November 2020

Dendriplex - Cyclodextrin conjugates for Gene delivery to Retina

Durga Deepak Puro
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Dendriplex-Cyclodextrin Conjugates For Gene Delivery To Retina

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Nanotechnology
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Date of Approval: November 20, 2020

Keywords: Non-viral vectors, Nanoparticles, Transfection, Posterior eye diseases,

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ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. Subhra Mohapatra for giving me this opportunity and my deepest gratitude towards Dr. Shyam Mohapatra for his support during this MSPN program. I would like to thank all my lab members for their immense help and support. I would especially like to thank Dr. Eleni Markoutsa for guiding me throughout the project. A very special thanks to Dr. Sweta, Karthick and Alejandro for helping me throughout my project.
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ABSTRACT

Gene therapy holds promise for treating a wide range of diseases including cancer and several other polygenic and monogenic disorder arising in different organs. Due to its immune privilege nature, eye is an ideal organ for gene therapy, as various visual pathologies arise from gene defect and can lead to partial or complete vision loss. The last decade, several nanoparticles have been developed and tested for their transfection efficiency in vitro and in vivo. Among them, dendrimers with small size, good physicochemical properties and endosomal escape activity have shown high transfection efficiency in various cell lines. In an attempt to further increase the transfection efficiency of the dendrimers, other molecules can be conjugated on the surface of dendrimers to facilitate more efficient cell entry and transfection. Herein, β-cyclodextrin was conjugated on the surface of the PAMAM dendrimer and the effect of this conjugation was tested for transfection efficiency, cellular uptake and toxicity of PAMAM dendrimer in Retinal Epithelium Pigment cells (ARPE). The successful conjugation of cyclodextrin on the PAMAM surface was confirmed by NMR. Different cyclodextrin to PAMAM ratios were also tried in an attempt to optimize the nanoformulation. The cyclodextrin-conjugated dendrimers were then characterized for particle size, zeta potential and morphology. Furthermore, cyclodextrin-conjugated dendrimers were complexed with plasmid and characterized using gel electrophoresis to confirm the successful formation of the complex. In conclusion, the cyclodextrin-PAMAM (CyD-PAMAM) conjugation improves the uptake and transfection efficiency of PAMAM dendrimer without increasing its cell toxicity significantly.
CHAPTER 1 : INTRODUCTION

1.1. Gene Therapy

Gene therapy was first used as a promising approach to treat incurable or inherited diseases that cannot be aided by drugs or other treatment. The applications of this approach have been expanded to target several other monogenic and polygenic disorders including cancer. [1] The origin of gene therapy can be dated back to 1960 with the understanding of transduction that is introduction of foreign DNA into a cell using viruses. After that, gene therapy took pace, and the 1st clinical trial was conducted in 1990 on 2 pediatric patients with adenosine deaminase deficiency (ADA-SCID). [2] Generally, the approaches were developed to achieve gene insertion into healthy cells (i) by replacing the mutated gene (ii) by inactivating the mutated gene or (iii) by instituting new gene. Currently this breakthrough technology is widely researched and many therapies are in different phases of clinical trials. [3]

![Gene therapy diagram](Created with BioRender.com)

To achieve gene insertion at desired target site, various viral and non-viral vectors have been investigated. As mentioned above, the process of inserting gene into the cell using viral vector is called transduction and that with the non-viral vector is called transfection, and both the vectors
have been exploited. Most of the clinical studies utilizes viral vectors for gene therapy. The viral vectors, as the name suggests are comprised of viruses with modified genomes. This is achieved by deleting some areas from their genomes to hinder their replication and make them safer. The broad spectrum of viral vectors has been developed as a delivery vehicle for both transient short-term and permanent long-term expression. Both DNA and RNA types of vectors are signified by either single-stranded (ss) or double-stranded (ds) genomes. But there are few drawbacks associated with these vectors, which include immunogenicity, production of toxin, mortality, mutagenesis after insertion. Moreover, they have lower loading capacity which necessitates the need of alternatives.[4] Several polymeric and lipid nanoparticles have been investigated as possible vectors for gene delivery. The advantage of these non-viral vectors over viral vectors are higher loading capacity, they are non-immunogenic and can be attached with a ligand to target a specific tissue. But they show lower transfer efficiency as compared to viral vectors which results in increase dose administration. In addition, the non-viral vector should withstand the endosomal environment after entrapment and consequently release the DNA intracellularly to ensure the desired gene function.[5]

