be observed that the BDNF expression and the number of retinal ganglion cells (nuclei) is higher in the ganglion cell layer of (Figure 5a) Sham mice compare to (Figure 5b) in TBI mice.

*Figure 5: Visualizing BDNF expression in TBI mouse model.* BDNF (green) expression and the number of Retinal Ganglion cells (Blue- nuclei) in the GCL of (a) Sham and (b) TBI mice. (GCL- Ganglion cell layer, ONL- Outer Nuclear Layer, INL- Inner Nuclear Layer)
3.2 Synthesis and Characterization of PAMAM-β-CyD nanoparticles

β-cyclodextrin was conjugated to the surface of the dendrimer to form a PAMAM – β-CyD conjugate. The conjugate synthesis was catalysed by NHS, EDC ester formation(Fig 6). The amine groups present in PAMAM reacts with the carboxyl group present in β- cyclodextrin through amidation reaction forming an amide bond. The dendrimers were conjugated with β- Cyclodextrin in 1:1 ratio. The unreacted cyclodextrin was removed by dialyzing the resultant reaction mixture against deionized water for 48 hours. The resultant mixture was lyophilized overnight.

**Figure 6: Synthesis of PAMAM-β-CyD Nanoparticles.** PAMAM-β-CyD nanoparticles were synthesized by amidation reaction. The carboxyl group of β- Cyclodextrin reacts with the amide group of the PAMAM dendrimers forming β- Cyclodextrin- PAMAM conjugate.

To confirm the conjugation of PAMAM with β- Cyclodextrin, $^1$H NMR was performed (Fig 7). On application of higher magnetic fields, the protons enter the higher energy state. When they return to their lower energy state, the emit energy and it is detected. NMR spectrum provides information about characteristic peaks representing the position of the hydrogen in the molecule. The β-Cyclodextrin (Fig 7a) had characteristic peaks at 3.7 and 5.4 ppm corresponding to the methyl groups present in the cyclodextrin ring. The NMR spectrum of PAMAM dendrimers (Fig 7b) showed characteristic peaks at 2.3, 2.5 and 2.7 ppm corresponding to the methyl groups present...
in the polymer chain. When the NMR spectrum was analysed for the synthesized nanoparticles (Fig 7c), there were peaks at 2.3, 2.5, 2.7, 3.7 and 5.4 ppm which confirms the conjugation of PAMAM with β- Cyclodextrin.

Figure 7: NMR spectra of (a) β- cyclodextrin (b) PAMAM and (c) PAMAM -β-Cyclodextrin conjugate.
The morphology of the PAMAM-β-CyD nanoparticles was analysed using transmission electron microscopy (TEM) at 60,000x magnification under JEOL 1400 TEM Scope. The PAMAM-β-CyD nanoparticles looked like round speckles at 60,000x magnification. (Fig 8).

The size of the PAMAM-β-CyD nanoparticles (NP) was determined using a Zeta Sizer (Fig 9). While the dendrimers alone had a particles size of $5.016 \pm 1.312$ nm, the PAMAM-β-CyD complex was determined to be $6.074 \pm 2.080$ nm.

### 3.3 Complexation of BDNF-SEP Plasmid

Gel electrophoresis was done to confirm the complexation of β-Cyclodextrin conjugated PAMAM dendrimers with BDNF-SEP plasmid. It was observed that higher the N:P ratio better the complexation (Figure 10). Dendrimers showed complete complexation in 5:1 group only. There were free plasmids in 1:1 and 2:1 lane of the dendrimer complexed plasmid. The PAMAM-β-CyD nanoparticles showed complete complexation in 1:1, 2:1, 5:1 and 10:1 group. While N:P ratios 1, 2, 5 and 10 complexed the plasmid perfectly in the nanoparticle group, the same was not observed in the dendrimers group.

