

**REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
INSTITUTE OF HEALTH SCIENCES**

**IN VITRO/IN VIVO EVALUATION OF DENDRIMERIC
SYSTEMS FOR RETINAL DRUG DELIVERY**

Pharm. (M.Sc.) Burçin YAVUZ

**Pharmaceutical Technology Program
PhD THESIS**

**ANKARA
2014**

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**SUPERVISOR
Assoc. Prof. Sibel PEHLİVAN**

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ABSTRACT

Yavuz, B. In Vitro/In Vivo Evaluation Of Dendrimeric Systems for Retinal Drug Delivery. Hacettepe University, Institute of Health Sciences, PhD Thesis in Pharmaceutical Technology, Ankara, 2014. Ocular drug delivery is still a challenge due to its complex structure with many anatomical and physiological barriers. Delivering drugs to the back of the eye is especially harder because of barriers like cornea, sclera, blood-retina barrier; acellular vitreous structure, tear turnover. Thus retinal drug delivery can be only achieved by intravitreal route, which is an invasive technique that might cause serious ocular damage with repeated applications. However, posterior segment of the eye has many serious diseases, which increase the risk of blindness unless treated, such as age related macular degeneration, choroidal neovascularization and diabetic retinopathy. Dexamethasone (DEX) is a corticosteroid has been used for most of the ocular diseases including DR and macular edema. It is desirable to have a drug delivery system that can deliver DEX in a non-invasive manner, and yet achieve sufficiently high concentration in retina, or prolong the residence time and reduce the application frequency. Dendrimers are nanostructured polymers with high capability of interacting especially with hydrophobic drugs and improve their solubility and tissue permeation. In this study, various types of PAMAM dendrimers were used to prepare DEX complex or conjugate formulations in order to investigate the effect of dendrimers with different generations and charges, on DEX delivery to the retina, in terms of corneal and scleral permeability or the duration of the stay. DEX-PAMAM complex formulations are expected to show immediate release and a possible delivery system for topical or subconjunctival application. On the other hand DEX-PAMAM conjugates were designed to obtain an extended release following subconjunctival or intravitreal injection. Thus, these formulations have been evaluated in different terms, based on their purpose of development.

Keywords: Ocular drug delivery, Retinal drug delivery, Dexamethasone, PAMAM dendrimers, in vivo.

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ÖZET

Yavuz, B. Retinaya İlaç Taşınması Amacıyla Dendrimerik Sistemlerin İn Vitro/İn Vivo Olarak Değerlendirilmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Farmasötik Teknoloji Programı Doktora Tezi, Ankara, 2014. Göze ilaç uygulanması, gözün anatomik ve fizyolojik bariyerler bulunduran kompleks yapısı nedeni ile halen zorluklar içermektedir. Gözün arkasına ilaç taşınması özellikle kornea, sklera, kan-retina bariyeri gibi bariyerler, vitrözün hücresiz yapısı, göz yaşı salgısı nedenleri ile daha zordur. Bu nedenle ilaçlar retinaya genellikle sadece intravitreal yoldan uygulanabilmektedir, ancak bu girişimsel yol tekrarlanan uygulamalarda göze ciddi zararlar verebilmektedir. Diğer yandan gözün arka kısmında görülen, yaşa bağlı maküler dejenerasyon, koroid nörovaskülerizasyone diyabetik retinopati gibi hastalıklar tedavi edilmediği takdirde körlüğe yol açabilmektedir. Deksametazon (DEX), diyabetik retinopati, maküler ödem gibi birçok oküler hastalığın tedavisinde kullanılan kortikosteroid bir ilaçtır. Bu nedenle DEX'u retinaya yeterli konsantrasyonda taşıyabilecek, girişimsel olmayan ya da gözde kalış süresini uzatarak uygulama sıklığını azaltacak bir taşıyıcı sisteme ihtiyaç duyulmaktadır. Dendrimerler, özellikle hidrofobik ilaçlarla yüksek oranda etkileşerek, çözünürlük ve dokudan permeasyonlarını arttırabilen, nanoyapılı polimerlerdir. Bu çalışmada farklı PAMAM dendrimer türlerinin, DEX ile kompleks ve konjugat formülasyonları hazırlanarak, farklı boyut ve yüke sahip dendrimerlerin DEX'un retinaya taşınması ve gözde kalış süresi üzerindeki etkileri araştırılmıştır. DEX-PAMAM kompleks formülasyonları hızlı salım yapan, permeabilite arttırıcı sistemler olarak topikal ve subkonjunktival yoldan, DEX-PAMAM konjugat sistemleri ise uzun süreli salım yapmak üzere subkonjunktival ve intravitreal enjeksiyon yolu ile uygulanmak üzere tasarlanmıştır. Formülasyonlar kullanılış amaçlarına uygun olarak farklı şartlar altında incelenmiş ve değerlendirilmiştir.

Anahtar Kelimeler: Oküler ilaç taşınması, Retinaya ilaç taşınması, Deksametazon, PAMAM Dendrimer, in vivo.

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SYMBOLS AND ABBREVIATIONS

AH	Aqueous humor
AMD	Age related macular degeneration
ARPE 19	Human retinal pigment epithelial cell line
BAB	Blood-aqueous barrier
BRB	Blood-retina barrier
CNV	Choroidal neovascularization
CRPE	Choroid – retinal pigment epithelium
CV%	Coefficient of variance percentage
DAD	Diode array detector
DEX	Dexamethosone
DMEM : F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMF	Dimethyl formamide
DR	Diabetic retinopathy
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
HPLC	High performance liquid chromatography
LCMS	Liquid chromatography/ Mass spectroscopy
LOD	Limit of detection
LOQ	Limit of quantitation
PEG	Poly(ethylene glycol)
PAMAM	Poly(amidoamine)
P_{app}	Apparent permeability coefficients
RPE	Retinal pigment epithelium
SCRPE	Sclera-choroid-retina pigment epithelium
SD	Standard deviation
SDS	Sodium dodesil sulphate
TA	Triamcinolone acetone
TEER	Trans epithelial electrical resistance
VEGF	Vascular endothelial growth factor

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1. INTRODUCTION

Ocular drug delivery is still one of the most challenging tasks for pharmaceutical scientists. The eye has a complex structure with high resistance to the foreign substances including drugs. Upon an ocular application, the anterior and posterior segments of the eye function both independently (1) . Thus, ocular drug delivery can be classified into two segments, anterior and posterior. Conventional drug delivery systems are not capable of meeting the requirements in the treatment of ocular diseases (2) . However, “more than 90% of the marketed ophthalmic formulations” are in the form of eye drops, and most of them target the “anterior segment eye diseases” (3) .

Most of the ophthalmic diseases affect vitreous, choroid, and neural retina. For example, glaucoma, diabetic retinopathy (DR), age-related macular degeneration (AMD), and various forms of retinitis pigmentosa are damaging the back of the eye, that may result in impaired vision and even blindness (4) . Unfortunately, treatment of “posterior segment diseases” is still an unsolved issue. Delivery of drugs to the posterior segment is more challenging than to the anterior segment, due to the ocular barriers, acellular nature of the vitreous body and the longer diffusion distance (5) . Thus, posterior segment of the eye have become an important therapeutic target with unmet medical needs. The main goal in the posterior segment disease treatment is the delivery of the drug to the tissues in the therapeutic range, while reducing the side effects. Systems developed to achieve this goal range from simple solutions to novel drug delivery systems, including liposomes, micelles, nanoparticles, dendrimers, iontophoresis, and gene delivery systems (6,7) .

Diabetic retinopathy (DR), a specific microvascular complication of diabetes, is the leading cause of blindness in working-age people. Pharmacological medication is essential for diabetic retinopathy treatment besides controlling the diabetes related physiological factors. The main pharmacological drug groups used for this purpose are corticosteroids, vascular endothelial growing factor (VEGF) inhibitors, protein kinase C inhibitors, somatostatin agonists and cyclooxygenase-2 (COX-2) inhibitors. Dexamethasone (DEX) is a commonly used corticosteroid drug for the treatment of most of the retinal diseases including DR. DEX with its high potency and effectiveness on multiple diseases was chosen for this research.

Current treatment of diabetic retinopathy requires intravitreal injection or intravitreal implants which are invasive and risky methods. Repeated intravitreal injection, reduces patient compliance and it has the risk of causing eye damage. Implantation of drugs such as steroids, which may cause serious side effects, is too risky because discontinuation of drug is only possible by surgical intervention. Even the subconjunctival application is a promising delivery route for retinal drug delivery, low trans-scleral transmission of most drugs is a limitation for reaching the efficient drug concentration at the target area.

Dendrimers are “tree-like,” nanostructured polymers that have been an important research field in ocular drug delivery. They are attractive drug delivery systems due to their nanosize range, ability to display multiple surface functional groups that allows for targeting and easy preparation (8) . Given their structural features, most of the ocular diseases would benefit from long-lasting drug delivery of dendrimers and dendrimer-based drug delivery systems. It was already reported that dendrimers present practical solutions to drug delivery issues by enhancing solubility and biodistribution as well as ocular permeability. Since it is easy to control the features of dendrimers such as their size, shape, generation, branching, molecular size, and surface functionality, these polymers are ideal carriers in pharmaceutical applications. Polyamidoamine (PAMAM) dendrimers, especially those with –OH and -COOH terminal groups are non-cytotoxic and are cleared intact through the urine at lower generations. Ongoing studies in developing improved ocular dendrimeric systems may not only serve to enhance the drug delivery to the ocular surface, but also may provide effective delivery of therapeutic agents to intraocular tissues, such as the retina or choroid, using noninvasive delivery methods (9) .

The aim of this study is to evaluate the effect of various PAMAM formulations on DEX delivery to the back of the eye, especially to retina via different delivery routes. Thus, different PAMAM dendrimer types have been used to prepare physical complexes or chemical conjugates of DEX in order to compare their effects on the ocular permeability, ocular tissue distribution and duration of stay in the eye to investigate if it is possible to deliver DEX to retina using dendrimeric systems either via intravitreal application with reduced frequency or a less invasive route such as subconjunctival or topical applications.

2. THEORETICAL PART

2.1. Structure and Physiology of Eye

The globe of the eye consists essentially of three coats enclosing the transparent refractive media. The outer, protective layer is made up of the sclera and cornea. The middle coat is mainly vascular consisting ciliary body, choroid and iris. The inner layer is retina, which contains the essential nerves responsible for vision. The cornea and the crystalline lens are the only tissues in the body in addition to cartilage which have no blood supply, whereas the choroid and the ciliary processes are highly vascularized and exhibit very high blood flows (10) . A schematic diagram of the human eye is given in Figure 2.1.

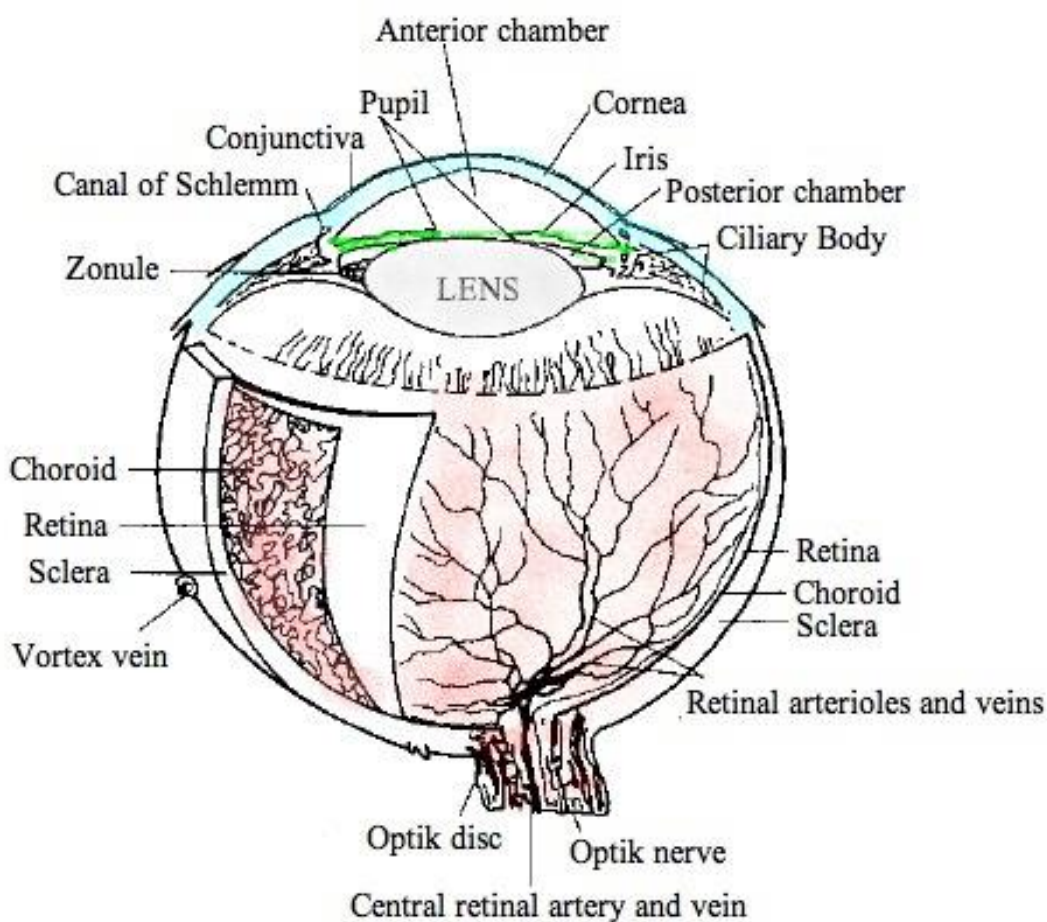


Figure 2.1. Schematic diagram of the human eye (11)

The external part of the eye is covered by eyelids, which protect the eye from mechanical or chemical injuries. In addition, the lids also keep out excessive light and spread the secreted tear film over the cornea as well as preventing evaporation from the surface of the eye. The upper lid is the more mobile and when it is open, it covers about 1mm of the cornea (12) .

Eye has two segments, which are anterior and posterior. Anterior segment includes pupil, cornea, iris, ciliary body, aqueous humor, and lens. The posterior segment of the eye is comprised of sclera, choroid, vitreous, and retina.

Cornea protects the eye against infection and physical damage to the inner parts and also refracts and transmits the light to the lens and retina. It has about 70% of the refractive power of the human eye. The cornea of an adult human eye has an average horizontal diameter of about 11.5 mm and a vertical diameter of 10.5 mm (13) . There are five layers in the human cornea: epithelium, Bowman's membrane, lamellar stroma, Descemet's membrane and endothelium. In addition to these layers, there is a basement membrane of the epithelium, which is located between the epithelial cell layer and Bowman's membrane (Figure 2.2) (14) . These layers make cornea the strongest barrier in the eye against foreign substances. Corneal epithelium is relatively impermeable compared to many other epithelial tissues such as intestinal, nasal, bronchial, tracheal. The corneal epithelium contains esterase enzymes, which can be utilized in the delivery of prodrugs. Although there are tight junctions between endothelial cells, this layer is weaker than epithelium and most drugs can penetrate endothelial cell layer from stroma (15) . The oxygen and nutrients are transported to cornea, which is a non-vascular tissue, by aqueous humor. The aqueous humor in human is approximately 300 μ L and fills the anterior chamber of the eye, where is the location in front of the lens. It normally contains no cells and its protein content is very low (16) .

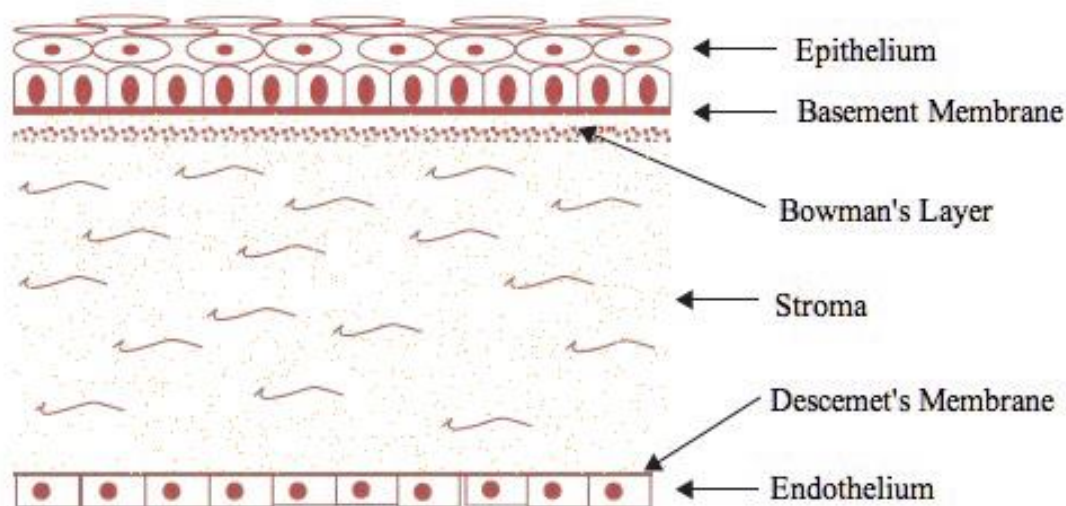


Figure 2.2. Schematic presentation of cornea layers (14)

Sclera is the outermost layer of the eye and forms a connective tissue coat that protects the eye from internal and external forces and maintains its shape. It is about 0.5–1.0 mm thick, consisting mainly of collagen bundles and elastic fibers. Cornea and sclera are connected at the limbus. The visible part of the sclera is covered by conjunctiva, which is a transparent mucous membrane. Unlike the cornea, the conjunctiva has a rich vasculature and a large amount of the administered drug crossing it is removed by the systemic circulation. Conjunctival epithelium is continuous with that of cornea and with the epidermis of the lids and has a surface area that is five times of the cornea (17,18) .

The middle layer of the eye is composed of the iris, the ciliary body and the choroid. Choroid is a highly vascularized tissue present between the retina and sclera. It forms the posterior part of the uvea, the anterior part consisting of the iris and ciliary body. The iris tissue is highly vascular, porous, and possesses a large surface. It consists of the pigmented epithelial cell layer, the iridial sphincter and dilator muscles, and the stroma. Color of iris depends upon the amount of melanin in its stroma. It controls the size of the pupil, and thus the amount of light reaching the retina. The ciliary body controls the shape of the lens and is the aqueous production site and it nourishes the lens, provides the muscle for accommodation and may

secrete the unique zonular fibers. The ciliary body comprises the ciliary muscle and the ciliary processes (18) .

The inner layer of the eye is the retina, a complex, layered structure of neurons that capture and process light. It is firmly attached to the ciliary body and consists of two major functional parts: the neural layer and the retinal pigment epithelium (RPE). RPE cells are connected by tight junctions and form a tight barrier between the choroid and retina. It selectively transfers nutrients to the retina from the choroid. The three transparent structures surrounded by the ocular layers are called the aqueous, the vitreous and the lens. Vitreous is a clear gel composed almost entirely of water (99%) and collagen fibrils and its pH is about 7.5. There is no flow through vitreous. Lens is located behind the iris in front of the vitreous and it is important for visual function. Lens enables protects the retina from the harmful ultraviolet radiation (18,19) .

Good visual function requires the surface to the eye to be covered by fluid to maintain a uniform surface. The eye is constantly cleansed and lubricated by the lacrimal apparatus structures, which are lacrimal glands, lacrimal canals, lacrimal sac and nasolacrimal duct. Muscles associated with the blinking compress the lacrimal sac, when these muscles relax; lacrimal fluids are released into the lacrimal sac and wash the foreign bodies out of the eye. Lacrimal fluid is an isotonic aqueous solution of bicarbonate and sodium chloride with pH 7.4, which also contains lysozyme. The amount of the lacrimal fluid in human eye is approximately 7 μ L (16) . The tear film consists of three main layers. The outermost layer is a thin lipid layer, the middle layer constitutes the tear fluid and the innermost layer is a mucoid layer, which is considered important for wetting the corneal and conjunctival epithelium. Drugs tend to be washed away quickly because of this lacrimal system and this affects bioavailability of the drugs negatively (20) . Anatomical and physiological parameters in human eyes were summarized in Table 2.1.