1.2 Gene therapy in ocular diseases

The unique structure of the eye, which is protected by the blood retinal barrier, sustains the introduction of antigen without eliciting any immunologic response. This immune privilege nature of the eye, makes it an ideal organ for gene therapy. [6] Most importantly, disease pathologies in the eye originate in the photoreceptor or the retinal pigment epithelium which are normally non-dividing, and this makes gene therapy a promising intervention as it would last for life. Moreover, the optical clarity of the eyeball helps to visualize different structure within the eye. Several clinical trials are currently being conducted for treating various pathologies arising in eye.
Table 1: Clinical trials for ocular gene therapy

<table>
<thead>
<tr>
<th>Viral vectors</th>
<th>Route of Administration</th>
<th>Clinical trial phase</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>Subretinal injection</td>
<td>Phase 1/2</td>
<td>Leber congenital amaurosis[7]</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Subretinal injection</td>
<td>Phase 1</td>
<td>Neovascular Age Related Macular Degeneration[8]</td>
</tr>
<tr>
<td>AAV</td>
<td>Subretinal injection</td>
<td>Phase 1/2</td>
<td>Choroideremia[9]</td>
</tr>
<tr>
<td>AAV</td>
<td>Subfoveal injection</td>
<td>Phase 2</td>
<td>Choroideremia[10]</td>
</tr>
</tbody>
</table>

Non-Viral vectors have not yet been used for ocular gene therapy trials but have achieved therapeutic effects in animal model.[11]

Figure 2: Histology of Retina (Created with BioRender.com)

As seen from the clinical trials, many ocular gene therapies are centered on the retina and its supporting cells. As shown in figure 2, the retina is stratified in layers and has a well-organized
architecture with different cells being interconnected. Retinal pigment epithelium (RPE) is a single layer of epithelial cells which play an important in the metabolism of photoreceptors and help in phototransduction of light. In a nutshell, all the retinal cells contribute towards the structure and biochemical functioning. [12]

Past few decades, genetic mutations in the eye have been extensively studied and linked to several retinal and RPE disorders. These wide range of inherited genetic defects in the retina are termed as Inherited retinal dystrophies (IRDs). The 1st gene therapy named Luxturna have been approved by FDA in 2017 for treating IRD caused by biallelic mutations in RPE65 in adult or pediatric patients which uses adeno-associated virus as a vector.[4, 13]

Apart from the inherited genetic defects, there are set of neurogenerative disorders such as glaucoma and age-related macular disorders which are not monogenic. Genetic treatments are being designed for such pathologies. In recent times, significant evaluation of the neurotrophic expression in the eye has been made to suppress the angiogenic factors. A study carried out to see the effect of brain derived neurotrophic factor (BDNF) mediated by AAV in retinal ganglion cells of rats with laser induced model of glaucoma have shown higher cell survival. [14] Other survival factor like pigment epithelial derived factor (PEDF) have shown preclinical therapeutic effect in ischemia reperfusion injury, retinitis pigmentosa and choroidal neovascularization and is currently in clinical trials for age-related macular degeneration treatment. [15].

In addition to the neurogenerative disorder, external injuries can also lead to visual pathologies. This external stress can cause the upregulation of certain gene leading to visual damage. To give an example, external brain injury or traumatic brain injury (TBI) is much prevalent and varies from 30 to 85%. It has been seen that optic nerve injury is one of the most common events after TBI.[16] A study carried out by Das et. al has shown the upregulation of a
chemokine CCL20 which plays an important role in inflammatory responses and can further cause retinal damage leading to partial or complete vision loss. Gene knockdown in such cases can significantly reduce the inflammation and restore the normal eye function. [17]

1.3 Viral Vectors used in gene therapy

So far, the viral vectors have found clinical success in gene delivery. The viral vectors which are currently being investigated are the lentivirus, adenovirus and adeno associated virus. [18] The viral vectors are brought into favorable stance by the progress being made in the vector engineering and safety development. It has been confirmed from the clinical and preclinical studies that there is no universal viral vector and the choice of vector is therefore influenced by factors such as the duration of expression, level of expression of the therapeutic agent, and even personal experience and preference of the researchers/clinicians. The other viruses which are used as viral vectors are lentivirus, retrovirus, flavivirus along with other viruses such as Newcastle, Rhabdo and measles virus[1]

The viral vectors are used to transduce the cells either by ex-vivo therapy or in vivo delivery. In case of ex vivo gene therapy there is no risk of vector propagation and infection to other cells as the target cells are isolated and then transduced.
However, in case of in vivo gene therapy, cell targeting is an important factor for efficient gene delivery. [19] The pre-existing tropism of the parent viruses are generally used to target the specific cells, but genetically engineered viruses with retargeting properties are also being developed and studied.