![Figure 8: Morphology of PAMAM-β-CyD Nanoparticles.](image)

The morphology of the nanoparticles was analysed under TEM at 60,000x magnification.
Figure 9: Size characterization. Size of (a) PAMAM dendrimers and (b) PAMAM dendrimer after conjugation with β-Cyclodextrin were measured to be 5.016 and 6.074 nm respectively.

Figure 10: Complexation with BDNF-SEP plasmid. Gel electrophoresis was done using 1% agarose gel. BDNF-SEP plasmid was complexed with dendrimers and PAMAM-β-CyD nanoparticles (NP) in different N:P ratios.
3.4 Cytotoxicity Studies

Next, Promega Cell TiterGlo assay was used to study the cytotoxicity of the PAMAM-β-CyD nanoparticles. The assay determines cell viability using the amount of ATP produced by the metabolically active cells to produce luminescence. The PAMAM dendrimer and PAMAM-β-CyD NP was conjugated with BDNF plasmid in two N/P ratios, 2:1 and 5:1. ARPE-19 cells were treated using these groups and the cell viability was calculated after 24, 48 and 72 hours.

**Figure 11: Cell viability studies.** ARPE-19 cells were treated with dendrimers and nanoparticles complexed with BDNF-SEP Plasmid. Cytotoxicity of PAMAM and PAMAM-β-CyD NP complexes after 24-, 48- and 72- hours. (*p<0.0.05, **p<0.005, #p<0.0001)

The cell viability of the PAMAM- plasmid complex with N/P 2:1 was the highest after control group (Figure 11). The PAMAM-β-CyD NP conjugated with BDNF-SEP plasmid showed lower cell viability compared to the dendriplex groups without β-Cyclodextrin. However, within the PAMAM-β-CyD NP groups, the treatment groups with N/P 5:1 showed better cell viability than the 2:1 group. The cell viability of all groups decreased over time.
3.5 Transfection efficiency

**Figure 12: Transfection efficiency of PAMAM and PAMAM-β-CyD complex in ARPE-19 cells.**

ARPE-19 cells were treated with PAMAM and PAMAM-β-CyD Nanoparticles complexed with TdTomato plasmid in the N:P ratio 2:1 and 5:1. PAMAM-β-CyD Nanoparticles showed higher transfection efficiency. Higher transfection efficiency was observed in groups with higher N/P ratio in both PAMAM and PAMAM-β-CyD NP treatment groups.

ARPE-19 cells were treated with different groups of PAMAM or PAMAM-β-CyD nanoparticle conjugated with TdTomato plasmid. TdTomato plasmid encodes red fluorescent protein (RFP) that act as the reporter gene to visualize transfected cells. After 24 and 48 hours of transfection, the cells were observed under the fluorescence microscope (Figure 12a). The group treated with
nanoparticles showed higher transfection efficiency compared to PAMAM dendrimers alone (Figure 12b). This is because of the presence of β-cyclodextrin on the surface of the PAMAM-β-CyD nanoparticles. The N:P ratio played a major role in increasing the transfection efficiency. The expression of the reporter gene, RFP, was higher in the groups treated with higher N:P ratio (5:1).

3.6 *Bdnf* mRNA regulation in ARPE-19 cells

**Figure 13: Bdnf mRNA regulation in ARPE-19 cells.** BDNF mRNA expression levels in ARPE-19 cells 24-, 48- and 72-hours post-transfection using PAMAM-β-CyD NPs complexed with BDNF-SEP plasmid. BDNF mRNA expression was upregulated in cells treated with BDNF expressing plasmid complexed with PAMAM-β-CyD. PAMAM-β-CyD by itself did not promote *Bdnf* mRNA upregulation. (*p<0.05, **p<0.005, ****p<0.0001).