Table 2.1. Anatomical and physiological parameters in human eyes (21)

Parameters	Value
Normal tear volume (μL)	7.0
Tear secretion rate ($\mu\text{L}/\text{min}$)	1.2
Solution drainage rate constant (min^{-1})	1.45
Corneal surface area (cm^2)	1.04
Conjunctival surface area (cm^2)	17.65
Corneal thickness (mm)	0.52
Volume of aqueous humor in anterior chamber (μL)	261-310
Aqueous humor section rate (%)	1-2
Distribution volume in anterior chamber (μL)	150-3000
Clearance in anterior chamber ($\mu\text{L}/\text{min}$)	1-30

2.2. Challenges in Ocular Drug Delivery

Eye structure is unique with protective barriers, which causes many challenges to the effective delivery of drugs to the eye. The eyeball is divided into 2 segments: the anterior segment containing the cornea, crystalline lens, iris, ciliary body, and fluid-filled aqueous humor and the posterior segment that includes the sclera, choroid vessels, retina, macula, optic nerve, and fluid-filled vitreous humor (2) . Eye is a well protected organ with several specialized cellular modifications that results in various barriers that partially isolate the eye from the rest of the body, which can be a drug delivery challenge (22) . These special processes/barriers are as follows:

- The “inner and outer blood–retinal barriers” separate the vitreous and the retina from the systemic circulation, and because of the acellular structure of the vitreous body, it reduces molecular convection (23) .
- The inner limiting membrane controls the particle exchange and entry from the vitreous to the retina.
- The “blood-aqueous barrier” limits the molecular transport from the blood to the inner part of the eye (24) .

- Corneal epithelium's intact structure with desmosomes and tight junctions causes resistance to the passage of most drugs due to the presence of layers: hydrophobic epithelium, hydrophilic stroma, and hydrophobic endothelium (25) .
- The tear film forms a mucoaqueous barrier, which washes the particles away continuously at the anterior surface of the eye (5) .

The anatomical and physiological barriers mentioned above are the challenges in drug delivery to the eye. Molecular size and shape, charge, and degree of ionization of the drug, solubility and lipophilicity affect the penetration rate to the eye (26) . Corneal efflux proteins, such as P-glycoproteins (P-gp) and multidrug resistance associated proteins prevent the effective drug retention and pump drug molecules out from the corneal epithelium (27) . Biocompatibility of the drug delivery systems is also relevant when ocular delivery is concerned. Factors that influence ocular permeability are summarized in Table 2.2.

The challenge of formulating an ocular therapeutic system is to achieve an efficient drug concentration at the target site long enough to provide the therapeutic efficacy (28,29) . An ideal topical formulation must be well tolerated and easy to administer, avoid systemic absorption and increase drug retention time in the eye.

Table 2.2. Factors that influence ocular permeability (30)

Membrane Factors	Drug Factors	Formulation Factors
Absorption area availability (cornea or sclera)	Concentration	Retention time
Thickness	Solubility	pH
Porosity	Partition coefficient	Tonicity
Tortuosity	Molecular weight	Viscosity
Liphophilicity/hydrophilicity balance	pKa	Release rate

The most common method of ocular drug delivery is the instillation of drops into the lower cul-de-sac. Typically “less than 5% of the topically applied drug” penetrates the cornea and reaches intraocular tissues, while most of the instilled dose

is often absorbed systemically via the nasolacrimal duct and conjunctiva (3) . The eye drops are easy to apply, but absorbed amount of drug into the target tissues are only a very small portion of the instilled dose. Ocular administration of irritating drugs or vehicles increases the drug loss from the precorneal area to a further extent due to induced lacrimation. It requires frequent application and large doses of drugs to achieve the effective therapeutic dose, which leads to an increase in both local and systemic side effects (31) . The passage of drugs from the anterior to the posterior segments of the eye is not very efficient due to the aqueous turnover. Therefore, ocular surface administered drugs usually cannot reach the posterior segments of the eye in sufficient therapeutic concentrations. Alternative approaches such as incorporation of permeation enhancers/cyclodextrins and increasing the viscosity of solutions did not provide any significant improvement (2) . Different approaches to increase ocular bioavailability, is given in Table 2.3.

Table 2.3. Different approaches to increase ocular bioavailability (32)

Improvement of penetration	Improvement of the formulation
Chemical modifications	Vehicle
*Prodrugs	*Suspensions & Ointments
* Penetration enhancers	* Viscous vehicles
* Ion pairs	*Bioadhesive vehicles
	*In situ gelling systems
Pharmaceutical modifications	Colloidal dispersion systems
*Cyclodextrins	*Liposomes
	*Nanoparticulate systems
	*Emulsions
Physical modifications	Solid polymeric matrix and devices
*Iontophoresis	*Degradable matrices
	*Non-degradable devices
	*Contact lenses
	*Collagen shields
	Implant devices

Since the penetration to cornea is often poor, systemic and intravitreal (injection or implant) administrations appear to be the main strategies for treating posterior segment infections/diseases. However, due to strong blood-ocular tissue barrier, systemic administration requires large doses, while intravitreal injections and implants are very invasive and are associated with a high degree of retinal damage risk, such as endophthalmitis and retinal detachment (33) . Thus, in order to deliver drug to the back of the eye, there has been growing attention to transscleral route (34) . Ocular penetration routes for drugs following topical, systemic and intravitreal administration is given in Figure 2.3.

Sclera is reported to be more permeable than cornea, even it is highly permeable to the large molecules of even protein size. However, retinal drug delivery is more complicated, because in case of transscleral application the drug must pass across the choroid and retina pigment epithelium (RPE) as well as sclera (35) . Furthermore, periocular (transscleral) routes such as subconjunctival, retrobulbar, sub-tenon have disadvantages such as hemorrhage at the injection site (36) .

Thus the major goal is to develop safe and efficient drug delivery systems with reduced side effects, improved bioavailability of drugs, increased retention time, cellular targeting, enhanced patient compliance, and providing extended therapeutic effects (37) . In the light of the recent studies, nanocarrier-based ocular drug delivery systems including dendrimers appear to be the most promising way to meet the requirements of an ideal ocular drug delivery system.

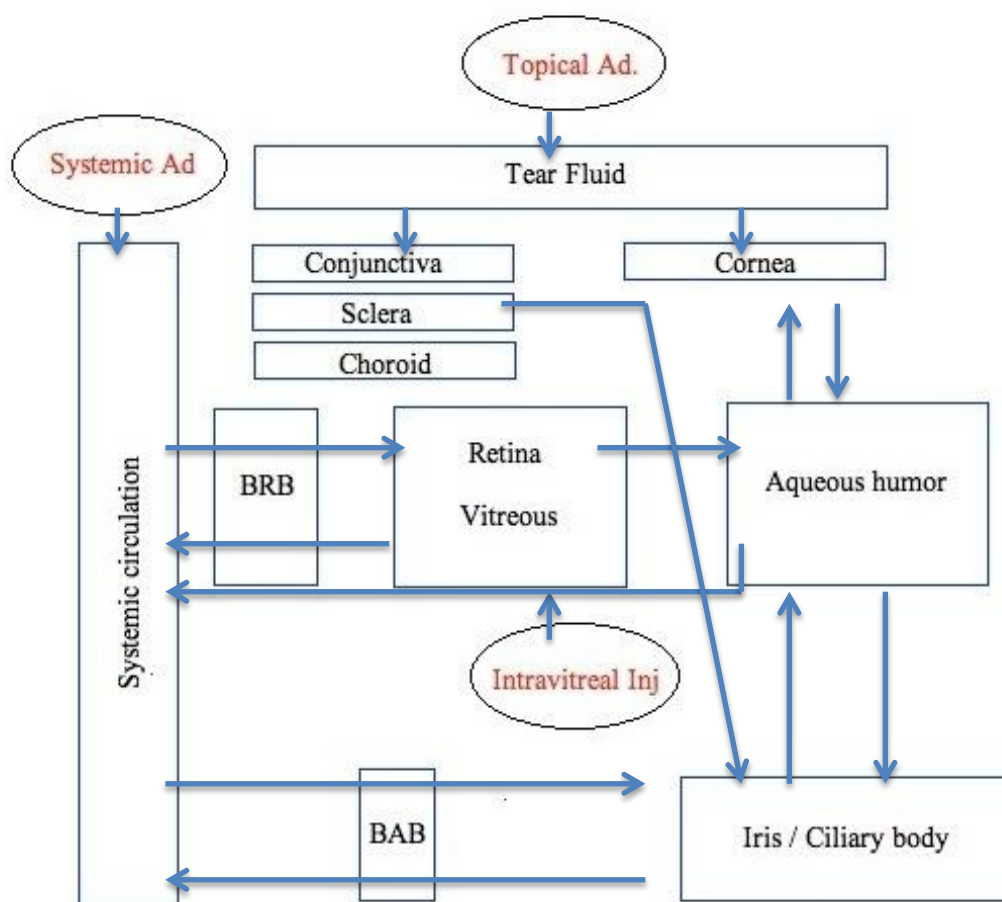


Figure 2.3. Ocular penetration routes for drugs following topical, systemic and intravitreal administration (38)

2.3. Ocular Diseases

2.3.1. Anterior Segment Diseases

Anterior segment diseases are also called periocular diseases and include conjunctivitis, keratitis, trachoma, cataract and dry eye.

Conjunctivitis is a condition with redness and sensitivity of the eye. Major cause of conjunctivitis is acute infection or allergy and bacterial conjunctivitis is the most common ocular infection. Keratitis is also an infection which patients have ocular pain, red eye, and often a decreased vision due to cloudy cornea. It might be a result of a bacterial, virus, fungus, protozoa or parasite infection. Trachoma is the infection of conjunctiva caused by *Chlamydia trachomatis* and usually seen in children. It is characterized with white lumps inside the upper eyelid and often

associated with thickening of the papillae. Dry eye is a common disorder of the tear film caused by decreased tear production or increased evaporation. It is characterized by chronic dryness of the cornea and conjunctiva, which is caused by unstable tear film associated with abnormality of the lipid, protein, and mucin profiles. Typical symptoms of dry eye include burning, stinging, and photophobia (16,39) .

Treatment approaches for anterior segment diseases are based on topical application. However short pre-corneal residence time might cause the treatment to fail. More than 90% of the dose is drained through the nasolacrimal duct to the nasal cavities and it is absorbed into the systemic circulation through nasal epithelium. Tears dilute the remaining drug in cul-de-sac, which reduces the transcorneal flux of the drug (25,40) . Topical formulations containing mucoadhesive or viscous materials can sustain and enhance drug delivery to target eye tissues. There are several marketed formulations to treat anterior segment diseases such as ocular solutions, emulsions, suspensions, gels, and in-situ gel forming solutions. (41) .

2.3.2. Posterior Segment Diseases

Diseases of the posterior segment of the eye are responsible for severe vision loss and blindness and the most prevalent posterior segment diseases include age related macular degeneration (AMD), diabetic retinopathy (DR), posterior uveitis and choroidal neovascularization (CNV) (42) .

Diabetes has many consequences in the eye, of which cataracts and DR are the most significant cause of blindness, and people with diabetes are 25 times more likely to become blind than the general population. Nearly 22% (0.9 million) of diabetic patients having vision-threatening DR and it is the most frequent cause of new cases of blindness among adults aged 20 –74 years. DR is a manifestation of a persistent inflammation in which an influx of inflammatory effectors, both cytokines and leukocytes, is responsible for the ischemia-induced neovascularization and damage to the retina. DR characterized by vascular closure, growth of new blood vessels on the retina and posterior surface of the vitreous. Macular edema, characterized by retinal thickening from leaky blood vessels, can develop at all stages of retinopathy (43-46) . DR can be divided into two clinical stages: nonproliferative and proliferative diabetic retinopathy. During nonproliferative DR,

the earliest visible sign of retinal damage results from abnormal permeability, which leads to the formation of microaneurysms (47) .

In the early stages of DR, no treatment is needed, unless macular edema is involved. To prevent progression of DR, people with diabetes should control their levels of blood sugar, blood pressure, and blood cholesterol. Proliferative retinopathy is treated with laser surgery, which is called scatter laser treatment and helps to shrink the abnormal blood vessels. Laser photocoagulation is found effective at slowing the progression of retinopathy and reducing visual loss, but the treatment usually does not restore lost vision (21) . The concept of DR as an inflammatory disease has led to direct clinical applications, with non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and anti-VEGF agents already in extensive use. Pegaptanib is an aptamer that binds VEGF and has been approved by FDA for the treatment of AMD. Early trials also have demonstrated its benefit in reducing diabetic macular edema. Bevacizumab and Ranibizumab are the other anti-VEGF molecules that have been studied for DR treatment (46,48) . It was also reported that celecoxib, a selective COX-2 inhibitor, inhibits diabetes-induced VEGF mRNA expression and vascular leakage in a diabetic rat model (49) .

The difficulties in drug delivery to the posterior eye segment, is mostly because of the long diffusion distance, the lens-iris barrier and the acellular nature of the vitreous body (5) . For the treatment of posterior segment diseases, various attempts have been made to improve drug bioavailability by increasing both drug retention in the precorneal area and drug penetration through the ocular barriers such as cornea and sclera. In addition, application route is very important parameter in terms of patient compliance and comfort considerations (50) .

All of the approaches are based on the premise that non-invasive topical methods to effectively deliver drugs, such as corticosteroids, to the posterior segment of the eye are not available, and invasive methods are the only alternative. The common choice for posterior segment disease treatment is intravitreal injection, which is a route bypassing the corneo-scleral barriers, but low patient compliance and possible complications are involved. Systemic administration is another major route for drugs to reach the retina through the blood circulation. However, the effective concentration can be reach only with high systemic side effects because of

the blood-retina barrier. The alternative application for posterior segment treatment is periocular route that delivers the drug via sclera. Several injection sites are available for periocular application, including subconjunctival, sub-tenon, peribulbar, posterior juxtrasclera and retrobulbar spaces (6,51) .

Even there are a large number of studies to achieve a successful treatment for posterior segment diseases, it's still a challenge to deliver drug to the back of the eye safely, with an effective dose. Advantages and disadvantages of the advanced drug delivery systems that have been studied for posterior segment diseases are given in Table 2.4.

Table 2.4. Advantages and disadvantages of the advanced drug delivery systems for posterior segment diseases of the eye (5)

Methods	Advantages	Disadvantages	Duration of Action
Non-biodegradable implants	<ul style="list-style-type: none"> *Controlled release of drug over a long period of time *Increased half-life of drugs * Drug stabilization * Improved patient compliance 	<ul style="list-style-type: none"> *Requires surgical implantation associated with ocular complications *Requires surgery to harvest the device once is depleted of the drug 	Years
Biodegradable implants	<ul style="list-style-type: none"> * Controlled release of drug over a moderate period of time * Increased half-life of drugs * Drug stabilization * Improved patient compliance *Removal not required *Various shapes 	<ul style="list-style-type: none"> *Surgical implantation or injection associated with ocular complications *Final uncontrolled ‘burst’ in the drug release profile 	Weeks to months
Intraocular liposomes, micro and nanoparticles	<ul style="list-style-type: none"> *Increased half-life of drugs *Decreased peak concentration resulting in decreased toxicity *Localized delivery of drugs * Improved patient compliance 	<ul style="list-style-type: none"> * Requires injection with associated risks *Vitreous clouding 	Days to weeks
Transscleral iontophoresis	<ul style="list-style-type: none"> *Non-invasive technique *Easy to use * Short application process * May be combined with other drug delivery systems * Ability to modulate dosage *Good acceptance by patients 	<ul style="list-style-type: none"> *Does not increase drug half-life *Mild pain 	Hours to days

2.4. Dexamethasone

Dexamethasone (DEX) is a glucocorticoid with similar structure to the natural steroid hormone made by the adrenal glands in the body. It relieves eye inflammation and swelling, heat, redness, and pain caused by chemicals, infection, and/or severe allergies. It is also used to treat persistent macular edema in retina, which is a major cause of visual disabilities and blindness among individuals with diabetes (52) .

It's a white powder, which is practically insoluble in water. A schematic representation of DEX structure is given in Figure 2.4.

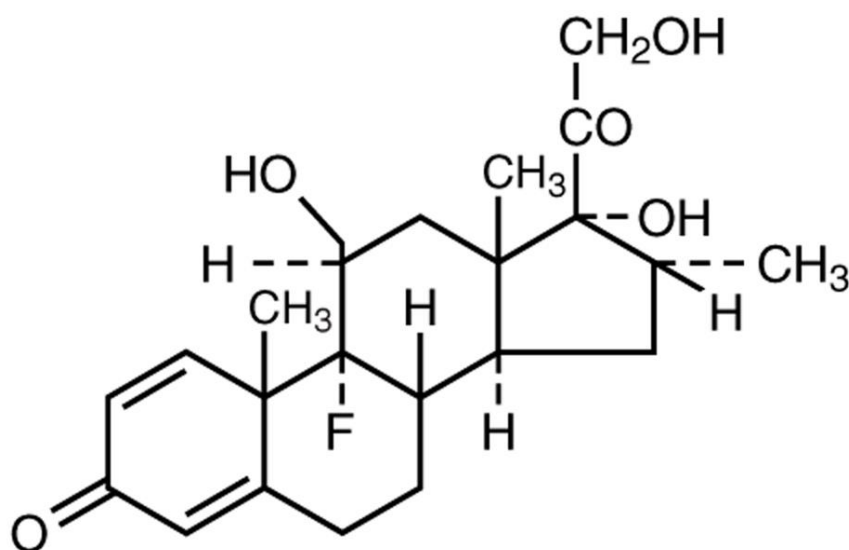


Figure 2.4. Schematic representation of DEX structure (21)

Oral or systemic administration of high-dose corticosteroids can produce therapeutic benefits but also adverse effects. Topical steroid preparations are commonly used for the treatment anterior segment diseases, and steroid delivery to the retina is currently undertaken with invasive intravitreal injections. Furthermore, it has been reported that continuous application of eye drops of 0.1% DEX for extended periods of time (3 weeks to 1 year) can cause glaucoma, cataract formation and thinning of the cornea or sclera (53) . It was determined the DEX concentration in in ocular tissues and serum after DEX administration through various routes such

as topical application of eye drop, subconjunctival injection and an oral dose. The results showed that the DEX concentration in the aqueous humor is far lower for eye drops compared to a subconjunctival injection even if an eye drop is instilled every 1.5 h. However the subconjunctival injection is not the optimal application route for DEX, because it needs to be applied daily to reach the therapeutic concentrations in the aqueous humor (54,55) . Therefore, a new, more effective delivery system, with fewer burdens, has been a goal attracting intensive research.

Since DEX is an important drug for ocular diseases, there are several attempts to improve ocular DEX delivery for both anterior and posterior segment diseases. A clinical study of 0.32% DEX-cyclodextrin-polymer complexes in humans showed 2.6-times higher AUC in the aqueous humor when compared with a DEX suspension (56) . DEX loaded poly(hydroxyethyl methacrylate) (PHEMA) contact lenses were also studied and bioavailability for DEX delivery was found much higher than eye drops. Controlled drug delivery of DEX to the eye through contact lens is also expected to be safer than delivery via drops because of reduction in the amount of drug that reaches other body tissues through systemic circulation (57) . DEX derivatives with different aqueous solubilities have been utilized in ocular studies including dexamethasone 21-disodium phosphate and dexamethasone 21-acetate and it was reported that the permeability rates of DEX derivatives through cornea, has been increased (58) .

There are also marketed DEX products for the treatment of ocular diseases. Ozurdex[®] is a biodegradable intravitreal implant containing 0.7 mg of DEX and was approved by the FDA in 2009 for the treatment of macular edema. In September 2010, the FDA approved Ozurdex[®] also for the treatment of noninfectious uveitis involving the posterior segment. Surodex[®] is an anterior segment implant of DEX for treating postoperative inflammation after cataract surgery. Given the distinct pharmacokinetics and pharmacodynamics of the injectable DEX implant, it is difficult to compare safety data (41,59) .

2.5. Dendrimeric Systems

2.5.1. Dendrimer Structure, Synthesis and Properties

Dendrimers are branched synthetic polymers that can be synthesized to provide macromolecules. Their tree-like branched architecture with several reactive end groups that surround a small molecule and form an internal cavity, is in particular very promising for biomedical applications (60) . Especially low generation dendrimers have the ability to encapsulate hydrophobic drug molecules into their internal cavities. Because of this unique structure, dendrimers are able to improve solubility of poorly water-soluble drugs (61) . In addition to the extraordinary structural control, another outstanding feature of dendrimers is their actual mimicry of globular proteins. They are referred to as “artificial proteins,” based on their electrophoretic, systematic, dimensional length scaling and other biomimetic properties (62,63) .

Dendrimers are built from a starting atom, such as nitrogen, after a repeating series of chemical reactions, carbon and other elements was added into it; produce a spherical branching structure. The central core molecule should have at least two reactive groups and the repeated branches are organized in a series of “generations” (64) . A schematic representation of a generation 2 dendrimer is given in Figure 2.5.