1.4 Non-Viral vectors in gene therapy

Although the viral vectors have shown clinical success, there is still room for further improvement. The limitation associated with viral vectors are being alleviated in newer generation, but safety is still a concern for delivery in eye. [20] Non-viral vectors are an alternative for gene deliveries with lesser side effects. Various physical and chemical methods have been used to introduce gene to targeted sites. Physical methods such as DNA bombardment using gene gun, are feasible when the cells are transfected ex-vivo, but for in-vivo delivery, the genes needs to be targeted to the specific site of interest and also protected from the physiological environment. [21]

<table>
<thead>
<tr>
<th>Viral Vectors</th>
<th>Non-Viral Vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Physical Methods</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>DNA bombardment</td>
</tr>
<tr>
<td>Adeno-Associated Virus</td>
<td>Electroporation</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Ultrasound</td>
</tr>
</tbody>
</table>

Table 2: Examples of Viral and Non-Viral Vectors

The chemical methods include the cationic liposomes/micelles or cationic polymers. The polycationic particles condense the negatively charged nucleic acid and act as a vector carrier. The complexes between a cationic liposome or micelle and nucleic acids are called lipoplex and that
with cationic polymer are called as polyplex. These complexes are stable enough to protect the nucleic acids from degradation and are able to enter cells by endocytosis. These non-viral vectors have low toxicity and are non-immunogenic when compared to the viral vectors. They show long term expression with reduced risk of mutagenesis and oncogenesis.\cite{1} Even then these non-viral vectors have low efficacy than viral vectors and have investigational scope to improve these vectors.

1.5 Dendrimers as gene vectors

Several nanoparticles have been developed for efficient delivery of genes to targeted tissue. Among them, dendrimer has shown high transfection efficiency in multiple cell lines and have also proven to be effective \textit{in-vivo}.\cite{22, 23}

Dendrimers are polymeric structures with branches comprised of repeating units forming the generations. The efficacy of the dendrimer has seen to be increased with the increasing number of branching, but on the other hand the higher generation are more cytotoxic.\cite{24}

![Figure 4: Structure of Dendrimer and Cyclodextrin](image)

Depending on the terminal group and the core, dendrimer properties differ. The primary amines present at the terminal ends of the branches, result in a high surface charge density is accountable for the successful ionic condensation of nucleic acid and the cell penetration. Once the dendrimers are inside the cell through endocytosis, they are entrapped in endosomes where
they release DNA via proton sponge effect into the cytosol. After the DNA is released in cytosol, the DNA molecules are trapped in the nucleus to give the required expression.[25]

Several molecules have been conjugated on dendrimers including PEG, RGD (Arginin Glycil Aspartic acid), hyaluronic acid, to prolong the systemic circulation as well as to target the region of interest. Cyclodextrins have been widely used in commercial eye drops and other formulations to ensure the successful delivery of drugs. Cyclodextrins are naturally found compounds containing glycopyranose units. The most common used cyclodextrins are α, β and γ with 6, 7 and 8 units, respectively.[26] Due to their unique structure which comprises of a hydrophobic cavity and the hydrophilic outer ring, they have been widely used for solubility enhancement of various drugs.[27] Among the 3 cyclodextrins, β-cyclodextrin has shown better physiological tolerance owing to its non-toxic nature. It has been commercially used for increasing the solubility of various drugs including dexamethasone and other corticosteroid which are mainstay in various eye diseases. [28]

Other researcher has also tested the conjugation of different cyclodextrins on the surface on nanoparticles for more effective gene therapy.[22, 29] Arima et. al have conjugated various cyclodextrins to different generations of dendrimers [24]. In another study, PAMAM dendrimers conjugates with α-CyD showed luciferase gene expression approximately 100 times higher than free dendrimers or non-covalent mixtures of dendrimer [30, 31]. It has been seen that the higher transfection efficiency by cyclodextrin is caused by fast disruption of the endosome [32]. The ability of cyclodextrins to penetrate through cell membrane is another factor that improves the transfection efficiency of the vector. Thus, conjugation of PAMAM with cyclodextrin can be used as a non-viral vector approach. [33]
1.6 Hypothesis