PCR was performed to check the *Bdnf* mRNA regulation in ARPE-19 cells after treatment with PAMAM-β-CyD NPs conjugated with BDNF-SEP plasmid. PAMAM-β-CyD NPs were chosen for this experiment because they had better transfection efficiency compared to the PAMAM group. The results indicated that over different time points, the PAMAM-β-CyD NP complexed with BDNF plasmid with N/P as 5:1 showed higher *Bdnf* expression in the ARPE-19 cells (Figure 13). During the first 24 hours, *Bdnf* mRNA levels are lower compared to the control group.
However, the \textit{Bdnf} mRNA levels increased with time in PAMAM-\(\beta\)-CyD NP- complexed with plasmid. The PAMAM-\(\beta\)-CyD NPs by itself did not show an increase in \textit{Bdnf} mRNA level. This implies that the plasmid encoding for BDNF resulted in the transcription of \textit{Bdnf} gene indicating successful gene delivery.
CHAPTER IV: DISCUSSION

Gene therapy refers to introducing a foreign nucleic acid construct into host cells for treatment purposes [33]. Treatment for various disease and disorders are now possible due to the efficiency of gene therapy. Gene therapy is a promising approach to treat retinal degeneration because of the immune privileged nature of the eye. One of the limitations for gene therapy is that the carriers for gene delivery are not safe [34]. Viral and non-viral vectors have been used to deliver genetic material to the host cells. Viral vectors have the disadvantage of eliciting an immune response and have a low loading capacity. Non-viral vectors can overcome this disadvantage. Non-viral vectors have high loading capacity, are biocompatible and non-immunogenic. [35]

PAMAM dendrimers are the most researched group of dendrimers. Holden et al show that PAMAM dendrimers are more effective than PBS based eye drops in delivering drugs for glaucoma [36]. Mastroakas et al used hydroxyl-terminated PAMAM dendrimers conjugated with triamcinolone acetonide to increase transfection efficiency in RPE cells [37]. Dendrimers have been used to treat retinal degeneration by conjugating with drugs or genetic materials in vitro and in vivo but they have not been used in clinical applications. This may be attributed to the surface positive charge that when interacted with negative charged membrane, results in membrane disruption and eventually, necrosis [38].

In this study, we used a dendrimer-based nanoparticle to complex the DNA for drug delivery to ARPE-19 cells. ARPE-19 cells are retinal pigmented epithelial cells. These cells were used because they are present in the posterior segment of the eye. In-vitro drug delivery using ARPE-
19 cells can be later translated ocular for drug delivery to the posterior segment in-vivo. PAMAM dendrimers were conjugated with β-Cyclodextrin through amidation reaction catalysed by NHS and EDC forming an ester intermediate. The conjugation was confirmed using NMR spectroscopy. The NMR of the synthesised PAMAM-β-CyD nanoparticle had characteristic peaks at 5.4, 3.7 ppm belonging to the Cyclodextrin and 2.7, 2.5 and 2.3 ppm belonging to the PAMAM were identified, confirming that the conjugation between β-cyclodextrin and PAMAM nanoparticles, this was similar to what was found by Saraswathy et al.[39]. The size of the PAMAM-β-CyD nanoparticles was determined to be 6.074 ± 2.080 nm after conjugation. conjugation ratio between PAMAM dendrimer and β-cyclodextrin can also be varied to test cytotoxicity and transfection efficiency levels.

The PAMAM-β-CyD nanoparticles were tested on ARPE-19 cells for the cytotoxicity. PAMAM dendrimers are inherently toxic to the cells because of the positive surface charge. Dendriplexes with higher N/P show higher cytotoxicity because of the presence of higher number of amine groups.[40] However, the comparison between PAMAM-β-CyD nanoparticles complexed with plasmid DNA shows that N/P of 5:1 has better cell viability than 2:1 group. This can be attributed to the stability of the PAMAM-β-CyD nanoparticle complex and successful condensation of the plasmid DNA to the PAMAM-β-CyD nanoparticle.[41] The lower cell viability of the PAMAM-β-CyD nanoparticle compared to the dendrimer may be contributed to the conjugation of β-Cyclodextrin to the dendrimers.