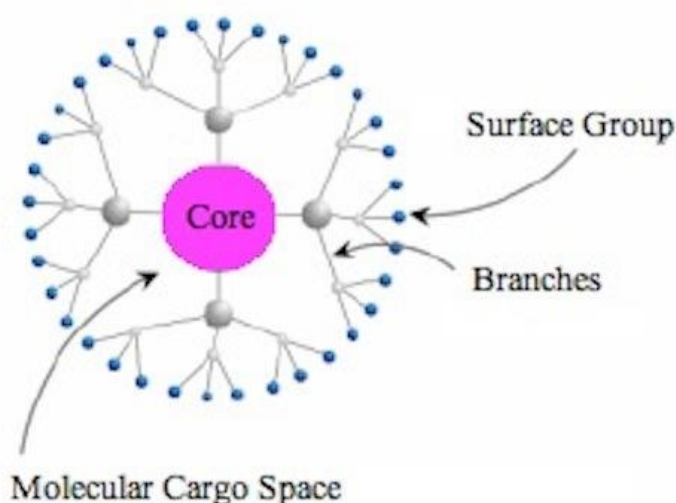


Figure 2.5. Schematic representation of a generation 2 dendrimer (9) .
(Image has been reprinted from the referred article with author’s permission).

Dendrimers can be constructed using either a divergent method or a convergent one (65). Regarding the divergent method, dendrimer grows outwards from a multifunctional core molecule. On the other hand, in the convergent approach, synthesis starts from the periphery of dendrimers toward the central core in which this part is termed “dendron”. When the dendrons grew enough, they are attached to a multifunctional core molecule (66). A schematic representation of divergent and convergent methods, are given in Figure 2.6. Theoretically monodisperse dendrimer size can be obtained by both synthetic methods. As the two methodologies have advantages and disadvantages, the most appropriate choice will depend mainly on the type of monomer used in the architecture of the polymer target.

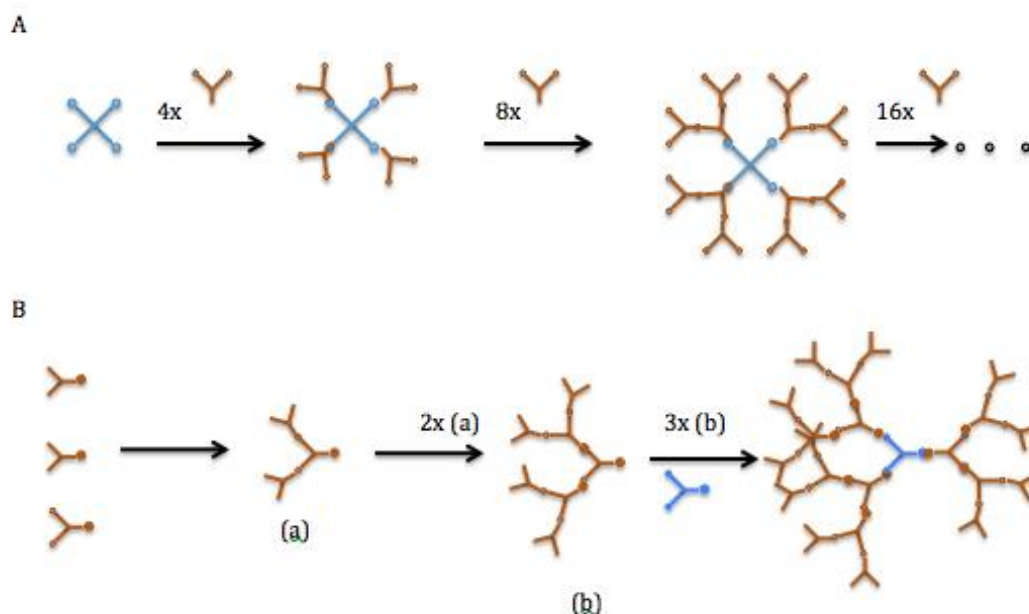


Figure 2.6. Schematic representation of divergent and convergent methods: (A) the divergent growth method (B) the convergent growth method (9). (Image has been reprinted from the referred article with author’s permission).

Other approaches have been developed based on the divergent and convergent methods such as double exponential growth, lego chemistry, and click chemistry. Monomer preparation from a single starting material for both divergent and convergent methods is possible using double exponential growth approach. Then a trimer is obtained by the reaction of the two result products, which can be used to repeat the growth again (67). In lego chemistry strategy, phosphorus dendrimers are prepared from highly functionalized cores and branched monomers. A scheme is developed that allows multiplications of the number of terminal surface groups from “48 to 250” in one step, after several variations in general synthetic scheme (68).

Dendritic polymers provide an additional approach to nanomedicine technologies and also provide some points of differentiation when compared with more traditional liposomal or nanoparticulate systems. They have many advantages such as their nanosize ranging from 1 to 100 nm with lower polydispersity index that allows them to avoid RES uptake. Dendrimers generally have greater solubility in common solvents as compared to linear polymers. However, the solubility depends on various components in addition to the surface groups as the generation number, nature of repeating units and even the core. Furthermore, multiple functional groups are present on outer surface of dendrimers, which can be used to attach vector devices for targeting anywhere in the body (69,70). Dendrimers also have the ability to encapsulate drug molecules into their internal cavities that leads to enhanced solubility, permeability, and retention effect depending on their molecular weight. It was reported that drug absorption is increased with dendrimers association in the cationic > uncharged > anionic order based on their permeability enhancer effects. Cationic dendrimers show permeability enhancement due to their ability of interacting lipid bilayers, while smaller generation dendrimers enhance the permeation since they have a better ability to move between cells (71).

Dendrimer cytotoxicity is related to the core chemistry; the nature of the dendrimer surface is the most influencing factor, because the interaction between surface cationic charge of dendrimers and biological membranes with negatively charge is the main reason of toxicity. Interaction of dendrimers with biological membranes results in membrane disruption, membrane thinning and erosion. Dendrimer toxicity in biological system is generally characterized by hemolytic

toxicity, cytotoxicity and hematological toxicity. Lower generation dendrimers with anionic or neutral polar surface groups were reported to have lower toxicity as compared to higher generation dendrimers with neutral and cationic surface groups. It was also reported that following repeated systemic or topical ocular application of cationic dendrimers are often toxic, whereas anionic dendrimers are not. To minimize this toxicity two strategies have been utilized; designing and synthesis of biocompatible dendrimers; and/or masking of peripheral modify the surface amine groups of dendrimers by surface engineering (72-74) . Many toxic effects of dendrimers are attenuated at their surfaces with hydrophilic molecules and poly(ethylene glycol) (PEG), which masks the surface charge cationic dendrimers improving biocompatibility and increasing the solubility of the polymers (71) .

Several studies have been published recently, that were reporting ocular dendrimeric formulations were developed without cytotoxicity or irritation (75,76) . Safety of the dendrimers are very important for ocular drug delivery, since serious side effects may occur due to cytotoxicity at the ocular tissues. Safe ocular dendrimeric formulations should be carefully designed and evaluated to provide properties such as biocompatibility and low immunogenicity. Furthermore, contribution and participation of ophthalmologists to the scientific process, alongside with chemists, formulation scientists and engineers, in order to overcome the potential toxicity of the dendrimers is very important.

2.5.2. Types of Dendrimers

Poly(amidoamine) (PAMAM) dendrimers (Figure 2.7) were the first “dendrimer family” that was synthesized, characterized, and commercialized. It is the most widely studied and characterized dendrimer, and so the better understood so far. (62) . They are synthesized by the “divergent” method and their structure starts from an ammonia (NH_3) or ethylenediamine ($\text{C}_2\text{H}_8\text{N}_2$) molecule as a core that binds to the amine groups of branches (R-NH_2) and amide ($-\text{CONH}_2\text{R}$). Dendrimers growth reaches a critical point at G7 where the branching arms limit their development into higher generations due to steric effect. This effect prohibits the synthesis of any larger dendrimers than G10 (77,78) . PAMAM dendrimers have a size range between 1.1 and 12.4 nm as their generations grow through 1–10 and each

new generation, PAMAM dendrimer doubles the number of functional groups and weight also increases in 1 nm diameter of its structure (79) . These dimensions have been compared to proteins (3–8 nm), linear polymer-drug conjugates (5–20 nm), and viruses (25–240 nm). PAMAM dendrimers are commercially available as methanol solutions and in generation G 0-10 with 5 different core type and 10 functional surface groups (70) .

Overall, PAMAM dendrimers are considered as ideal drug delivery systems due to their large variety of surface groups, unique architecture and high aqueous solubility. The most widely studied PAMAM dendrimers for medical applications have been the derivatives with $-NH_2$, $-COOH$ and $-OH$ surface groups (80) . Some physicochemical properties of PAMAM dendrimers are shown in Table 2.5.

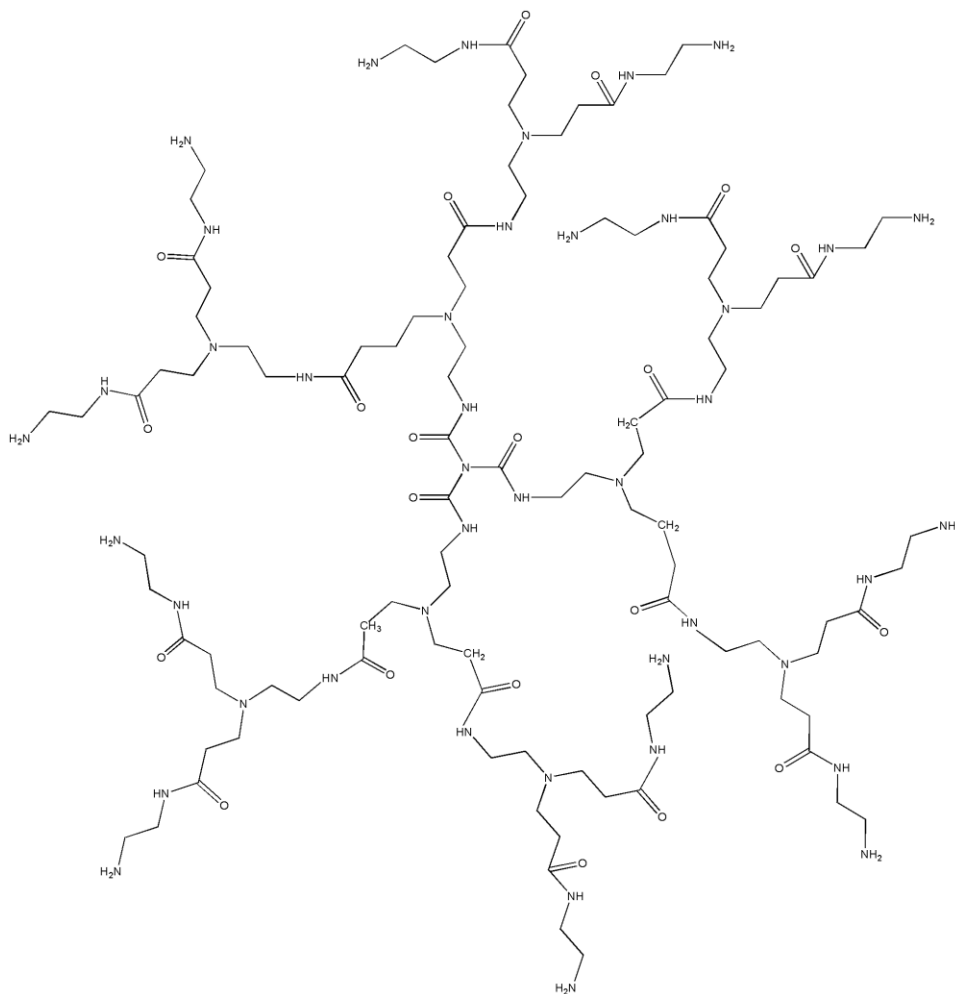


Figure 2.7. Structure of PAMAM dendrimers (9)

Table 2.5. Physicochemical properties of PAMAM dendrimers (66,81)

Generation	Molecular Weight	Measured Diameter (Å°)	Surface Groups
0	517	15	4
1	1430	22	8
2	3256	29	16
3	6909	36	32
4	14215	45	64
5	28826	54	128
6	58048	67	256

Poly(amidoamine) organosilicon (PAMAMOS) dendrimers are silicon containing first commercial dendrimers which are inverted unimolecular micelles that consists of hydrophilic, nucleophilic polyamidoamine interiors and hydrophobic organosilicon exteriors (82) .

Polypropyleneimine (PPI) dendrimers have been studied for their medical applications, but it was reported that the presence of multiple cationic amine groups in their structure causes significant toxicity. These dendrimers are generally having poly-alkyl amines as end groups, and numerous tertiary tris-propylene amines present in interior portion and they are commercially available up to generation 5 as AstramolTM (83) . Polyaryl ether dendrimers also have been investigated for drug delivery, but it was found that due to their poor water solubility, solubilizing groups are required at the periphery of their structure (84) .

Surface engineered dendrimers were developed as a strategy for reducing dendrimer toxicity. Functionalization also helps the dendrimers to gain some properties that can be beneficial for their use as a drug delivery system, as well as reducing the inherent toxicity (74) . PEGylation is one of the most popular modifications of dendrimer surface, which offers so many advantages such as improved bioavailability/oral delivery application related to improved biodistribution and pharmacokinetics, enhanced solubility, increase in drug loading, sustained and controlled delivery of drugs, better transfection efficiency, and tumor localization as well as cytotoxicity reduction. PEG is typically conjugated to the surface of a

dendrimer to provide a hydrophilic shell around a hydrophobic dendritic core to form a unimolecular micelle (85) . Acetylation is another approach to reduce toxicity based on modification of surface amino groups with acetyl groups (86) . In addition, biodegradable dendrimers have been designed (polylysine, poly(disulfide amine), polyether, polyester dendrimers) and after suitable surface modifications they have been developed as promising antibacterial, antiviral, chemotherapeutic, and vaccine carrier candidates.

Glycodendrimers, that include carbohydrates in their architecture, also have great potential as drug carriers. Most of the glycodendrimers have saccharide residues on their outer surface, but glycodendrimers with a sugar central core have also been described (87) . Amino acid-based dendrimers, peptide dendrimers, hydrophobic dendrimers, and asymmetric dendrimers were also investigated for a variety of pharmaceutical applications (67,88) .

Several dendrimer-based FDA approved products are already in the market. For example, *Stratus CS Acute Care* (Dade Behring), was launched for “cardiac diagnostic testing,” and it contains dendrimer-linked monoclonal antibody. *SuperFect* (Qiagen) is another product based on modified “Tomalia-type PAMAM” dendrimers, is a well-known gene transfection agent available for a wide range of cell lines (89,90) . In addition, a formulation of “polyanionic lysine G4 dendrimers” with an anionic surface of “naphthalenedisulfonate (SPL7013) in a Carbopol gel” that shows antiviral activity against HIV and HSV for the treatment, has already been taken into clinical trials by Starpharma, according to FDA requirements. It’s called *VivaGel* and it is currently in Phase III clinical trials Durex condoms also has a license agreement with VivaGel to use it as a condom coating (91,92) .

2.5.3. Interaction between Dendrimers and Drug Molecules

The external surfaces of dendrimers have been investigated as potential sites of interaction with drugs. Modification of the dendrimer end group functionality may offer molecules with novel biological properties such as cooperative receptor-ligand interactions that will help dendrimers to interact with hydrophobic drugs. Dendrimers can increase bioavailability, the cellular uptake, and therapeutic efficacy,

and they are also able to optimize the biodistribution and to reduce the systemic toxicity (93) .

Two methods have been reported for dendrimeric drug delivery. Dendrimers interact with drug molecules physically by absorption on surface by electrostatic interactions or by conjugation with the surface groups for *covalent bonding* or by *encapsulation* of the drug into the cavities of the dendrimer. The covalent conjugation of the drugs was often used for targeting and achieving the higher drug payload, while the noncovalent interactions have resulted in higher solubility of insoluble drugs.

Drug gets trapped inside the dendrimer using the interaction between drug and the dendrimer in encapsulation method and drug encapsulation may be either a simple physical entrapment or it might be the result of nonbonding-specific interactions within the dendrimer. The presence of large numbers of ionizable groups on the surface of dendrimers provides an opportunity for electrostatic attachment of numerous ionizable drugs. On the other hand, the drug is attached to the exterior end groups of the dendrimer in conjugation method and conjugates are usually prodrugs. The covalent drug attachment to the surface groups of dendrimers through hydrolysable or biodegradable linkers offers controlled drug release. (60,94) . A basic schematic representation of drug encapsulated and drug conjugated dendrimers is given in Figure 2.8. Dendrimers and dendrons are also ideal to prepare cross-linked covalent gels, and for the self-assembled noncovalent gels due to their unique structure (95) .

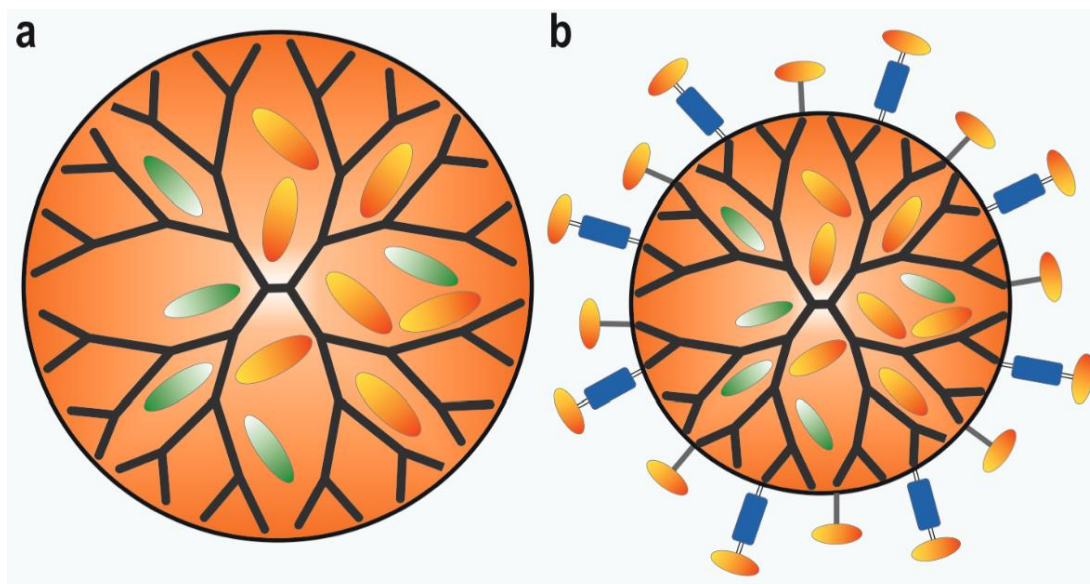


Figure 2.8. Schematic representation of drug encapsulated (a) and drug conjugated dendrimers (b) (9) . (Image has been reprinted from the referred article with author's permission).

Drug dendrimer interactions are affected from the generation, structure, concentration, and surface modifications of the dendrimers. For example, PAMAM and PPI have a slightly different dendritic framework which makes PPI dendrimers relatively more hydrophobic compared to PAMAM dendrimers and that causes different solubilizing power (96) . Furthermore, dendrimer surface modification can improve the therapeutic efficacy by targeting and reducing toxicity.

i- Encapsulation of Drugs within Dendritic Structure (Complexation).

The encapsulation of drugs may be a purely physical entrapment or involve interactions with specific structures within the dendrimer. The acid-base reaction between the dendrimers and the drugs with coulomb attractions pulls the drug molecules inside the host structure, whereas the hydrogen bonding keeps them together.

Jansen et. al. reported the first encapsulation of a dye inside a dendrimer in 1994, the so-called “dendritic box” (97) . It is possible to entrap guest molecules in the dendritic cavities during the synthetic process, with the help of a shell preventing diffusion from the structures, even after prolonged heating, sonication, or solvent

extraction (98,99) . Following dye encapsulation, encapsulation of anticancer drugs was the focus of the research. Kojima and coworkers encapsulated the anticancer drugs methotrexate and doxorubicin using G3 and G4 ethylenediamine-based poly(amidoamine) (PAMAM) dendrimers with poly(ethyleneglycol) monomethyl ether (MPEG) grafts (100) . Methotrexate and folic acid was also attached to the exterior of the dendritic structure and targeted the tumor cells using drug-dendrimer conjugates by the same group (101) .

Dendrimers with an polar shell and apolar core have been referred to as “unimolecular micelles,” whereas dendrimer concentration has no effect on dendritic structure unlike conventional micelles (102) . However, the disadvantage of this approach is that it is difficult to control the release of drugs from the dendrimer core. PEG has been conjugated to dendrimer surface to form a unimolecular micelle by providing a hydrophilic shell around the dendritic core. PEGylated dendrimers are of particular interest in drug delivery because of their biocompatibility, high water solubility and ability to modify the biodistribution of carrier systems (103,104) .

Linear hydrophilic blocks and a hydrophobic dendritic block have been used to synthesize “dendrimeric block copolymers” and their ability to complex “poorly water soluble” molecules have been studied. Kim and coworkers have been synthesized a series of “G1–G5 PAMAM-block-PEG-block-PAMAM triblock copolymers” and studied as potential polymeric gene carrier (105) .