The conjugation of cyclodextrins on the surface of PAMAM has been shown to improve their transfection efficiency in gene delivery, but its implication in the eye has not been investigated. The cavity of the cyclodextrin have affinity for certain lipids in cell membrane which helps in internalization of the dendrimers thus increasing its transfection efficiency. Our hypothesis is that conjugation of β-cyclodextrin on the surface of PAMAM-G4 dendrimer will result in the successful delivery of genes to the Retinal Pigment Epithelium cells which are involved in pathogenesis of most of the retinal and posterior eye diseases.
CHAPTER 2 : MATERIALS AND METHODS

2.1 Materials

Polyamidoamine dendrimer (PAMAM-G4, 64 terminal amino groups, MW 14215 Da) purchased from Dendritech, (Midland, MI, USA). Carboxymethyl-β-cyclodextrin (CM-β-CyD) was purchased from Sigma Aldrich (Milwaukee, WI, USA), 1-ethyl-3-(3-dimethlaminopropyl) carbodiimide (EDC) was purchased from Acros organics (Geel, Belgium), N-hydroxysuccinimide NHS, fluorescein (FITC) and dialysis membrane (MW 3500) was purchased from Thermofisher Scientific (Waltham, MA, USA), Plasmid plus mega kit was purchased from Qaigen (Hilden, Germany) for isolation of the plasmid, Agarose was purchased from fisher chemicals which was used in gel preparation for electrophoresis, Purple loading dye was purchased from new England biolabs (Ipswich, MA, USA). DNA ladder 1kb and Ultrapure water were obtained from Invitrogen (Carlsbad, CA, USA). Deuterated water for NMR was obtained from Cambridge isotope laboratories (Andover, MA, USA). Ultrapure water was used in all the experiments.

2.2 Methods

2.2.1 Preparation of PAMAM/Cyclodextrin conjugation

For the conjugation of β-cyclodextrin to dendrimers, 1ml of phosphate buffered saline pH 7.4 was used to dissolve Carboxymethyl β-cyclodextrin (57.31 mg, 41.62 µmol) and stirred well with equimolar amounts of EDC (41.62 µmol, 6.46 mg). This reaction was allowed to occur for 4 hours in order to activate the carboxyl group of CM-β-CyD. PAMAM (50 mg, 3.52 µmol) was
added to the reaction mixture and stirred well at room temperature (RT) for 24 h. The reaction mixture was then dialyzed (MWCO 3500) against deionized water for 48 h to remove the unconjugated β CyD and lyophilized to obtain beta-cyclodextrin conjugated PAMAM (β-CyD PAMAM)[33]. The cyclodextrin was conjugated at 4 different molar ratios with PAMAM ranging from 1:1, 1:2, 1:4 and 1:8 to compare the effect of the conjugation.

![Figure 5: PAMAM/Cyclodextrin conjugation (created with Biorender.com)](image)

2.2.2 Characterization of the conjugated CyD/PAMAM

The particle size is an important parameter used for measuring the hydrodynamic diameter of the nanoparticulate which gives the information about the size of the nanoparticle in solution. Whereas the zeta potential gives the information about the charge present at the surface of the nanoparticle. The CyD-PAMAM conjugate was diluted at the ratio of 1:10 with distilled water and was added to the foldable capillary cuvette to measure the size and zeta potential. Malvern zetasizer was used with the light scattering angle of 173°. Transmission electron microscopy (TEM) was used for morphological characterization of the CyD-PAMAM conjugates. The conjugates were negatively stained using ammonium molybdate. Around 30 μl of sample was placed on the 300-mesh copper grid and air dried for 30 minutes followed by addition of 3% ammonium molybdate for 2 mins, after which the stain was removed, and the grid was washed thrice with distilled water and air dried for 48 hours before analyzing.
2.2.3 Plasmid isolation

The gene encoding Tdtomato fluorescent protein was used to determine the transfection efficiency of the CyD-PAMAM conjugate. The bacterial colonies of Tdtomato/E.Coli were grown on laura broth agar plate containing ampicillin. The bacterial culture was grown overnight in culture media using ampicillin as a selective antibiotic. The plasmid was then isolated using the plasmid plus mega kit by Qiagen according to manufacturer instruction. Nanodrop spectrophotometer was used to measure the concentration of the plasmid and its purity was determined from the 260/280 ratio.