The transfection efficiency of β-Cyclodextrin conjugated PAMAM nanoparticles were higher than the PAMAM itself. As expected, the transfection efficiency increased with higher N:P ratio.[42] The presence of cyclodextrin increased the transfection efficiency compared to PAMAM- pDNA complex.[43].
The cell viability and therapeutic effects of the PAMAM-β-CyD nanoparticles were not tested on TBI-like conditions in vitro. This can be done through Lipopolysaccharide (LPS) stimulation\[44\], which creates an inflammatory environment that is a hallmark of TBI. Only two different N:P ratios were used in this study. Higher N:P ratios were not explored due to the cytotoxicity caused by higher amine groups. However, literature suggests that a higher N:P ratio has better transfection levels\[45\]. The therapeutic effects of BDNF complexed PAMAM-β-CyD nanoparticle can be tested using different N:P ratios. The transfection efficiency of the PAMAM-β-CyD nanoparticle was not tested using BDNF-SEP plasmid. This was because the green fluorescent signal from the SEP could not be observed in ARPE-19 cells. Literature suggests that the SEP signal can be observed when the cells are treated with NH4Cl with a basic pH \[46\]. However, the signal observed in these studies lasted only for a few seconds. Due to this reason, the GFP signal from BDNF-SEP could not be observed. To confirm the transfection of BDNF Plasmid, another plasmid coding for a red fluorescent reporter gene could be complexed with it to confirm the transfection. The transfection efficiency of dendrimers conjugated with a GFP encoding pDNA \[47\] is similar to what has been observed in this study. Cellular uptake and transportation of PAMAM-β-CyD nanoparticles is controlled by the molecular structures. This serves as a barrier for the PAMAM-β-CyD nanoparticles to enter and exit the cell. Since the PAMAM-β-CyD nanoparticle used in this study had not been covered with stealth coating like Polyethylene glycol to escape the in vivo environment, the PAMAM-β-CyD nanoparticles have the ability to interact with the biological proteins and form aggregates\[48\]. Mukherjee SP et al showed that the dendrimers in serum rich culture media depleted the proteins, which leads to cytotoxic effects \[49\].

The PAMAM-β-CyD nanoparticles complexed with BDNF expressing plasmid was tested for *Bdnf* mRNA regulation in the ARPE-19 cells. There was a significant increase in the mRNA levels...
in the groups treated with N/P 2:1 and 5:1, 48- and 72- hours post transfection compared to the control group. Within these groups, PAMAM-β-CyD NP complexed with BDNF- SEP plasmid in 5:1 N/P ratio showed higher mRNA levels. This indicated that the transfection efficiency of the PAMAM-β-CyD nanoparticles is proportional to the N/P ratio. It was found that the PAMAM-β-CyD nanoparticle by itself was not able to increase the Bdnf mRNA levels. The protein levels of BDNF need to be estimated to ensure translation of Bdnf mRNA. ELISA or western blotting needs to be run, this experiment could not be completed due to limited time and resources. The experiments were not done in repetitions which can contribute to errors. Multiple repetitions are required to ensure data reproducibility.

These findings indicate that the PAMAM-β-CyD nanoparticle synthesised to transfect cells with BDNF Plasmid can upregulate BDNF expression and can be an effective approach to treat retinal degeneration due to TBI. This study can be further validated by translating it into animal models of TBI. For future studies, the hydrophobic cavity of β-Cyclodextrin can be utilized to encapsulate drugs to decrease inflammation, oxidative stress, and intraocular pressure in the eye.
CHAPTER V: CONCLUSION

It may be concluded that the synthesized PAMAM-β-CyD nanoparticles complexed with BDNF encoding plasmid are able to transfect the cells and upregulate Bdnf. The nanoparticles mediated gene therapy may be a promising approach for gene therapy for retinal degeneration post-TBI, in the future.
REFERENCES


3. Centers for Disease Control and Prevention. National Center for Health Statistics: Mortality data on CDC WONDER.