“Cored dendrimers” with modified dendritic architecture have been synthesized to encapsulate the drug by Zimmerman et al. Following the synthesis, the core was removed via cleavage of ester bonds, while the rest of the structure remained the same as a consequence of robust ether linkages (106,107) .

Dendrimer-mediated complexation has advantages in terms of high drug loading, release control, lower toxicity of entrapped drugs stability. However, the noncovalent complexation often results in lower drug encapsulation and complex stability compared to covalent conjugation (108) .

ii- Dendrimer Drug Conjugation

The outer surfaces of dendrimers have been investigated as potential interaction sites with drugs to increase the loading capacity. The number of available surface groups for drug interactions increases in two folds with each higher generation of dendrimer. Covalent interaction method offers many advantages, such as allowing multiple drugs to be attached to dendrimers through the groups of the surface. Furthermore, covalent bonds between the drug and the polymer are likely more difficult to break giving them greater control over the drugs. Drugs may be covalently bound to the surface of a dendrimer via ester, amide, or some other labile chemical linkages, which can be hydrolyzed by endosomal or lysosomal enzymes, inside the cell. Disulfide, peptide or ester linkers are common examples of linkers that can be nonspecifically cleaved *in vivo* (93,94) . It was reported that the release of the free drug can be enhanced by a suitable linker choice, especially, the linker/spacer length and flexibility. Some of the linkers are pH-sensitive and have proven to enhance intracellular release of the free drug (109) . Several ionizable groups on the surface of dendrimers are also present where ionizable drugs can attach electrostatically. The main used points of attachment to conjugate drugs to dendrimers are amides, esters, disulfides, hydrazones, thiol-maleimide, and sulfinyl (80) .

The covalently conjugated drug and noncovalent inclusion complex were compared by Patri et. al. in terms of release kinetics and efficacy, using generation 5 PAMAM dendrimers for targeting methotrexate. This study revealed that the dendrimer complex releases the drug immediately and drug is active *in vitro*, whereas covalently conjugated drug is more suitable for specifically targeted drug delivery (110) .

Encapsulating drugs into the dendrimer has the advantage of reducing the number of steps taken to prepare the drug-dendrimer system in contrast to covalent drug association and therefore reducing the cost of synthesis. On the other hand, controlled drug release can be achieved by the covalent drug attachment using biodegradable linkages than electrostatic drug dendrimer complexes. However, the major disadvantage of the conjugation is that the drug might be released in less active form or the drug release might be too slow to be effective *in vivo* (111) .

iii- Dendritic Gels

Hydrogels have been attracting because of their architectural properties, drug loading capacity, and controlled drug release capability. Hydrogels are hydrophilic and “three-dimensional polymeric networks” have found application in drug delivery due to their high water absorbing capacity (112) . “In situ forming gels” have been investigated for a variety of applications including ocular, oral, nasal, vaginal, rectal, and injectable (113) . Dendrimers have been used in both ‘traditional’ polymer-type gels, as well as in the self-assembly of supramolecular gels. Dendritic molecules, as a consequence of their inherent branched structure, can act as multivalent crosslinking units (95) .

Usually it's required to use a crosslinker during polymerization to obtain a “polymer network”. Synthesis of hydrogels has been a function of the multivalent crosslinker behavior of dendritic molecules (114) . It has been shown that the dendritic branching can play unique roles in controlling the self-assembly process with their steric impact. It was also proposed that hydrogen bonds between alcohol groups on the dendritic head groups were important in the self-assembly process. Furthermore, the repeated structure can lead to multiple interactions between branched units and strengthen the noncovalent interactions responsible for the self-assembly process (115) .

2.5.4. Ocular Applications of Dendrimeric Systems

Dendrimers use for ophthalmic drug delivery has been investigated due to their various advantages as a carrier system. It has been reported that dendrimers were used for several purposes in ophthalmology, such as drug delivery, antioxidant delivery, peptide delivery, gene delivery biomedical imaging, and genetic testing (116) . A list of the ocular applications of dendrimers is given in Table 2.6.

Dendrimers can transport into and out of the cells and there are different cell entry pathways for PAMAM dendrimers, depending on their surface functionality. Anionic PAMAM dendrimers are endocytosed primarily through a caveolin-mediated process, whereas neutral and cationic dendrimers were reported to internalize in cells following a clathrin-mediated process. This process can offer

many advantages in terms of crossing the epithelial and retinal barriers in the cornea and retina (117,118) .

Several ocular application routes such as topical, intravitreal, subconjunctival etc. have been studied for dendrimeric drug delivery and it was reported that better water solubility, permeability, bioavailability, and biocompatibility has been achieved. Vandamme and coworkers have evaluated a series of poly(amidoamine) (PAMAM) dendrimers to develop an ocular drug delivery system with controlled release and increased residence time of pilocarpine nitrate and tropicamide. It was reported that anionic dendrimer solutions had longer ocular residence time. Results showed that these PAMAM formulations improved bioavailability of pilocarpine nitrate compared to the control based on the “miotic activity test” on albino rabbits. It was also reported prolonged reduction of intraocular pressure (IOP) was achieved (119) .

Spataro et al. have synthesized “phosphorus containing dendrimers,” from generation 0 to generation 2, with one quaternary ammonium salt as core and several carboxylic acid terminal groups. Ocular carteolol delivery has been evaluated in vivo in a rabbit model and an increase of the carteolol amount in the aqueous humor was observed. Even after several hours of cationic dendrimer application, no irritation has been reported (120) .

Table 2.6. Ocular applications of dendrimers and dendrimeric delivery systems (9)

DRUG	DENDRIMER TYPE	ADMINISTRATION	TREATMENT	OUTCOMES	REF
Pilocarpine nitrate & Tropicamide	PAMAM G1.5-4	Topical	Myosis and mydriasis	Increased corneal residence & prolonged reduction of IOP	(119)
Carteolol	Phosphorus containing dendrimers	Topical	Glaucoma	Increased corneal residence, reduced toxicity & IOP	(120)
Gatifloxacin	Dendrimeric polyganidilyated translocators	Topical	Conjunctivitis & intraocular infections	Enhanced corneal transport & increased antimicrobial activity	(121)
Puerarin	PAMAM	Topical	Ocular hypertension & cataract	Increased bioavailability	(122)
-	Anionic and cationic carbosilane dendrimers	Topical	Tolerance	Hydrogen bonding between mucin and PAMAM – enhanced retention time	(123)
-	Modified G1, G2 and G3 dendrimers	Topical (corneal hydrogel adhesive)	Corneal wounds	Wound sealing & no scar formation	(124)
Brimonidine & Timolol maleate	PAMAM hydrogel (G3)	Topical	Glaucoma	Increased uptake	(125)
Brimonidine & Timolol maleate	Hybrid PAMAM dendrimer hydrogel/ PLGA nanoparticle	Topical	Glaucoma	Increased uptake	(126)
-	Lys _x Cys _y dendritic polymers – in situ gel	Topical	Cataract incisions	Wound sealing	(127)

Glucosamine & Glucosamine 6-sulfate	PAMAM G3.5- COOH	Subconjunctival injection	Antiangiogenic in glaucoma surgery	Reduced inflammation & no scar formation	(75)
Carboplatin	PAMAM G3.5-COOH (dendrimeric nanoparticles)	Subconjunctival injection	Retinoblastoma	Increased half life & bioavailability Reduced drug toxicity & tumor mass	(76)
Fluocinolone acetonide	PAMAM G4-OH	Intra vitreal injection	Retinal neuroinflammation	Reduced inflammation	(128)
VEGF-ODN	Lipophilic amino-acid dendrimer	Intra vitreal injection	CNV	Prolonged suppression of VEGF & neovascularization	(129)
-	Polypropyleneimine octaamine G2	Corneal scaffold	Corneal tissue engineering	Enhanced human corneal epithelial cell growth	(130)
-	Surface modified -COOH ending dendrimers	Corneal scaffold	Corneal tissue engineering	Promoted adhesion & proliferation of human corneal epithelial cells	(131)
Concanavalin A	Prophyrin glyco-dendrimers	Topical - Photodynamic therapy	Intraocular tumors & retinoblastoma	Enhanced targeting & reduced toxicity	(132)
-	Porphyrin dendrimers	Topical - Photodynamic therapy	AMD & CNV	Selective accumulation in inflammatory cells & Prolonged retention time	(133,134)
DNA	Phtalocyaninedendrimers			Accumulation in photoirradiated areas & increased transgene expression	(135)
Photosensitizer	G 3 aryl ether dendrimer zinc porphyrin	Intra venous injection - photodynamic therapy	CNV	Accumulation in neovascularized area	(136)

Durairaj and coworkers studied on dendrimeric polyguanidilyated translocators (DPTs), which are a class of dendrimers with tritolyl branches and surface guanidine groups. They investigated DPTs as potential ophthalmic carriers for gatifloxacin, a “fourth generation fluoroquinolone” which was approved for conjunctivitis treatment. It was reported that the DPT forms stable gatifloxacin complexes and enhances solubility, permeability, anti-MRSA activity, and in vivo gatifloxacin delivery. Based on the results of the study, DPTs were reported as a potential delivery system allowing once a day dosing (121) .

Puerarin–dendrimer complexes were prepared using PAMAM dendrimers (G3.5, G4, G4.5, and G5) by Yao et. al. Their physicochemical properties, in vitro release, corneal permeation, and ocular residence times were evaluated. Corneal permeation and ocular residence time in rabbits were evaluated using diffusion cells with excised corneas. Puerarin-dendrimer complexes were reported to exhibit longer residence time in rabbit eyes than puerarin eye drops, without damaging corneal epithelium or endothelium. In vitro release studies also indicated that puerarin release was much more slower from complexes than the free puerarin in PBS. However, corneal permeation studies showed that there was no significant difference between puerarin-dendrimer complexes and puerarin eye drops in terms of drug permeability coefficient (122) .

In vitro and in vivo tolerance of carbosilane dendrimers (G1 and G3, anionic and cationic), were investigated by Bravo-Osuna and coworkers for topical ophthalmic administration. Formulations were applied to New Zealand albino rabbits and it was reported that animals did not show any discomfort or clinical signs following the administration of dendrimer solutions. Nonionic interactions between mucins and the PAMAM dendrimer surface moieties were observed via hydrogen bonding. It was also reported that anionic dendrimers were nontoxic for both conjunctival and corneal cells based on the MTT test results (123) .

A series of dendrimeric adhesives have been developed by Grinstaff to repair corneal wounds, composed of generations 1, 2, and 3 (G1, G2, and G3) with a combination of PEG, glycerol, and succinic acid. The polymer was modified to contain terminal methacrylate groups, $([G1]-PGLSA-MA)_2$ -PEG. Ocular adhesives were formed using two techniques: a photocrosslinking reaction and a peptide

ligation reaction to couple the individual dendrimers together. Both hydrogels were reported to be adhesive, soft, elastic, transparent, and hydrophilic. Photocrosslinkable ([G1]-PGLSA-MA)₂ - PEG adhesive has applied to chicken eyes and it was reported that it completely sealed the wound on postoperative day. Histological studies also showed that 28 days after the application, sealing of the wounds were appeared to be more complete when treated with adhesive gels as compared to sutured wounds. The advantage of photo-cross-linked gels, is the light-induced ability of the polymer to crosslink and adhere to the tissue; however, there is a potential ocular damage risk due to the light (124) .

Holden et. al. have developed a PAMAM dendrimer hydrogel that is made from “ultraviolet-cured PAMAM dendrimer” linked with PEG-acrylate chains for the delivery of brimonidine (0.1% w/v) and timolol maleate (0.5% w/v) which were two antiglaucoma drugs. Dendrimeric hydrogel was obtained by crosslinking of the reactive acrylate groups, triggered by UV light. The dendrimeric hydrogel was reported to be mucoadhesive and nontoxic to epithelial cells of human cornea. Higher uptake from “human corneal epithelial cells” and significantly enhanced “bovine corneal transport” were reported for both drugs, compared to the eye drops. Dendrimeric hydrogel formulations’ higher uptake explained the temporary decomposition of the corneal epithelial tight junctions (125) . The same group also developed a novel “hybrid PAMAM dendrimer hydrogel/poly(lactic-co-glycolic acid) (PLGA) nanoparticle platform (HDNP)”, again for co-delivery of brimonidine and timolol maleate. In vitro potential toxicity of the formulations was also investigated and it was found that the formulation was non-cytotoxic to human corneal epithelial cells. HDNP was administered topically to adult “normotensive Dutch-belted male rabbits,” and formulation was found to be effective and maintained significantly higher concentrations of both drugs up to 7 days in aqueous humor and cornea compared to saline. Furthermore, the results showed that HDNP was not inducing any ocular inflammation or discomfort. This study demonstrated that this new platform can enhance drug bioavailability, and it is capable of sustaining drug activity following topical administration (126) . Wathier et. al. also developed an in situ gel formulation using “Lsy_xCys_y dendritic polymers” to be used in cataract incisions as a replacement of nylon sutures. It was reported that the

hydrogel sealant procedure was simple and required less surgical time than conventional suturing and no additional tissue trauma was inflicted (127) .

Water soluble conjugates of D(+)-glucosamine and D(+)-glucosamine 6-sulfate with anionic PAMAM (G3.5) dendrimers have been synthesized by Shaunak and coworkers to obtain synergistic “immunomodulatory and antiangiogenic effect.” When a combination of dendrimer glucosamine and dendrimer glucosamine 6-sulfate conjugates were used in a clinically relevant scar tissue formation rabbit model after glaucoma filtration surgery, it was reported that the long-term success of the surgery has increased from 30% to 80%. In addition, neither microbial infections nor clinical, biochemical, or hematological toxicity was observed in all animals (75) .

There is a high risk of complications with high metastatic potential for intraocular tumors such as retinoblastoma. One of the studies that have been investigating drug delivery to intraocular tumors has explored the use of anionic PAMAM dendrimers with carboxyl end groups (G3.5-COOH) for extended half-life, sustained carboplatin delivery and reduced toxicity. A transgenic murine retinoblastoma model was used to explore carboplatin-loaded PAMAM dendrimer complexes following subconjunctival administration. The results indicated that the carboplatin-loaded dendrimer nanoparticles not only crossed the sclera, but were also retained for an extended period of time in the tumor vasculature, providing a sustained therapeutic dose of carboplatin (76) .

Targeted drug delivery for retinal neuro-inflammation treatment was investigated, using “G4.0 hydroxyl-terminated PAMAM dendrimer-drug conjugate nanodevices” by Iezzi and coworkers. Fluocinolone acetonide was conjugated to the dendrimers and in vivo efficacy study was performed for over a 4-week period, using the “Royal College of Surgeons rat retinal degeneration model.” The results showed that following intravitreal administration, PAMAM dendrimers were selectively localized within “activated outer retinal microglia” in two retinal degeneration rat models and even 35 days after administration the dendrimers were detected in the target cells (128) .

Marano et. al. have developed biocompatible conjugates of lipophilic amino-acid dendrimers with collagen scaffolds to obtain enhanced physical and mechanical properties and adhesion ability. Dendrimers-based approach was used for anti

vascular endothelial growth factor oligonucleotide (VEGF-ODN) delivery and successfully tested in a rat model to treat choroidal neovascularization (CNV). It was reported that dendrimer/ODN-1 complexes suppressed VEGF expression in cell level studies around 40 to 60% significantly. Examinations of injected rat eyes also showed that injections of complex formulations had no significant toxicity (129) .

The repair of wounds such as corneal wounds that arise from surgical procedures has a significant clinical importance. Therefore, Duan et. al. have generated highly crosslinked collagen using G2 polypropyleneimine octaamine dendrimers to use it as a tissue-engineering corneal scaffold. The dendrimer crosslinked collagen, EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride), and glutaraldehyde crosslinked collagen thermal gels were compared in terms of optical transparencies and the transparency of dendrimer crosslinked collagen was reported to be significantly higher. It was reported that dendrimer crosslinked collagen gels improved “human corneal epithelial cell growth” and adhesion without cell toxicity (130) . “Surface modified dendrimers” were conjugated with cell adhesion peptides by the same group to be used as corneal tissue engineering scaffolds and the material has been incorporated into both bulk structures of the gels and onto the gel surface. Dendrimer amine groups were modified using carboxyl group and it was reported that the surface modification promoted human corneal epithelial cell adhesion and proliferation (131) .

Photodynamic therapy (PDT) is an efficient treatment candidate for retinoblastoma along with the other various solid tumours. Makky and coworkers have designed a photosensitizer, porphyrin-based glycodendrimers with the mannose specific ligand protein Concanavalin A conjugated on to their surface, to target the tumor cells in the retina specifically. The results indicated that mannosylated dendrimers demonstrated specific interactions with the receptors in the lipid bilayer and malignant ocular tissue accumulation was enhanced (132) . Dendrimers were also investigated as drug carriers and photosensitizers for exudative age related macular degeneration (AMD) and CNV treatment. Porphyrin-based dendrimers were explored by Nishiyama et al.. for their efficacy in treating retinal tumors and exudative AMD associated with CNV. The results showed that the formulations were selectively accumulated in the CNV lesions within 24 h, when

injected into a CNV rat model (133,134) . The same group also developed phthalocyanine core-based dendrimer photosensitizers, which can be used to compact and deliver therapeutic genes with a targeting approach. Transgene expression in the irradiated areas was monitored upon subconjunctival injection of the dendrimer formulation and followed by laser irradiation (135) .

Sugisaki and coworkers have studied the accumulation of dendrimer porphyrin (DP), DP encapsulated polymericmicelles, and the efficacy of photodynamic therapy (PDT) using a mice corneal neovascularization model. In this study a 3rd generation “aryl ether dendrimer zinc porphyrin” with carboxyl ending groups and polymeric micelles composed of the DP and PEG-poly(L-lysine) was used for PDT as a photosensitizer formulation. It was reported that both DP and DP micelle were accumulated in the neovascularized area in 1 hour to 24 hours following administration (136) .