2.2.4 Complexation of plasmid with CyD-PAMAM [34, 35]

The plasmid was complexed with CyD-PAMAM by adding the plasmid while vortexing the conjugate and incubating the complex at room temperature for 15 mins. The complex was then characterized for size and zeta potential.[36]

Batches with various N/P ratios ranging from 5, 10 ,20 and 30 were prepared. This is an important physicochemical property as it validates the complete condensation of DNA into the dendrimers, which helps it to protect from the physiological conditions when introduces into the host body. The formula by which this N/P ratio is calculated is given as follows[21, 37]:

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

2.2.5 Gel electrophoresis

Gel electrophoresis was carried out to check the complexation of the DNA plasmid with the CyD PAMAM conjugate. Various ratios of DNA/PAMAM were used to see the complexation.
1% Agarose gel was prepared using 1X Tris-acetate EDTA (TAE) buffer, to which ethidium bromide was added. Samples were prepared using 5 μl purple loading dye and the gel was allowed to run at 100 mV for 30 mins. The gel was then analyzed under UV to see the DNA bands.

2.2.6 Synthesis of FITC labelled CyD-PAMAM conjugates

The FITC-NHS corresponding to 5% molar ratio of the amine group of the CyD-PAMAM and PAMAM was allowed to react in 1 ml distilled water overnight at 4°C in dark (figure 6). The reacted product was then dialyzed (3.5 kDa) against deionized water for 24hrs to remove any unreacted FITC.

2.2.7 Cellular uptake studies for the complexes

The synthesized conjugates of FITC with CyD-PAMAM and PAMAM were used to monitor the cellular uptake. Around $1-4 \times 10^3$ were plated in 96 well and incubated for 24hrs at
37°C with 5% CO₂ before adding the complex. Before analyzing the cells were washed three times with phosphate buffer saline (PBS) to remove the FITC conjugate from cell surface. The cells were analyzed under fluorescence microscope at the time points of 2, 4, 6, 24, 48 and 72 hours.

2.2.8 Cytotoxicity studies for the conjugate

The cytotoxicity study was carried out using Cell TiterGlo (CTG) luminescent cell viability assay (Promega). The CTG reagent was prepared according to manufacturer’s instructions and 100 μl was added to the well containing 100 μl of media. The plate was covered in the aluminum foil and placed on the shaker for 15mins. After 15mins the supernatant media was added to another plate and luminescence was measured using BioTek synergy plate reader.

2.2.9 In-vitro transfection ARPE19 cells with complexed plasmid

Around 1-4 × 10³ cells per well were plated in 96 well plate and were incubated for 24hrs at 37°C with 5% CO₂. After 24hrs the media was removed, the cells were washed with PBS and serum free media was added to the wells. The batches with the selected N/P ratios were added to the cells and the cells were incubated at same condition as given above. After 24hrs the cells were stained with nucBlue dye and incubated for 45 mins and then analyzed for transfection using the fluorescence imaging microscope (Keyence).
2.2.10 Statistical Analysis

The given data are presented as means with standard deviations. Statistical analysis was performed using the unpaired Student’s t-test. Differences were considered statistically significant at a p value of less than 0.05. The multiple comparison was done using one-way anova tukeys HSD test.
CHAPTER 3 : RESULTS

3.1 Preparation and characterization of the dendrimer complex

The PAMAM G4-amine end with 64 terminal amines was used as a nonviral gene vector, because of the optimal efficacy and lower toxicity in the live cells. It has been seen that higher the generation of PAMAM, greater is the transfection efficacy. The β-cyclodextrin was conjugated on to the PAMAM by amidation reaction. The carboxymethyl- β-cyclodextrin was used as starting reagent and the carboxylic groups were activated using EDC and NHS added in equimolar quantity. The unreacted β-cyclodextrin was removed by dialyzing the product was dialyzed against deionized water. As shown in figure initially the EDC reacts with the carboxylic group of the cyclodextrin to form an active O-acylisourea intermediate which can also directly react with the amines at pH 4.5. N-hydroxysuccinimide (NHS), on the other hand is included in EDC coupling protocols to improve efficiency of the intermediates. The intermediate formed by EDC couples with NHS to form NHS ester which is more stable than O-acylisourea intermediate and helps to form an efficient conjugation to primary amines.[33]

Figure 8: Gel electrophoresis (created with Biorender.com)
Figure 9: Reaction of EDC and NHS with carboxylate molecule

Table 3: Characterization of the molar ratios of CyD:Dendrimer

<table>
<thead>
<tr>
<th>molar ratio (CyD:Dendrimer)</th>
<th>Particle Size (nm)</th>
<th>PdI</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>9.34±0.5</td>
<td>0.171±0.02</td>
<td>70</td>
</tr>
<tr>
<td>1:2</td>
<td>3.22±0.73</td>
<td>0.163±0.004</td>
<td>54</td>
</tr>
<tr>
<td>1:4</td>
<td>55.2±0.89</td>
<td>0.246±0.05</td>
<td>43</td>
</tr>
<tr>
<td>1:8</td>
<td>191±0.23</td>
<td>0.238±0.01</td>
<td>42</td>
</tr>
</tbody>
</table>

The table 3 shows the conjugated batches along with percent yield and particle size. Conjugates with molar ratio 1:1 and 1:2 had highest percent yield and smaller article size. These batches were used for further experiments.

The conjugation was confirmed using the Nuclear Magnetic Resonance ($^1$H NMR) which gives the number of protons associated to the carbon present in the molecule. The conjugated product was lyophilized and about 1 mg was dissolved in 500 μl of deuterated water and characterized using NMR. The characteristic peaks for cyclodextrin and PAMAM were identified and integrated to get the amount of conjugation. The characteristics peaks for PAMAM were seen at 2.74, 2.5 and 2.3 ppm (fig. 10(a)).
Figure 10: NMR spectra of (a) PAMAM (b) Carboxymethyl-β-Cyclodextrin (c) 1:1 conjugation (d) 1:2 conjugation

Whereas the characteristic peaks for cyclodextrin were determined at 5.19 and 3.7 ppm (fig. 10(b)). The conjugation was confirmed from the peaks obtained at 5.19, 3.7 and 2.7 ppm (fig. 10(c)). The conjugation was quantitated by integrating the peak 3.7 representing the proton of hydroxyl group on cyclodextrin and the peak 2.7 representing methylene group of PAMAM.[33, 36, 38]

The conjugated dendrimers were characterized for particle size and zeta potential using Malvern zeta sizer which are among the important parameters in gene therapy. The conjugation of molar ratio 1:1 had shown complete conjugation of the amine groups with cyclodextrin which was confirmed the NMR. Similarly, the conjugation of molar ratio 1:2 had shown conjugation of cyclodextrin on 50% of the amine groups. The particle size for 1:1 conjugation was seen to be 9.34±0.59 nm while that of 1:2 batch was seen to be 3.22±0.73 nm (Fig.11 (a) and (c)). The zeta
potential was reduced for both the batches showing the occupancy of terminal groups of PAMAM with cyclodextrin (Fig.11 (b) and (d)).

![Graphs](image)

Figure 11: (a) and (b) Particle size and zeta potential for 1:1 conjugation, (c) and (d) Particle size and zeta potential for 1:2 conjugation

The morphology of the conjugate was analyzed using the Transmission Electron Microscopy (TEM) at 200000V and magnification of 29000x. The circular nanoparticles can be seen with average particle size of 10nm. After complexation with the plasmid the particle size was increased and was seen in the TEM image as well.
Figure 12: TEM image of (a) CyD-PAMAM conjugate (b) CyD-PAMAM complexed with Plasmid

3.2 Complexation and characterization of conjugated PAMAM with plasmid

The electrostatic interaction between the positive terminal group of PAMAM and the negative plasmid DNA forms the complex. For the complexation, the plasmid was added to the dendrimer under vortexing followed by incubation at room temperature for 15 minutes. The complex was then characterized for particle size and zeta potential were the particle size was obtained to be and zeta potential was found to be for 1:1 and 1:2 respectively. The n/p ratio is an important parameter which gives the condensation of the plasmid DNA in the dendrimer structure.