3. MATERIALS AND METHODS

3.1. Materials

Acetonitrile (HPLC grade)	Sigma Aldrich, USA
Alexa 488 labeling kit	Life Technologies, USA
Ammonium phosphate	Merck, USA
ARPE 19 human corneal epithelium cells	ATCC, USA
Calcium chloride	Merck, USA
Dexamethasone	Deva Pharmaceutical Company, TURKEY
Disodium hydrogen phosphate	Merck, USA
DMEM: F12	Biochrom AG, GERMANY
DMF	Merck, USA
Fetal Bovine Serum (FBS)	Biochrom AG, GERMANY
Glucose	Sigma Aldrich, USA
HEPES	Sigma Aldrich, USA
Isoflurane	Isoflurane USP, PPC, USA
L-glutamin 2	Biochrom AG, GERMANY
Magnesium sulfate	Merck, USA
Methanol (HPLC grade)	Sigma Aldrich, USA
PAMAM Dendrimer, Generation 3 solution	Sigma Aldrich, USA
PAMAM Dendrimer, Generation 3.5 solution	Sigma Aldrich, USA
PAMAM Dendrimer, Generation 4 solution	Sigma Aldrich, USA
PAMAM Dendrimer, Generation 4.5 solution	Sigma Aldrich, USA
PAMAM-OH Dendrimer, Generation 3 solution	Sigma Aldrich, USA
PAMAM-OH Dendrimer, Generation 4 solution	Sigma Aldrich, USA
Pentobarbital sodium	Fatal-Plus
Penicillin–streptomycin	Biochrom AG, GERMANY
Phosphoric acid	Sigma Aldrich, USA
Potassium chloride	Merck, USA
Potassium dihydrogen phosphate	Merck, USA
Rabbit eyes	Pel-Freez Biologicals, USA

SDS	Merck, USA
Sodium bicarbonate	Sigma Aldrich, USA
Sodium chloride	Sigma Aldrich, USA
Sprague Dawley rats	Colorado University Vivarium, USA
Sterile pipette	Greiner bio-one, GERMANY
Tubes	Eppendorf, USA
Trypsin-EDTA	Biochrom AG, GERMANY

3.2. Equipment

220 Volt pipette (Labopet 240)	Greiner bio-one, GERMANY
¹ H-NMR	Varian 400 MHz
Cell culture 0.1 µm insert	Greiner bio-one, GERMANY
Cell culture flask	Greiner bio-one, GERMANY
Cell culture well	THINCERT Plate-24, Greiner bio-one, GERMANY
Centrifuge	Eppendorf Centrifuge 5415 C, USA
Digital scale	Shimadzu, JAPAN
Elisa plate reader	ASYS Hiteck GmbH, Austria
Fluorospectrometer	SpectraMax M5, Molecular Devices, USA
Fourier transform infrared spectroscopy (FTIR)	Perkin – Elmer Spectrum BX FT-IR, USA
High performance liquid chromatography (HPLC) system	Agilent 1200 Series, GERMANY
Homogenizator	Tissue Tearor, Biospec Product Inc., USA
Incubator	Sanyo MCO-18AIC, JAPAN
Incubator with shaker	Max Q 4000, Barnsted Lab Line, USA
Laminar flow cabin	BHG 2000 S/D, D:Group Company, ITALY
Light microscope	Leica, GERMANY

Liquid chromatography/ Mass spectroscopy (LC/MS)	AB Sciex Q-trap 4500 LC/MS, USA API-3000 triple quadrupole mass spectrometry (Applied Biosystems, Foster City, CA, USA) coupled with a PerkinElmer series-200 liquid chromatography (Perkin-Elmer, Waltham, Massachusetts, USA)
Lyophilizator	Heto PowerDry PL 3000, DENMARK
Micropipette	Eppendorf, GERMANY
Multipoint magnetic stirrer	Variomag Multipoint HP, GERMANY
NanoZS Zetasizer	Zetasizer NanoSeries ZS, Malvern Instruments, USA
Nitrogen evaporator	Organometron Associates Multivrap 118 Nitrogen Evaporator, USA
Ocular fluorophotometer	Fluorotron Master, Ocumetrics, USA
Osmometer	Advanced Instruments Model 3250, USA
Reverse phase C18 colon (for HPLC)	4.6mm x 250mm ACE 5µm Extend C18, USA
Reverse phase C18 colon (for LCMS)	4.6mm x 50mm Agilent Zorbax 5µm Extend C18, USA
Trans epithelial electrical resistance (TEER) system	Millicell® ERS, Millipore, USA
Ultrapurified water system	Milli-Q Integral 5, Millipore, USA
Vortex	VWR VX-2500 Multitube Vortexer, USA
Water bath	SBD 300, Şimşek Labor Teknik, TURKEY

3.3. Methods

3.3.1. High Performance Liquid Chromatography (HPLC) Quantification Method for Dexamethasone

HPLC system (Agilent 1200 Series) was used to develop a quantification method for DEX in order to analyze some of the in vitro study samples. Properties of the developed method is as follows:

- Mobile phase: ACN: Water (50:50)
- Flow rate: 1mL/min
- Column: C18 reverse phase column (22 cm)
- Injection volume: 20 μ L
- Detector: Diode array (DAD) detector
- Wavelength: 246 nm
- Retention time: 5.8 min
- Analyze time: 8 min
- Column temperature: 25 $^{\circ}$ C

Analytical Method Validation

The method was validated to meet requirements for a global regulatory filing and the validation parameters were:

- Specificity
- Range/Linearity
- Accuracy
- Precision
- Stability
- Limit of Detection (LOD)
- Limit of Quantitation (LOQ) (137)

Specificity

Polymers and solvents, that have been used to formulate DEX-PAMAM complexes, have been analyzed to show that method is specific for DEX only. All the dendrimer (PAMAM G3, PAMAM G3.5, PAMAM G3-OH, PAMAM G4,

PAMAM G4.5, PAMAM G4-OH) solutions, acetonitrile and methanol have been tested to prove there are no peaks that could interfere with DEX (137) .

Range/Linearity

The range of an analytical procedure is the interval between the upper and lower concentrations of analyte in the sample for which has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. A range has been determined in order to quantify the DEX amount, in the samples of in vitro studies.

The linearity of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration of analyte in the sample (137) . The linearity of this range has been tested by analyzing a series of DEX solutions in a concentration range of 10-500 $\mu\text{g/mL}$ with 6 replicates. Peak area against concentration has been plotted to obtain the calibration curve.

Accuracy

Samples at 10, 75 and 500 $\mu\text{g/mL}$ concentrations were prepared for accuracy testing. Three preparations were made for each concentration. Recovery of DEX was determined for each sample. Six samples were prepared for each concentration. Accuracy was reported as percent recovery by the assay of known amount of analyte in the sample (137) .

Precision

Precision is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision was evaluated by performing repeatability (instrument and method precision) and intermediate precision (variation between days). The results were expressed as standard deviation (SD) and coefficient of variation (CV%) (137) .

Six samples prepared at the same concentration (75 $\mu\text{g/mL}$) to evaluate method precision and coefficient of variation (CV%). Six injections of the same sample (75 $\mu\text{g/mL}$) were made to evaluate instrument precision. Samples (75 $\mu\text{g/mL}$)

prepared on three separate days with 3 replicates to evaluate the intermediate precision.

Stability

Samples (75 µg/mL, n=3) prepared and analyzed right after preparation and 24 hours after preparation to show if drug is stable during the analysis.

LOD and LOQ

Determination of LOD and LOQ were measured based on the signal-to-noise ratio. A signal to noise ratio 3 was considered as LOD and 10 as LOQ, respectively.

3.3.2. LC/MS Quantification Method for Dexamethasone

LC/MS system (AB Sciex Q-trap 4500) was used to develop a quantification method for DEX in order to analyze ex-vivo and in vivo study samples that were not fluorescent labeled. In vitro release study samples were also quantified by LC/MS. Properties of the developed method is as it follows:

- Machine: Qtrap 4500 (ABSciex)
- Ion mode: Positive
- Internal standard: Triamcinolone acetonide (TA)
- Column – Zorbax C18 (4.6 X 50 mm)
- Mobile phase:
 - Aqueous phase – 5 mM ammonium formate adjusted to pH 3.5
 - Organic phase – Acetonitrile with 0.1% formic acid
 - Gradient method
- Run time: 3.6 minutes

DEX standard curve was developed with a linear range from 0.5 ng/mL to 500 ng/mL. 100 µL of vitreous humor or 50 µg of tissue (either from blank rabbit tissue or samples) was added with 0.25 mL of double distilled water containing 100 ng/mL of triamcinolone acetonide as internal standard. Standards/samples were vortexed for 10 minutes on multi-vortexer, and were added with 0.75 mL of acetonitrile. Subsequently, sample tubes were vortexed for 20 minutes, and

centrifuged at 13000 g for 5 minutes. Supernatant liquid was separated into glass tubes and the organic solvent was evaporated under nitrogen stream. Residue was dissolved in 0.25 mL or 0.1 mL of ACN: water (1:1) for standards or samples, respectively. Subsequently, all the tubes were centrifuged at 13000 g for 5 minutes. Supernatant free of any tissue was injected into LC-MS. A standard curve was prepared accordingly before every analysis.

3.3.3. Preparation of DEX-PAMAM Formulations

Two approaches have been used to prepare DEX formulations with PAMAM dendrimers, which were physical complexation and chemical conjugation. Six different types of PAMAM dendrimers, with both anionic and cationic structures, were used in order to compare the effect of the charge and the generation of dendrimers. The dendrimer types that have been used to prepare the formulations and the abbreviations for formulation codes were given in Table 3.1.

Table 3.1. Dendrimer types that have been used in the formulations and the abbreviations for formulation names

Type of PAMAM	End Group	DEX-PAMAM Complexes	DEX-PAMAM Conjugates
PAMAM G3	-NH ₃ (cationic)	G3 complex	
PAMAM G3-OH	-OH (anionic)	G3-OH complex	
PAMAM G3.5	-COOH (anionic)	G3.5 complex	G3.5 conjugate
PAMAM G4	-NH ₃ (cationic)	G4 complex	
PAMAM G4-OH	-OH (anionic)	G4-OH complex	
PAMAM G4.5	-COOH (anionic)	G4.5 complex	G4.5 conjugate

DEX-PAMAM complex formulations are expected to show immediate release and a possible delivery system for topical or subconjunctival application. On the other hand DEX-PAMAM conjugates were designed to obtain an extended release following subconjunctival or intravitreal injection. Thus, these formulations will be evaluated in different terms, based on their purpose of development.

i- Preparation of DEX-PAMAM Physical Complex Formulations

Excess amount of DEX (1:5 DEX:PAMAM molar ratio) weighed into vials and dissolved in 1 mL of methanol (138). PAMAM solutions in methanol (purchased from Sigma Aldrich, USA), equivalent to 25 mg of PAMAM, was added and stirred on a magnetic stirrer for 24 hours at room temperature. Calculated DEX and PAMAM amounts were given below:

- 7.1 mg DEX +1 mL methanol → 125 μ L PAMAM G 3 (20%, w/v)
- 7.07 mg DEX +1 mL methanol → 125 μ L PAMAM G 3-OH (20%, w/v)
- 3.8 mg DEX +1 mL methanol → 250 μ L PAMAM G 3.5 (10%, w/v)
- 3.45 mg DEX +1 mL methanol → 250 μ L PAMAM G 4 (10%, w/v)
- 3.44 mg DEX +1 mL methanol → 250 μ L PAMAM G 4-OH (10%, w/v)
- 0.93 mg DEX +1 mL methanol → 250 μ L PAMAM G 4.5 (5%, w/v)

The solvent is evaporated under nitrogen and then reconstituted with 1.5 mL water and stirred for 6 hours to remove excess amount of DEX. Samples were centrifuged at 7500 rpm for 5 minutes and supernatant was lyophilized for 24 hours. FTIR spectrum has been used to confirm the complexation.

ii- Preparation of DEX-PAMAM Conjugate Formulations

PAMAM G3.5 or PAMAM G4.5 dendrimer solution (50.0 mg, 0.0019 mmol) and DEX (47.84 mg, 0.1219 mmol) in anhydrous DMF:DMSO (3 mL, 9:1) was added dropwise a solution of N,N'-dicyclohexylcarbodiimide (27.7 mg, 0.1341 mmol) and 4-dimethylaminopyridine (14.9 mg, 0.1219 mmol) in anhydrous DMF:DMSO (1 mL) under nitrogen at 40 °C. The reaction was stirred for 72 h. The solvent was removed under vacuum. The crude mixture was dissolved in purified water and extracted with dichloromethane (4 x 30 mL). The aqueous phase was lyophilized to yield yellowish product, dexamethasone attached PAMAM G3.5 and PAMAM 4.5 dendrimer (49.5 mg, 50.6 % yield). Number of drug molecules attached to the dendrimer is determined by ¹H-NMR. DEX-PAMAM G 3.5 molar ratio was found 1:10 and DEX-PAMAM G 4.5 molar ratio were found 1:4 respectively. The conjugation reaction was schematized in Figure 3.1.

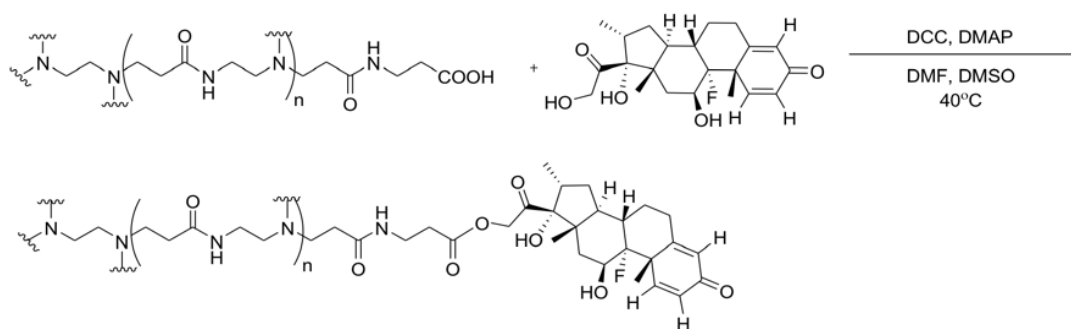


Figure 3.1. DEX-PAMAM dendrimers conjugation reaction

3.3.4. Characterization of DEX-PAMAM Formulations

i- FTIR Analysis of DEX-PAMAM Complex Formulations

The use of the dendrimers as drug delivery vehicle depends on their ability to form a complex with the drug and that complexation depends on the nature of the core–surface groups of the dendrimer and electrostatic interaction between the dendrimer and the drug. So the drug–dendrimer complex formation between DEX and dendrimers used in this project were characterized by their Fourier transform infrared (FTIR) spectra (139). FTIR spectra of free DEX, PAMAM dendrimers and DEX-PAMAM complex formulations were taken with a Perkin-Elmer BX FT-IR spectrophotometer (USA) between 800 and 4000 cm^{-1} in order to show the complexation by indicating the absence of free DEX in the formulations

ii- $^1\text{H-NMR}$ Analysis of DEX-PAMAM Conjugate Formulations

Following the synthesis of the conjugate formulations, number of drug molecules attached to the dendrimers was determined by $^1\text{H-NMR}$ (Varian 400 MHz). Calculation of the drug content was achieved by comparing the integration value of proton “e” placed at 2.19 ppm ($\text{CH}_2\text{-N}$ at the very core of the PAMAM dendrimer) and of protons “g” and “h” placed at 6.28 ppm ($\text{CH}=\text{CH}$ at the double bond of the conjugated drug molecule) and 7.44 ppm ($\text{CH}=\text{CH}$ at the double bond of the conjugated drug molecule) respectively.

iii- Particle Size and Zeta Potential Measurements

Mean diameter and polydispersity index values of PAMAM dendrimers and DEX-PAMAM formulations were determined by quasi-elastic light scattering technique using Malvern NanoZS. Surface charge of the formulations was also determined by zeta potential measurements using Malvern NanoZS. Analyses were performed in triplicate at 25°C.

iv- Loading Efficiency of The DEX-PAMAM Complex Formulations

DEX-PAMAM complex formulations were accurately weighed and dissolved in 1.5 mL purified water by stirring at room temperature for 1 hour. All samples were prepared with three replicates and DEX amounts were quantified by the HPLC method, which was previously described. Calculations were made based on the amounts added for preparation (140) .

3.3.5. In Vitro Release Studies for DEX-PAMAM Complex Formulations

0.5 mg of DEX-PAMAM complex formulations (n=3) were incubated in 5 mL PBS at 37 ± 0.5 °C using an incubator with shaker for up to 3 hours. The release study has ended at 3 hours, since complex formulations were designed as fast release formulations which will be applied either topical or subconjunctival routes and their ocular retention time will be shorter (119,141) . Sampling time points were 5, 15, 30, 60, 90, 120, 180 minutes and equal amount of the fresh medium was replaced with samples at that time points. Experiments were performed under sink conditions. 100 ng/mL TA was added as internal standard and samples were quantified by LC/MS.

The difference factors (f_1) and the similarity factors (f_2) were calculated to compare release profiles. The difference factor, f_1 was determined with the Eq. 3.1 and the similarity factor, f_2 is defined in Eq. 3.2. f_1 value between 0 and 15 and f_2 value between 50 and 100 suggests the two dissolution profiles are similar (142) .

$$f_1 = \left\{ \frac{\sum |R_t - T_t|}{\sum R_t} \right\} \times 100 \quad \text{Eq. 3.1}$$

$$f_2 = 50 \log \left\{ \left(1 + \frac{1}{n} \sum (R_t - T_t)^2 \right)^{-0.5} \times 100 \right\} \quad \text{Eq. 3.2}$$

3.3.6. Hydrolysis Studies of DEX-PAMAM Conjugates

Hydrolysis studies were performed to investigate the drug release behavior of DEX-PAMAM conjugates in presence of ocular esterase enzymes (143) . Cornea and SCRPE was isolated from fresh New Zealand albino rabbit eyes. Whole cornea and SCRPE of one eye was used per sample to prepare the hydrolysis medium. Cornea or SCRPE tissues were added to assay buffer and tissues were homogenized to prepare the medium. Ingredients of the assay buffer was as follows:

NaCl	7.14 g
NaHCO ₃	2.1 g
K ₂ HPO ₄	0.0696 g
MgSO ₄	0.296 g
CaCl ₂	0.154 g
HEPES	2.38 g
Glukoz	1.8 g
Distilled water	q.s. 1 L

10 µg/mL DEX:PAMAM G 3.5 (1:10) and 5 µg/mL DEX:PAMAM G4.5 (1:4) conjugates solutions were prepared in 3 mL hydrolysis medium. Sample volume was 100 µL and sampling times were 1h, 2h, 4h, 6h, 24 h, 48h, 96h and 144h. ACN:MeOH (100 µL) with 100 ng/mL TA (internal standard) was added to the samples as enzymatic activity terminator (144) . Samples were incubated at 37 °C during the analysis (n=3). Samples were centrifuged at 10000 rpm for 5 minutes to remove the tissue remains and then analyzed with LC/MS for quantification.

3.3.7. Cell Culture Studies

i- MTT Cytotoxicity Assay

MTT cytotoxicity test was performed on ARPE 19 (human retinal pigment epithelial) cell line for DEX solution, DEX-PAMAM complexes and DEX-PAMAM conjugates. ARPE-19 cells medium was prepared by adding fetal bovine serum (FBS) and penicillin–streptomycin to DMEM:F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) to have the final concentration of 10% (v/v) and 50 Unit/mL respectively.

The medium in the flasks was changed in every two days and confluent cell monolayers were trypsinized. Cells in the exponentially growing phase were used in the cytotoxicity experiments (passage number 22). ARPE 19 cells were seeded on 96-well tissue culture plate at the initial density of 5000 cells/well (n=3). After a 24 h stabilization of the cells, fresh medium containing 1 mg/mL of the formulations was added and incubated for 24 hours. After the incubation, media was removed and 100 μ L fresh medium and 25 μ L MTT solution (1 mg/mL) were added to each well. Incubation was allowed for another 4h in darkness at 37 °C. Since living cells metabolize the MTT and form blue formazan crystals, 80 μ L/well DMF-SDS (45%DMF and 25% SDS) solution was added to dissolve the formazan crystals. Plates were kept overnight in the incubator. Absorbance values were measured by reading the plates at 570 nm on an ELISA plate reader (ASYS Hiteck GmbH, Austria), and percentage of viability was calculated. The viability of the treated cell cultures was expressed as a percentage of control untreated cell cultures assumed to be 100%.

Statistical analysis was performed using One Way ANOVA using SPSS. Post-hoc comparison of means was performed by Tukey HSD test with post-hoc procedures and statistical significance was considered at $p < 0.05$.

ii- In Vitro Permeability Studies

In vitro permeability studies were performed on ARPE 19 (human retinal pigment epithelial) cell line for DEX solution, DEX-PAMAM complexes and DEX-PAMAM conjugates. ARPE-19 cells medium was prepared by adding fetal bovine serum (FBS) and penicillin–streptomycin to DMEM:F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) to have the final concentration of 10% (v/v) and 50 Unit/mL respectively.

Cells were seeded at 150000 cells per well apically in the 12-well inserts and then incubated at 37 °C in 5% CO₂. The medium of the flask was changed with fresh medium after every 2 days and trypsinized when near to confluency. Experiments were performed 15 days after seeding when the cell monolayer had reached confluence. Cell monolayer integrity was tested by measuring transepithelial

resistance with Millicel® ERS. When the resistance reached in the range of 200–250 Ωcm^2 , cell monolayer was used for transport studies.

0.5 mL of 1 mg/mL concentration of each formulation in PBS was added to the apical side of the monolayer and 1 mL PBS was used in the basolateral side (n=3). The wells were then placed on a shaker at 30 rpm and 37 °C for 2 hours after which samples from the basolateral side was analyzed by HPLC. Transepithelial resistance was also measured during the experiment to see the changes in cell monolayer integrity.

Apparent permeability co-efficient (P_{app} , cm/s) was calculated by the following equation (Eq. 3.3).

$$P_{app} = dQ/dt \times 1/AC_0 \quad \text{Eq. 3.3}$$

where dC/dt is the rate of drug permeation ($\mu\text{g/s}$); A is the surface area of the insert (cell monolayer) (cm^2); C_0 is the initial concentration of drug in the apical side ($\mu\text{g/mL}$). Statistical analysis was performed using One Way ANOVA using SPSS. Post-hoc comparison of means was performed by Tukey HSD test with post-hoc procedures and statistical significance was considered at $p < 0.05$.

3.3.8. Ex Vivo Transport Studies

In order to evaluate DEX transport across ocular barriers, ex vivo transport studies were performed for DEX-PAMAM complex formulations, which were designed to increase ocular permeation of DEX following topical, or subconjunctival applications. Transport studies across cornea and sclera-choroid-retina pigment epithelium (SCRPE) were performed for PAMAM-DEX complexes (G3, G3.5 and G3-OH, G4, G4.5 and G4-OH) in comparison with DEX solution. All transport experiments were carried out in quadruplicate.

One batch of produced complex formulation was diluted 10 times for transport studies. 100 μM Atenolol was added as paracellular marker to assay buffer, which was used to prepare the solutions (44) . 700 μL of prepared DEX-PAMAM complex solutions were diluted in 6.3 mL assay buffer with 100 μM atenolol. These

initial solutions were analyzed using LC/MS later to quantify the exact initial concentrations of the formulations.