Increase in the particle size of the dendrimer conjugate was seen after complexation with plasmid. Similarly, an increase in the zeta potential was seen after complexation which would be due the condensation of the DNA in the dendrimer cluster.[37] The dendrimers are smaller in size than the plasmid DNA and form a dendrimer cloud by electrostatic interaction. This interaction can expose the unconjugated surfaces of the dendrimer which can be the reason for the increase in the zeta potential.
Figure 13: (a) Particle size and (b) zeta potential of 1:1 conjugated batch (c) Particle size and (d) zeta potential of 1:2 conjugated batch after complexation with plasmid.

The complex was then further characterized using gel electrophoresis. Compaction of the plasmid within the dendrimer is an important factor which protects the dendrimer from the physiological degradation. Gel electrophoresis is a simple technique to see if the compaction of gene has taken place.

The complexation of plasmid was carried for both the batches. The ethidium bromide added to the gel complexes with the DNA and can be viewed under Ultraviolet light. Various N/P ratios i.e 5, 10 and 20 were used, and the complexes were allowed to run on 1% agarose gel. It was observed that for 1:2 conjugated batches the plasmid complexation was seen for all the N/P ratios, were as, for 1:1 conjugation the plasmid which was not complexed was seen on the agarose gel for N/P ratio 5:1 and 10:1 indicating some of the plasmid complexation with dendrimer, which is
because most of the amines group for this batch are conjugated with cyclodextrin leaving less room for plasmid complexation (Fig.14).

![Gel electrophoresis for (a) 1:1 conjugated batch (b) 1:2 conjugated batch](image)

3.3 Cellular uptake studies

The fluorescence intensity was normalized, and the images were taken at same exposure time to minimize the discrepancy in the data. The fluorescent intensity was measured using imageJ software and the intensity was plotted. The cellular uptake of the dendrimer was seen to be good for the 1:2 conjugated batch and that of PAMAM alone when compared to 1:1 batch. The highest cellular uptake was seen after 48 hrs of incubation time. The co-localization of the plasmid and FITC can be seen in the cells depicting the yellow color.
Figure 15: (a) Cellular uptake in ARPE cells and (b) Fluorescence intensity after 24hrs, 48hrs and 72hrs with statistical analysis at 48 hr time point (P>0.05)

Figure 16: Co-localization of FITC and RFP Plasmid (a) 1:1 conjugation (b) 1:2 conjugation
3.4 Cytotoxicity of the complexes

![Graphs showing cell viability over time](a) 24hrs, (b) 48hrs, (c) 72hrs.

Figure 17: Cytotoxicity of the complexes after (a) 24 hrs, (b) 48 hrs and (c) 72 hrs.

The cytotoxicity studies were carried out using CTG assay where the luminescence is directly proportional to the viable cells. The data showed that the complex was not toxic to the cells when compared to that of commercially available lipofectamine. The lipofectamine containing media was removed after 4hrs of incubation with the cells were as the dendrimers were incubated for 24hrs after which the media was replaced with fresh media. The treated groups were compared with the control and statistically analyzed using one-way annova.

3.5 *In-vitro* transfection ARPE19 cells with complexed plasmid

The smaller size of the dendrimer facilitates their entry into the cells and the lower pH inside the cell helps the detachment of plasmid from the dendrimer and facilitates its delivery to the desired site.[31] The selected N/P ratio from the gel electrophoresis, cellular uptake and
cytotoxicity studies were used for transfecting the ARPE (Retinal Pigment Epithelial) cells. The transfection was analyzed at three time points i.e 24 hours, 48 hours and 72 hours for 4 groups which included lipofectamine, dendrimers, 1:2 conjugated batch and 1:1 conjugated batch. After 72 hours the transfection efficiency was calculated using ImageJ software were the transfected cells and the live cells counted. The formula used was as follows:

Transfection efficiency = Transfected cells/Live cells × 100

Figure 18: (a) the % transfection efficiency of the 4 groups after 72 hrs (b) represents the ARPE transfection after 24, 48 and 72 hrs
CHAPTER 4: DISCUSSION AND CONCLUSION

Currently gene therapy is the foremost researched area with several viral vectors in clinical trials. But the overall production cost for these viral vectors is high making the treatment expensive. Moreover, the unpredicted mutagenesis is of great concern as it varies from patient to patient. Non-viral vectors, on the other hand, are a promising alternative for gene therapy. Among several lipid and polymer-based vectors, dendrimers have been widely used.