Cornea or SCRPE was isolated from fresh rabbit eyes (New Zealand albino). Tissue was placed on the needles, which were present on the acceptor side of the transport chambers, then donor side of the chambers sealed. Schematic representation of the diffusion chamber system was given in Figure 3.2. Assay buffer (200 μ L) was added to the chambers, while the other chambers getting prepared to keep the tissue hydrated. After placing all tissues to chambers the blank assay buffer was removed. Chambers were placed in to the transport system, as the acceptor side will stay on the right side. The thermostat was set to 37 °C and the air with 5% CO₂ was set to 4-5 to adjust the bubbling speed to obtain 2-3 bubbles per minute. 1.5 mL of DEX-PAMAM complex solutions (G3, G3.5 and G3-OH, G4, G4.5 and G4-OH) or DEX solution was added to the donor side of the chambers. 1.5 mL of preheated assay buffer was added to the acceptor side.

200 μ L samples were collected from acceptor side every 30 minutes for 3 hours and 200 μ L preheated assay buffer was added as replacement. The plate used to collect samples was pre-chilled. At the end of 3 hours, 200 μ L samples were collected from the donor side.

The pH of the medium was measured using pH sticks, both in the beginning and at the end of the experiment and the pH was approximately 7 and did not change during the experiment. DEX amount in the samples were quantified using LC/MS analysis.

Apparent permeability co-efficients (P_{app} , cm/s) were also calculated by the Eq. 3.3. Statistical analysis was performed using One Way ANOVA using SPSS. Post-hoc comparison of means was performed by Tukey HSD test with post-hoc procedures and statistical significance was considered at $p < 0.05$.

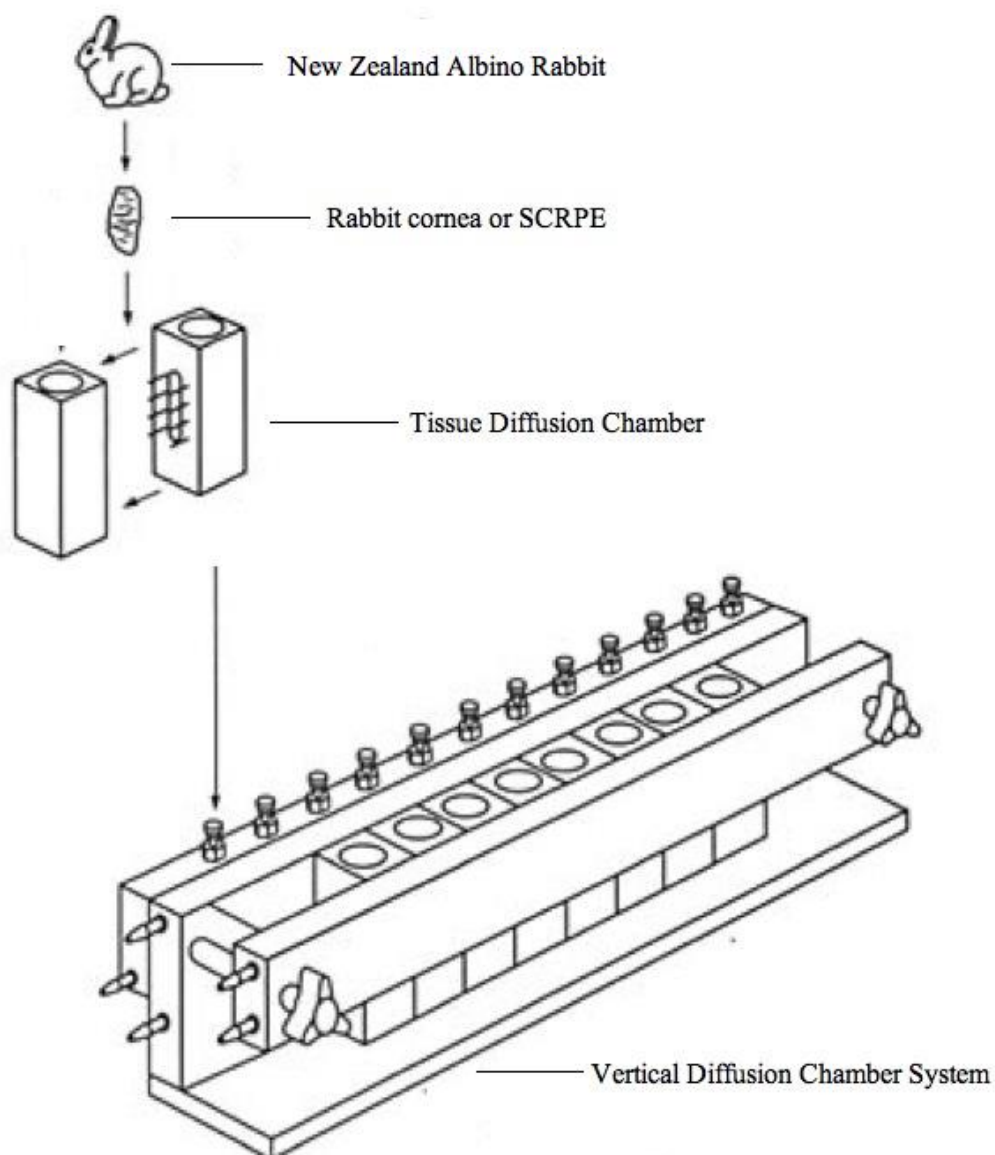


Figure 3.2. Diffusion chamber system for ex vivo transport studies (145)

3.3.9. In Vivo Studies

All in vivo experiments have been performed with the permission of Colorado University Institutional Animal Care and Use Committee (IACUC), under the protocols numbered 83411(09)1D and 83412(05)1D.

Healthy, male Sprague Dawley (SD) rats (300-400 g) were used for all in vivo studies and the applications were made to both eyes of 3 animals for each case ($n = 3 \times 2 = 6$ eyes). An application scheme for in vivo studies is given in Figure 3.3.

IN VIVO STUDIES

In Vivo Studies for DEX-PAMAM Complex Formulations		In Vivo Studies for DEX-PAMAM Conjugate Formulations	
<p style="text-align: center;">Topical Drops</p> <p><u>Barriers:</u> Cornea SCRPE</p> <p><u>Formulations:</u></p> <ul style="list-style-type: none"> • DEX suspension (control) • G3 complex • G3-OH complex • G3.5 complex • G4 complex • G4-OH complex • G4.5 complex <p>Animal: SD Rats (21) LC/MS quantification</p>	<p style="text-align: center;">Subconjunctival Injection</p> <p><u>Barriers:</u> SCRPE</p> <p><u>Formulations:</u> Selected formulations (Anionic vs. cationic)</p> <ul style="list-style-type: none"> • DEX suspension (control) • G4 complex • G4.5 complex <p>Animal: SD Rats (9) LC/MS quantification</p>	<p style="text-align: center;">Subconjunctival Injection</p> <p><u>Barriers:</u> SCRPE</p> <p><u>Formulations:</u> Alexa 488 Labeled</p> <ul style="list-style-type: none"> • G3.5 conjugate • G4.5 conjugate <p>Animal: SD Rats (6) Fluorotron analysis + Fluorospectrometric quantification</p>	<p style="text-align: center;">Intravitreal Injection</p> <p><u>Barriers:</u> -</p> <p><u>Formulations:</u> Alexa 488 Labeled</p> <ul style="list-style-type: none"> • G3.5 conjugate • G4.5 conjugate <p>Animal: SD Rats (6) Fluorotron analysis + Fluorospectrometric quantification</p>
		<p><u>Formulations:</u> Alexa 488 Labeled (Blank)</p> <ul style="list-style-type: none"> • PAMAM G3.5 • PAMAM G4.5 <p>Animal: SD Rats (6)</p>	<p><u>Formulations:</u> Alexa 488 Labeled (Blank)</p> <ul style="list-style-type: none"> • PAMAM G3 • PAMAM G4 <p>Animal: SD Rats (6)</p>

Figure 3.3. Application scheme for in vivo studies

Ocular application sites that DEX formulations have been applied to were schematized in Figure 3.4.

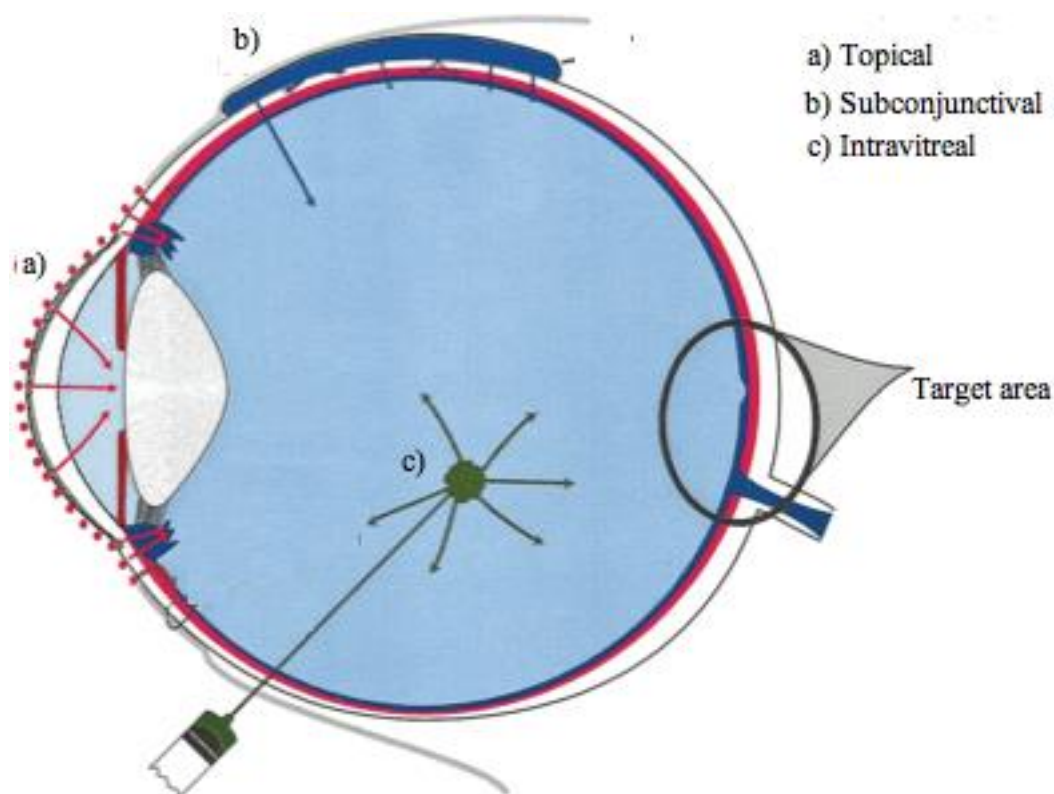


Figure 3.4. Schematic representation of the ocular application sites that DEX formulations have been applied (45) .

i- In Vivo Studies for DEX-PAMAM Complex Formulations

Ocular Distribution of DEX-PAMAM Complex Formulations Following Topical Application

0.1% DEX suspension is available in market. Thus, complexes equivalent to 0.1% (1mg/mL) DEX were prepared. DEX-PAMAM complex formulations were dried under nitrogen and reconstituted in PBS to obtain 1mg/mL concentration. 0.1% DEX suspension was also prepared in PBS.

Each formulation was applied to both eyes of 3 Sprague Dawley rats (n=6). The animals were anesthetized using isoflurane inhalation and then 5 μ L of the DEX suspension or DEX-PAMAM complex formulations, which were equivalent to 1mg/mL DEX, was applied to both eyes of the animals. Animals were euthanized 30

minutes after dosing by pentobarbital sodium (Fatal-Plus) injection and both eyeballs were collected and snapped frozen in dry ice/isopentane.

All the Eppendorf tubes for tissue isolation were weighed. 6 eyes from 3 animals were isolated for each formulation. Tissues were placed in pre-weighed Eppendorf tubes and then weight again to calculate the exact tissue weights. The isolated tissues were as follows:

- Vitreous
- Retina-CRPE
- Sclera
- Conjunctiva
- Cornea
- Aqueous humor
- Lens

TA solution (100 ng/mL) was used as internal standard. 250 μ L TA solution was added to the samples and homogenized. Samples were vortexed for 15 minutes, then 750 μ L ACN was added and vortexed for additional 15 minutes. Centrifuged for 5 minutes at 10000 rpm to remove the tissue residues. Evaporated under nitrogen and resuspended in 250 μ L ACN:water (1:1) and vortexed. Centrifuged again for 5 minutes at 10000 rpm and placed into LC/MS plates. DEX amount in the samples were quantified with LC/MS analysis.

Statistical analysis was performed using One Way ANOVA using SPSS. Post-hoc comparison of means was performed by Tukey HSD test with post-hoc procedures and statistical significance was considered at $p < 0.05$.

Ocular Distribution of DEX-PAMAM Complex Formulations Following Subconjunctival Application

Two of the DEX-PAMAM complex formulations, DEX-PAMAM G4 and G4.5 were selected for subconjunctival application, based on their ex vivo transport and in vivo topical ocular distribution results. Pre-trials indicated that cationic DEX-PAMAM G4 complex has the lowest ocular permeation and tissue distribution where

anionic DEX-PAMAM G4.5 complex has one of the highest ocular tissue permeation and ocular tissue distribution. Thus those two complex formulations were selected for subconjunctival injection for comparison. DEX-PAMAM G4 and G4.5 complex formulations were dried under nitrogen and reconstituted in PBS to obtain 1mg/mL concentration. DEX (0.1%) suspension was also prepared in PBS.

Each formulation was applied to both eyes of 3 Sprague Dawley rats (n=6). The animals were anesthetized using ketamine & xylazine (80/12 mg/kg) and then injected with 5 μ L formulations subconjunctivally using a 30 gauge needle. 1 hour after injections, animals were sacrificed using pentobarbital sodium (Fatal-Plus) injection and both eyeballs were collected and snapped freezed in dry ice/isopentane (49) .

All the Eppendorf tubes for tissue isolation were weighed. 6 eyes from 3 animals were isolated for each formulation. Tissues were placed in pre-weighed Eppendorf tubes and then weight again to calculate the exact tissue weights. Tissue isolation and extraction method was applied as it was given in section 3.3.9.1.1. DEX amount in the samples were quantified with LC/MS analysis.

Statistical analysis was performed using One Way ANOVA using SPSS. Post-hoc comparison of means was performed by Tukey HSD test with post-hoc procedures and statistical significance was considered at $p < 0.05$.

ii- In Vivo Studies for DEX-PAMAM Conjugate Formulations

Fluorotron Analysis for DEX-PAMAM Conjugate Formulations Following Subconjunctival Application

Fluorotron analysis was performed to investigate the clearance time of conjugates from vitreous and retina based on the fluorescent signals in animals' eyes following subconjunctival injection of fluorescent labeled formulations (42) . Alexa 488 was conjugated to both DEX-PAMAM G3.5 and G4.5 conjugates, in order to label the formulations for fluorotron analysis and fluorospectrometric quantification of the conjugates. DEX-PAMAM conjugate solutions (2 mg/mL, 0.5 mL) were prepared in purified water. 1 M sodium bicarbonate solution was prepared by adding 1 mL deionized water to the provided sodium bicarbonate vial. 50 μ L of 1M sodium bicarbonate solution was added to 0.5 mL of 2mg/mL conjugate solution. Reactive

dye was allowed to warm up to room temperature. Conjugate solution was added to dye vial and the reaction mixture was stirred for 1 hour at room temperature.

The purification column was assembled. Elution buffer (10x PBS) was diluted 10 fold in deionized water to obtain 1x elution buffer (1x PBS). The purification resin was stirred with a pipet to obtain a homogeneous suspension. The column was filled with the purification resin and the excess buffer was drained. The reaction mixture was added to the column. Then the vial was washed with 100 μ L elution buffer and added to the column. After the solution entered into the column, elution buffer was added to the column until the labeled conjugate has been eluted. The first of the two colored bands was collected as the labeled conjugate. Samples were placed into dialysis bags with 3500 MW cut off and placed in a beaker with 1 L PBS. The beaker was left in a dark place overnight to ensure all free dye was removed from the formulations. Samples were evaporated and resuspended in 250 μ L elution buffer to concentrate samples and obtain 4mg/mL.

Fluorotron analysis was performed for both eyes of the animals before injection to get the baseline peaks. Each formulation was applied to both eyes of 3 Sprague Dawley rats (n=6). The animals were anesthetized using ketamine & xylazine (80/12 mg/kg) and then injected with 5 μ L formulations subconjunctivally using a 30 gauge needle. Fluorotron analysis was performed on dosed animals at time points, which were 2 min, 1h and 2h. 2 hours after injection the animals were sacrificed using pentobarbital sodium (Fatal-Plus) injection, since the drug was cleared from the eyes. Both eyes were collected and snap freezed in dry ice/isopentane and stored at -80 °C until sample processing for ocular distribution study.

Ocular Distribution of DEX-PAMAM Conjugate Formulations Following Subconjunctival Application

Animals, which were injected with Alexa 488 labeled DEX-PAMAM conjugate formulations for fluorotron analysis, were used to evaluate the ocular distribution of conjugates. Following fluorotron analysis, 2 hours after injection the animals were sacrificed using pentobarbital sodium (Fatal-Plus) injection and both

eyes of the animals were collected and snap frozen in dry ice/isopentane and stored at -80 °C for ocular distribution study.

All the Eppendorf tubes for tissue isolation were weighed. 6 eyes from 3 animals were isolated for each formulation. Tissues were placed in pre-weighed Eppendorf tubes and then weight again to calculate the exact tissue weights. Tissue isolation and extraction method was applied as it was given in section 3.3.9.1.1. Fluorospectrofotometric analysis was performed (excitation: 495, emission: 519) to quantify the amount of the labeled conjugates in the tissues.

Statistical analysis was performed using One Way ANOVA using SPSS and statistical significance was considered at $p < 0.05$.

Fluorotron Analysis for DEX-PAMAM Conjugate Formulations Following Intravitreal Application

Fluorotron analysis was performed to investigate the clearance time of conjugates from vitreous and retina based on the fluorescent signals in animals' eyes following subconjunctival injection of fluorescent labeled formulations. Alexa 488 was conjugated to both DEX-PAMAM G3.5 and G4.5 conjugates, in order to label the formulations for fluorotron analysis and fluorospectrometric quantification of the conjugates. Alexa 488 labeling was performed as it was reported in section 3.3.9.2.1.

Fluorotron analysis was performed for both eyes of the animals before injection to get the baseline peaks. Each formulation was applied to both eyes of 3 Sprague Dawley rats (n=6). The animals were anesthetized using ketamine & xylazine (80/12 mg/kg) and then injected with 5 μ L formulations intravitreally using a 30 gauge needle. Fluorotron analysis was performed on dosed animals at time points, which were 2 min, 30 min, 1h, 2h, 4h, 24h. 24 hours after injection the animals were sacrificed using pentobarbital sodium (Fatal-Plus) injection, since the drug was cleared from the eyes. Both eyes were collected and snap frozen in dry ice/isopentane and stored at -80 °C until sample processing for ocular distribution study.

Ocular Distribution of DEX-PAMAM Conjugate Formulations Following Intravitreal Application

Animals, which were injected with Alexa 488 labeled DEX-PAMAM conjugate formulations for fluorotron analysis, were used to evaluate the ocular distribution of conjugates. Following fluorotron analysis, 24 hours after injection the animals were sacrificed using pentobarbital sodium (Fatal-Plus) injection and both eyes of the animals were collected and snap frozen in dry ice/isopentane and stored at -80 °C for ocular distribution study.

All the Eppendorf tubes for tissue isolation were weighed. 6 eyes from 3 animals were isolated for each formulation. Tissues were placed in pre-weighed Eppendorf tubes and then weight again to calculate the exact tissue weights. Tissue isolation and extraction method was applied as it was given in section 3.3.9.1.1. Fluorospectrofotometric analysis was performed (excitation: 495, emission: 519) to quantify the amount of the labeled conjugates in the tissues.

Statistical analysis was performed using One Way ANOVA using SPSS and statistical significance was considered at $p < 0.05$.

Fluorotron Analysis for Blank PAMAM Dendrimers Following Intravitreal Application

In order to compare the effect of the charge and generation of dendrimers on the duration of the ocular residence time, PAMAM dendrimers (PAMAM G3, G4, G3.5 and G4.5) were also investigated to shed light on future work and to improve the current conjugate formulations. Blank dendrimers with different generations and charges were tested in terms of their ocular clearance and ocular tissues to investigate the effects of dendrimer types. Results of this study might help to improve the formulation in further studies to extend the clearance time and enhance the retinal accumulation. Alexa 488 was conjugated to anionic PAMAM G3.5 and G4.5 dendrimers and cationic PAMAM G3 and G4 dendrimers, in order to label the dendrimers for fluorotron analysis and fluorospectrometric quantification of the conjugates. Alexa 488 labeling was performed as it was reported in section 3.3.9.2.1. Sample fluorescent signals were measured and concentrations were adjusted to obtain close fluorescent levels (6250-6750) since there is no drug

involved. The adjusted dendrimer concentrations that were applied to animals were 2mg/mL for PAMAM G3, 2.66 mg/mL for PAMAM G4, 0.2 mg/mL for PAMAM G3.5 and 0.5 mg/mL for PAMAM G4.5 respectively. Fluorotron analysis was performed as it was reported in section 3.3.9.2.3. Time points for fluorotron measurements were 2 min, 1h, 2h, 4h, 24h.

Ocular Distribution of Blank PAMAM Dendrimers Formulations Following Intravitreal Application

Animals, which were injected with Alexa 488 labeled PAMAM dendrimers for fluorotron analysis, were used to evaluate the ocular distribution of dendrimers. Following fluorotron analysis, 24 hours after injection the animals were sacrificed using pentobarbital sodium (Fatal-Plus) injection and both eyes of the animals were collected and snap frozen in dry ice/isopentane and stored at -80 °C for ocular distribution study.

All the Eppendorf tubes for tissue isolation were weighed. 6 eyes from 3 animals were isolated for each formulation. Tissues were placed in pre-weighed Eppendorf tubes and then weight again to calculate the exact tissue weights. Tissue isolation and extraction method was applied as it was given in section 3.3.9.1.1. Fluorospectrofotometric analysis was performed (excitation: 495, emission: 519) to quantify the amount of the labeled conjugates in the tissues.

Statistical analysis was performed using One Way ANOVA using SPSS. Post-hoc comparison of means was performed by Tukey HSD test with post-hoc procedures and statistical significance was considered at $p < 0.05$.

4. RESULTS

4.1. Validation of HPLC Quantification Method for Dexamethasone

Validation of the HPLC method for DEX quantification included specificity, range, linearity, precision, accuracy, LOD and LOQ. A sample chromatogram is given in Figure 4.1 for DEX solution with a concentration of 100 $\mu\text{g/mL}$, in a mobile phase.

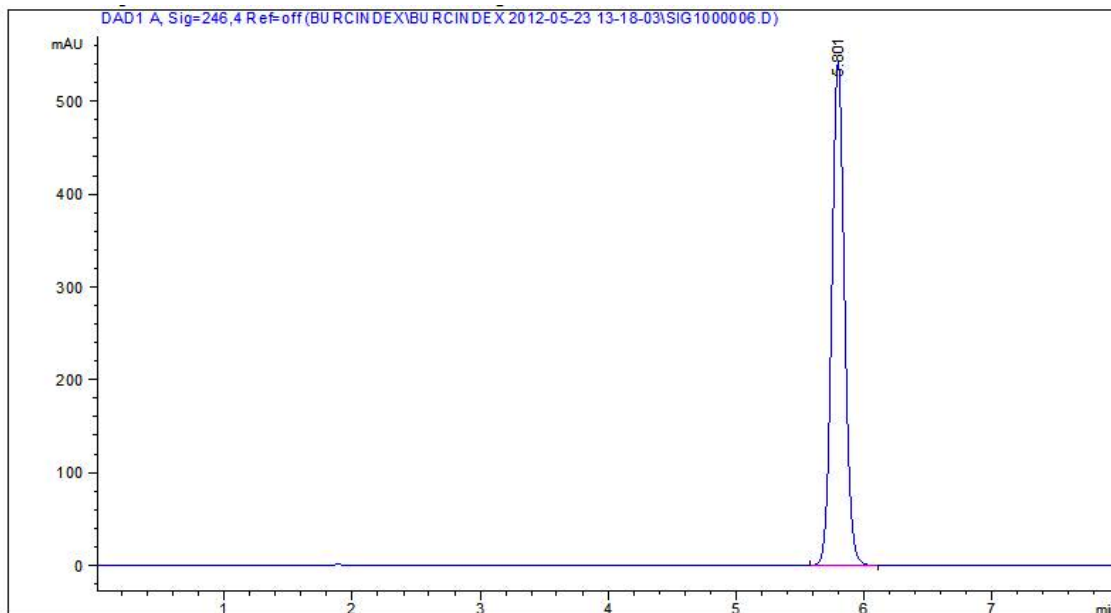


Figure 4.1 Sample DEX chromatogram

Specificity

Specificity was examined by analyzing solvents and solutions of dendrimers, which were used to prepare DEX formulations. Absence of interference was demonstrated. Sample chromatograms for acetonitrile (ACN), methanol (MeOH) and PAMAM G3.5 dendrimer is given in Figure 4.2.

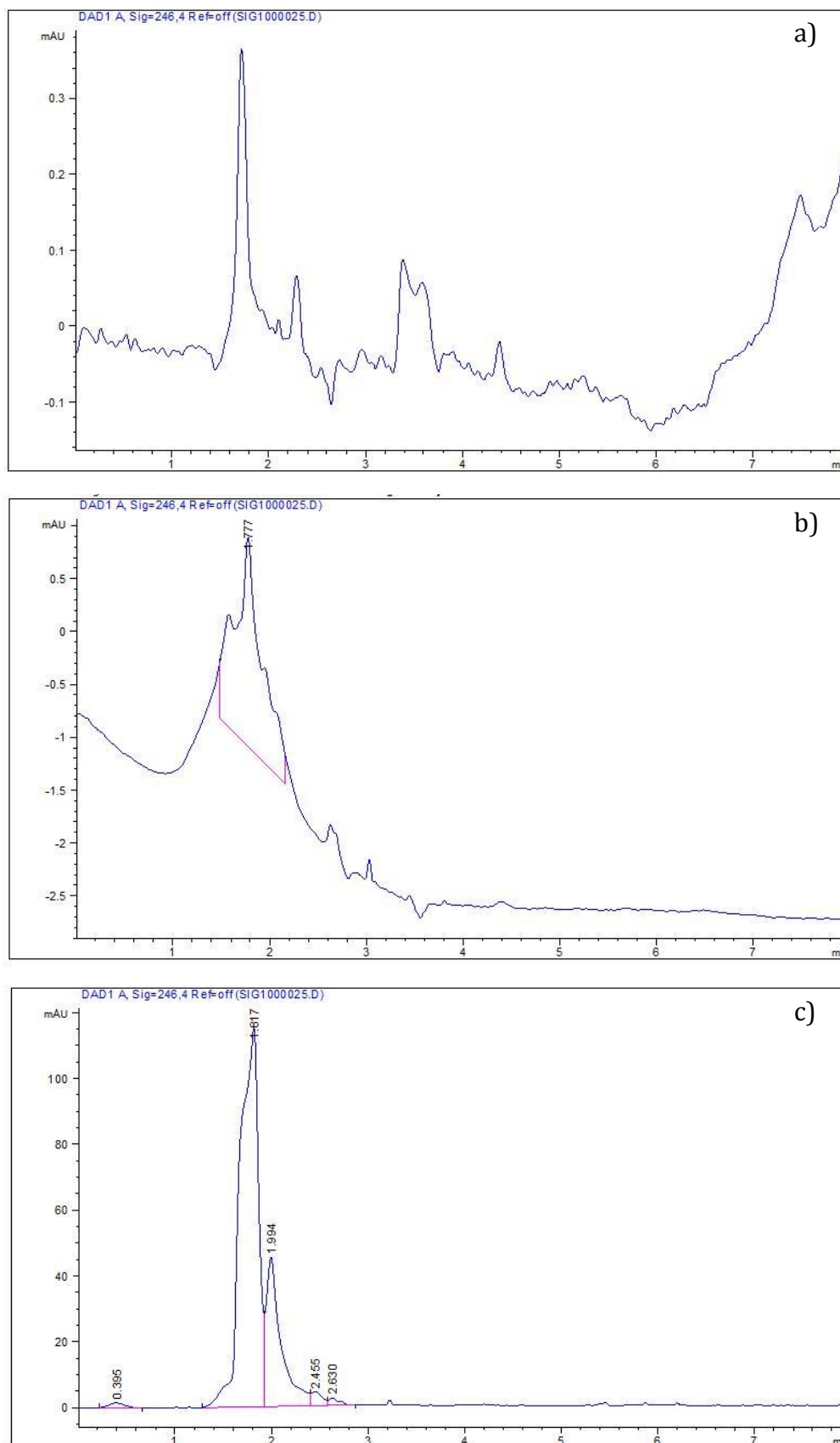


Figure 4.2. Sample chromatograms for a) ACN, b) MeOH, c) PAMAM G3.5

Range/Linearity

The range of an analytical procedure is the interval between the upper and lower concentrations of analyte in the sample. Data indicate that the DEX peak area is linear over the concentration range of 10–500 $\mu\text{g/mL}$. This range covers the in vitro working range for DEX. The R^2 for the regression line is 0.99925 with a slope of 40.437 and a y-intercept of -97.328. The regression line is given in Figure 4.3.

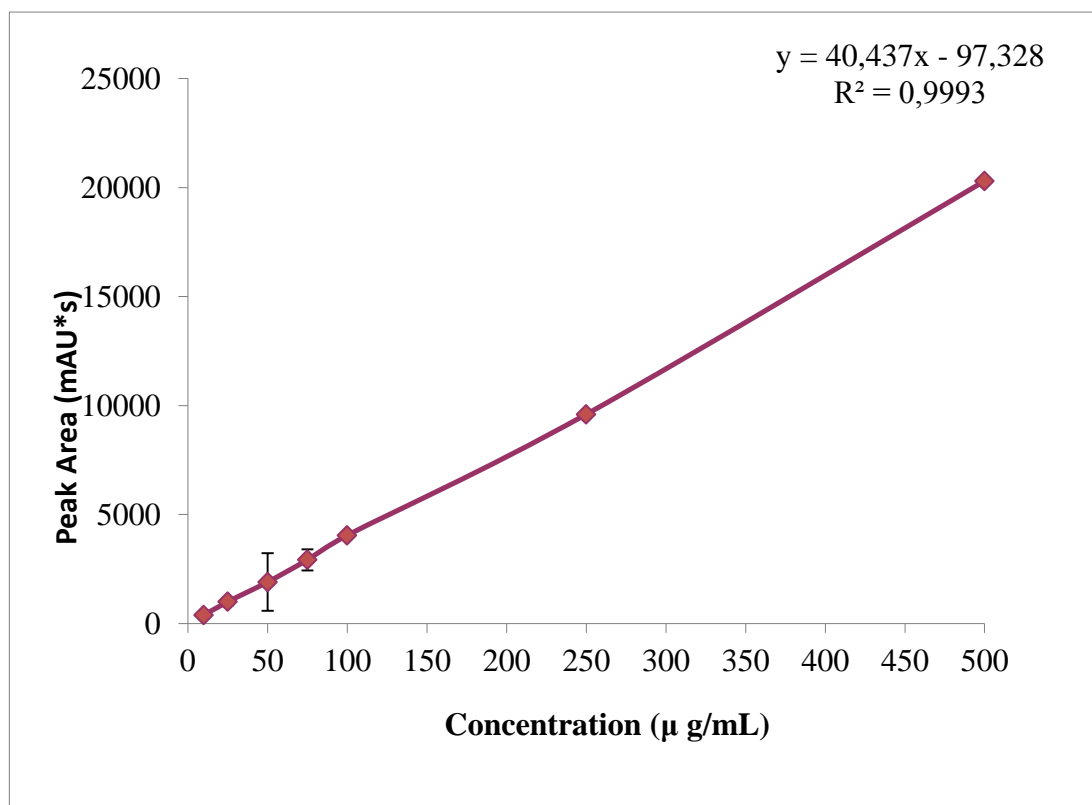


Figure 4.3. The regression line for DEX (10–500 $\mu\text{g/mL}$)

Accuracy

Six preparations were made for each concentration. Recovery of DEX was determined for each sample. Results are shown in Table 4.1. Coefficient of variations are $<2\%$ for each concentration which were considered acceptable. Average percent recoveries were calculated for each concentration and the results are 97.7% for 10 $\mu\text{g/mL}$, 102.1% for 75 $\mu\text{g/mL}$ and 101.3 % for 500 $\mu\text{g/mL}$.

Table 4.1. Average percent recoveries of DEX

	10 µg/mL	75 µg/mL	500 µg/mL
X	97.69	102.07	101.25
SD	1.45	0.52	1.24
CV%	1.49	0.51	1.22

Precision

Six samples were prepared at the same concentration (75 µg/mL) to evaluate method precision and coefficient of variation (CV%) being 0.66%.

Six injections of the same sample (75 µg/mL) were made to evaluate instrument precision. CV% was 0.42%.

Intermediate precision (variation between days) was evaluated to show the variation between the days. Samples (75 µg/mL) prepared with 3 replicates, on three separate days and CV% was 1.31%.

Stability

Samples with a concentration of 75 µg/mL (n=3) were prepared and analyzed right after preparation and 24 hours after preparation to show if it is stable during the analysis. The average recovery was 98.6% of the initial concentration, after 24 hours of incubation in room temperature.

LOD and LOQ

LOD and LOQ were measured based on the signal-to-noise ratio. LOD was found 78.2 ng/mL an LOQ was found 261.1 ng/mL.

4.2. Characterization of DEX-PAMAM Formulations

4.2.1. FTIR Analysis of DEX-PAMAM Complex Formulations

The results for the FTIR spectra were presented in Figure 4.4 to Figure 4.9 as a comparison of DEX, plain dendrimer and complex formulation. FTIR spectra of the complexes indicated the disappearance of typical bands of DEX, such as C=O stretch peak at 1700–1720 cm^{-1} . The O–H stretching bands (3200-3400 cm^{-1}) are present in both DEX and dendrimer structures, thus disappearance of C–H stretch peak was not expected upon complexation but any shifts of these bands might indicate the formation of hydrogen bonds between DEX and dendrimers. The results can be concluded as no free DEX peak was observed in complex formulations.

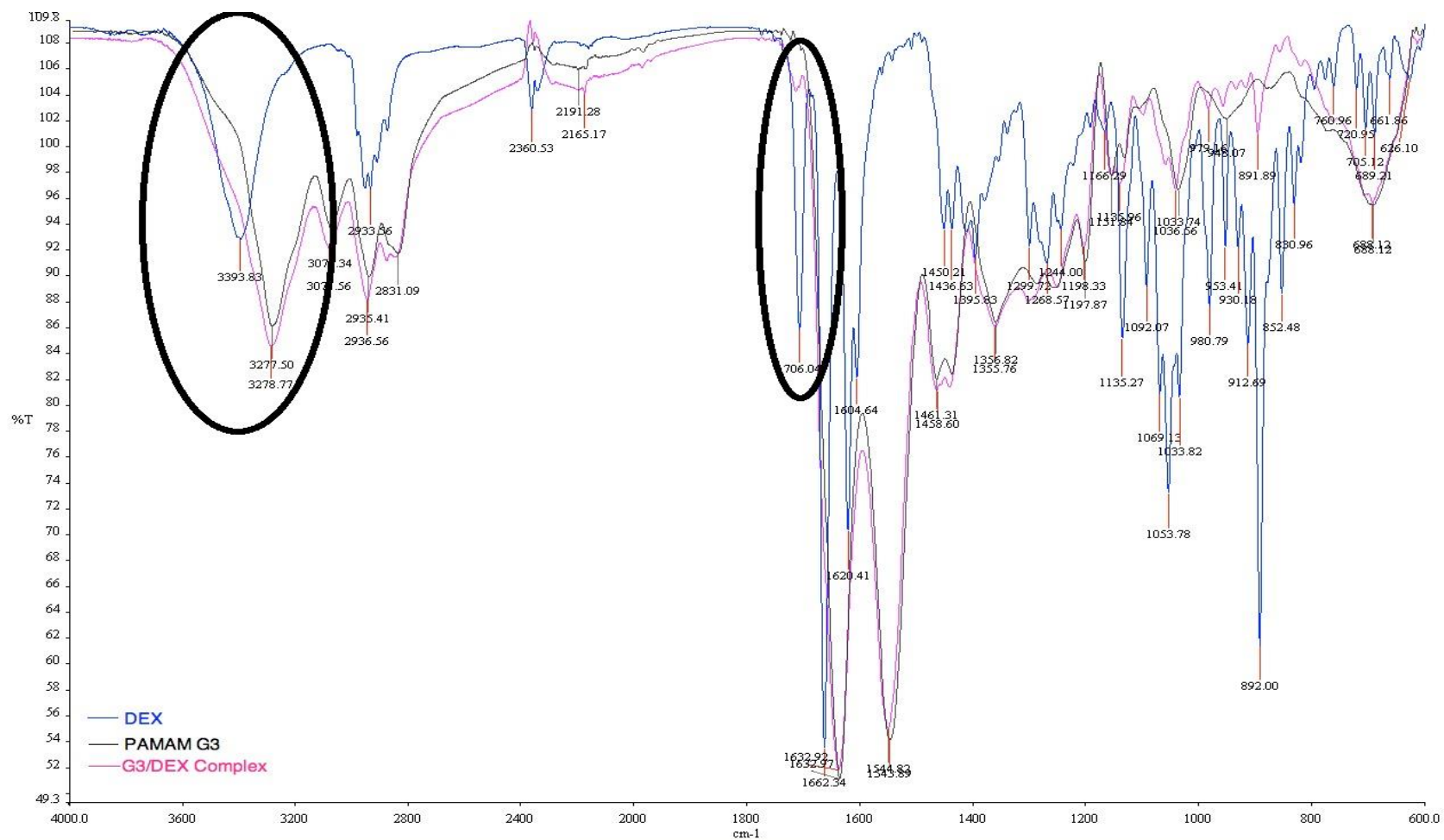


Figure 4.4. FTIR Spectrogram of DEX in comparison with PAMAM G3 and DEX-PAMAM G3 complex formulation.

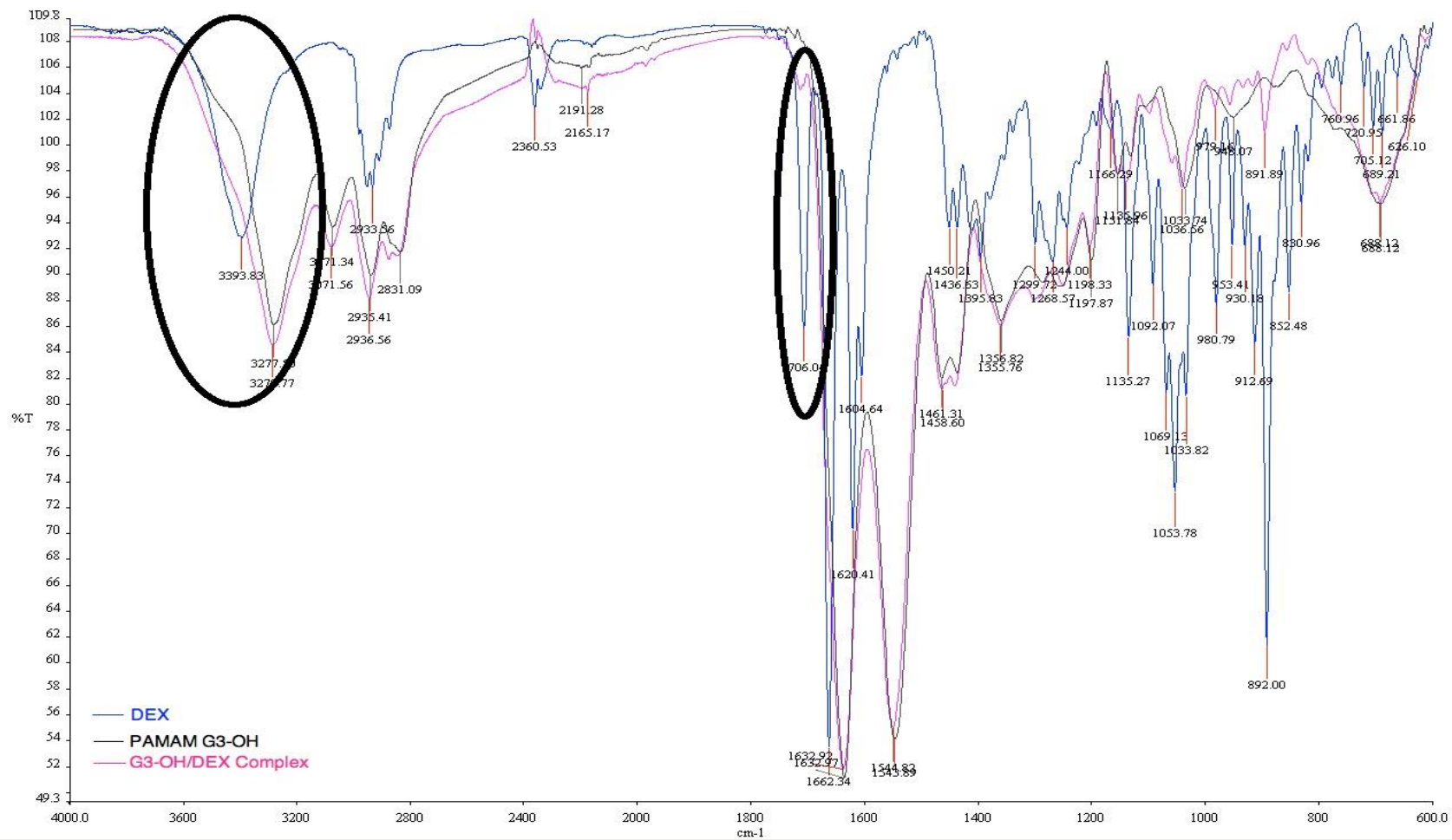


Figure 4.5. FTIR Spectrogram of DEX in comparison with PAMAM G3-OH and DEX-PAMAM G3-OH complex formulation.