A generation 4 PAMAM with 64 terminal amine groups was used for conjugation with β-cyclodextrin to obtain the dendriplexes with better transfection efficiency. According to literature higher the generation of the dendrimer, more cytotoxic they are because of which generation 4 PAMAM were used. Cyclodextrin, on other hand, facilitate the entry of dendrimer into the cells mainly by membrane disruption.[23] Thus, conjugation of dendrimer and β-cyclodextrin was carried out to increase the transfection efficiency.

The conjugation of the dendrimer and β-Cyclodextrin was carried out using the amidation reaction between the terminal amines of dendrimers and the carboxylic group of carboxymethyl-β-Cyclodextrin. The reaction was optimized for the reaction time and the time for which the dialysis was carried out. Various batches were prepared to optimize the amount of cyclodextrin conjugated on the dendrimers for achieving desirable complexation of pDNA and transfection efficiency. Batches were formulated with the ratio of 1:6, 1:3 and 1:1 of dendrimer: cyclodextrin ratio. The conjugation was confirmed by the nuclear magnetic resonance (NMR) by the characteristic peak at 5.12, 3.7 and 2.7 depicting the anomeric hydrogen, the hydroxyl group of β-Cyclodextrin and methylene group on the dendrimer respectively. For gene delivery, the size of
the DNA/dendrimer complex is critical. Small nanoparticulate are advantageous for in vivo experiments, due to their extracellular diffusion and endocytosis mechanism. The average size distribution was 7.2 nm and the zeta potential was +7.23 mV for 1:1 batch and the size distribution for 1:2 was found to be 4.34 nm and the zeta potential was +13.21 mV. The zeta potential of the batches reduced as the amount of the CyD was increased showing the conjugation of CyD on the terminal amines of PAMAM. The 1:1 and 1:2 conjugates were electrostatically complexed with the plasmid at different N/P ratios ranging from 5:1, 10:1, and 20:1 and were analyzed using the gel electrophoresis to confirm the complexation and the ionic condensation of the plasmid. The 1:2 batch had shown complete condensation of the plasmid in the dendrimer at all 3 N/P ratios, were as, for 1:1 batch the complete condensation was seen for 20:1 but some of the plasmid was condensed at 5:1 and 10:1 N/P ratio. Further the cytotoxicity studies were carried out to see the effect of N/P ratio on the ARPE cells. Along with the batches with 5:1, 10:1 and 20:1, N/P ratio of 30:1 batch was also prepared to see the effect of higher N/P ratio. There was significant difference in the cytotoxicity as the N/P ratios increased, which can be because of increasing number of the amine groups. [39] These N/P ratios were then utilized to observe the cellular uptake and transfection efficiency in the ARPE cells. The cellular uptake was examined by conjugating the FITC on the PAMAM and CyD-PAMAM conjugate and analyzed after 24, 48 and 72 hrs. It was observed that the cellular uptake of the PAMAM and the CyD-PAMAM was maximum after 48hrs. The uptake for 1:1 conjugated batch was seen to be low as compared to that of the PAMAM and 1:2 batch. This can be because of less amount of FITC being conjugated on this batch due to preoccupied amine groups. The highest cellular uptake was seen for 1:2 conjugated batch with N/P ratio of 10:1 which was similar as that of PAMAM. The transfection efficiency was calculated after 72 hrs of incubation, and it was seen that the 1:1 batch with N/P ratio of 5:1 had shown highest
transfection efficiency as compared to the 1:2, PAMAM and commercially available lipofectamine. Even though the cellular uptake for 1:1 batch was seen to be less, the endosome escape ability of the cyclodextrin might have facilitated the plasmid entry into the nucleus.[23] However, the commercially available lipofectamine have shown 20% of transfection efficiency but the cytotoxicity was also high. When compared to PAMAM, the transfection efficiency for 1:1 and 1:2 conjugated CyD:PAMAM was significantly higher, showing the effectiveness of this non-viral gene delivery.

Future experiments are needed to be carried out to see the in-vivo activity of this conjugation. Thus, animal studies are required to see the transfection efficiency in physiological conditions. This CyD-PAMAM conjugates with specific targeting ligand can be administered topically which would then travel to the posterior segment of the eye lowering the use of intravitreal injections which are most prominently used for drug delivery to the posterior segment of the eye.

In conclusion, this conjugation of CyD-PAMAM can be effectively used as a non-viral vector for gene therapy.
REFERENCES