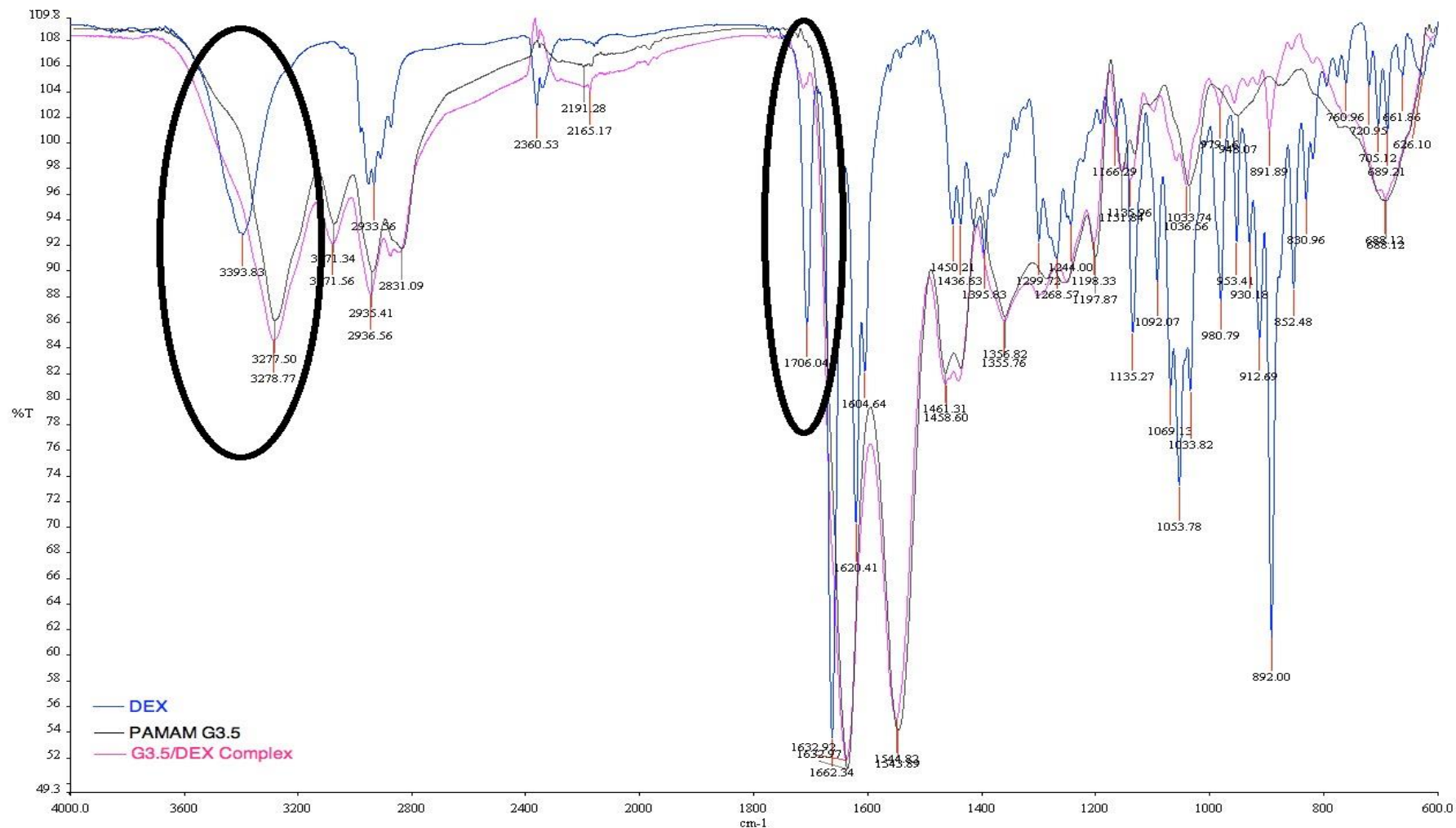


Figure 4.6. FTIR Spectrogram of DEX in comparison with PAMAM G3.5 and DEX-PAMAM G3.5 complex formulation.

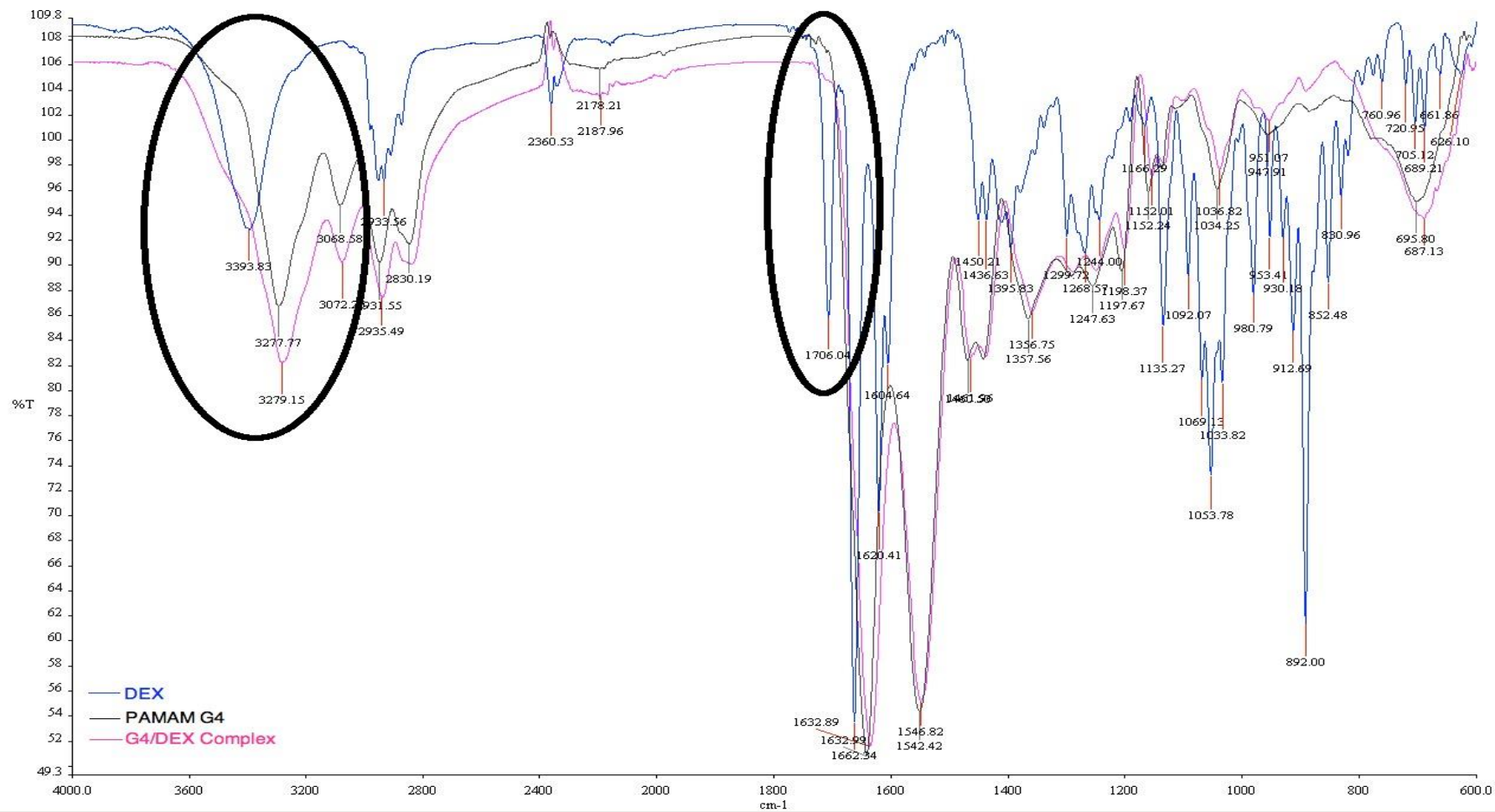


Figure 4.7. FTIR Spectrogram of DEX in comparison with PAMAM G4 and DEX-PAMAM G4 complex formulation.

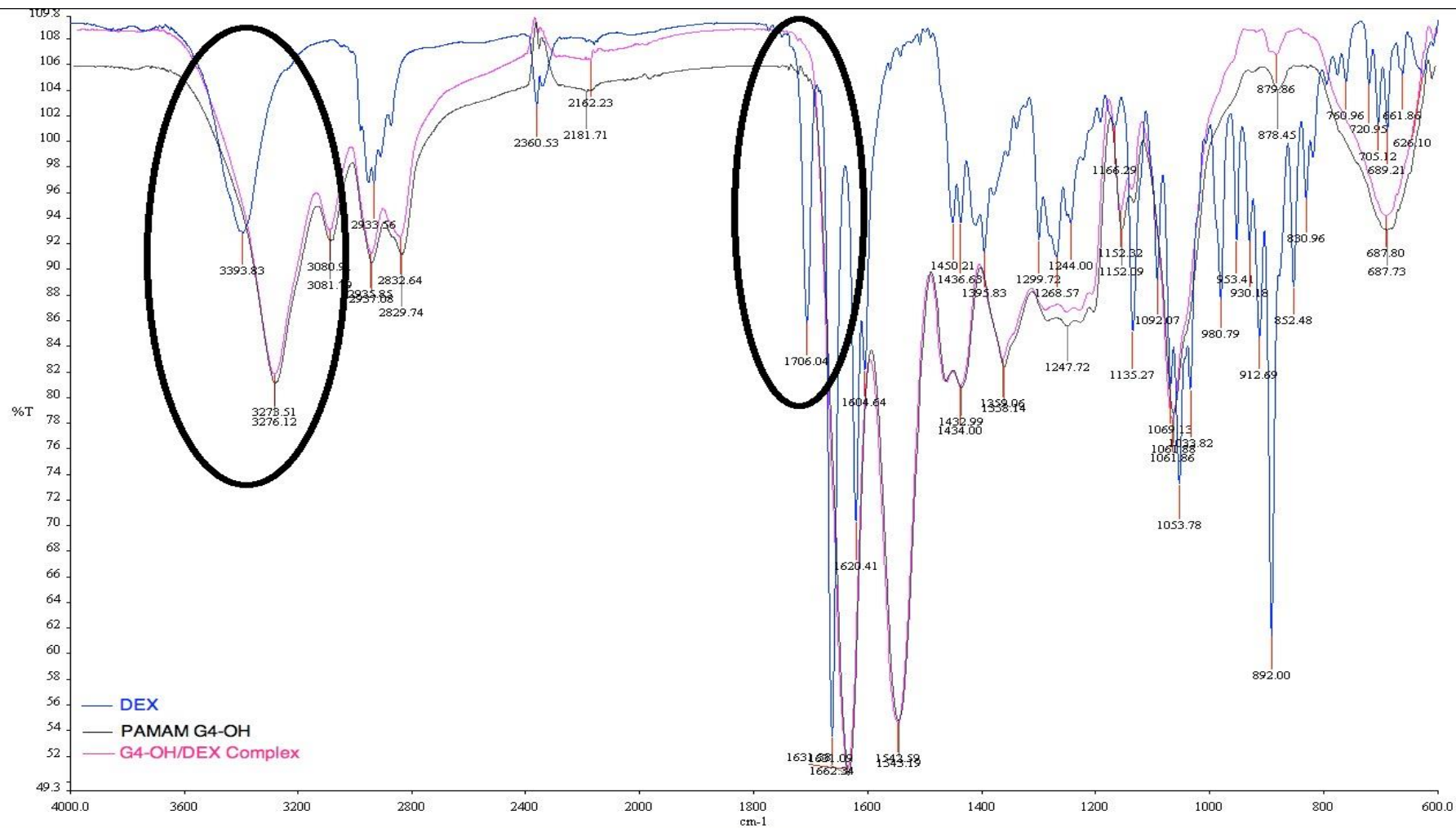


Figure 4.8. FTIR Spectrogram of DEX in comparison with PAMAM G4-OH and DEX-PAMAM G4-OH complex formulation.

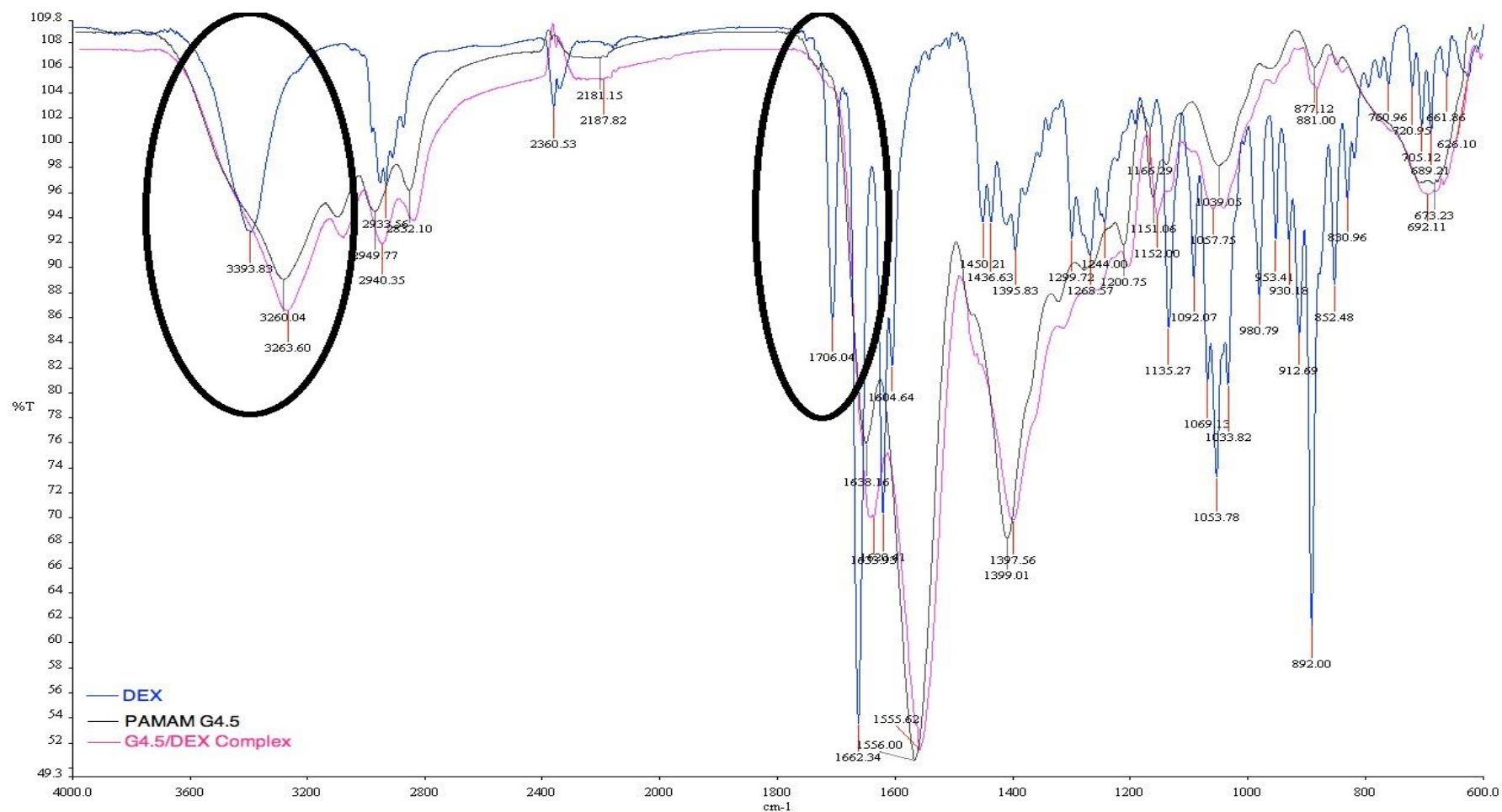


Figure 4.9. FTIR Spectrogram of DEX in comparison with PAMAM G4.5 and DEX-PAMAM G4.5 complex formulation.

4.2.2. ¹H-NMR Analysis of DEX-PAMAM Conjugate Formulations

Number of drug molecules attached to the dendrimers, were determined by ¹H-NMR (Varian 400 MHz) (146). Calculation of the drug content was achieved by comparing the integration value of proton “e” placed at 2.19 ppm (CH_2-N at the very core of the PAMAM dendrimer) and of protons “g” and “h” placed at 6.28 ppm ($CH=CH$ at the double bond of the conjugated drug molecule) and 7.44 ppm ($CH=CH$ at the double bond of the conjugated drug molecule) respectively. The final drug number per PAMAM 3.5 dendrimer was calculated as 10 and per PAMAM 4.5 dendrimer was calculated as 4 respectively.

Results of the ¹H-NMR study were summarized in Table 4.2. ¹H-NMR spectrum for PAMAM G3.5 and PAMAM G4.5 dendrimers was given in Figure 4.10 and spectrums for DEX-PAMAM G3.5 and DEX-PAMAM G4.5 conjugate formulations were given in Figure 4.11 and Figure 4.12.

Table 4.2. ¹H-NMR results for DEX-PAMAM conjugate formulations

H¹ NMR, ppm	3.3	2.4	2.8	2.6	2.19
	# of a	# of b	# of c	# of d	# of e
G 3.5	248	120	120	120	4
G 4.5	504	248	248	248	4

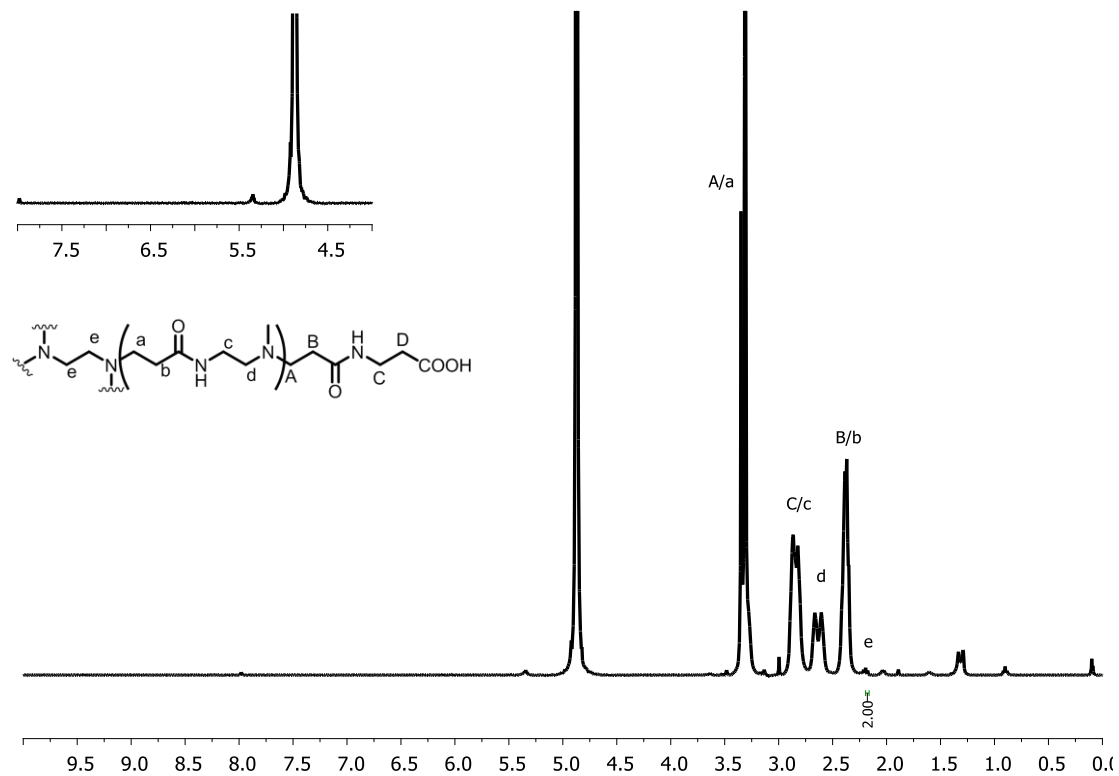


Figure 4.10. ^1H NMR spectrum for PAMAM 3.5 and PAMAM 4.5

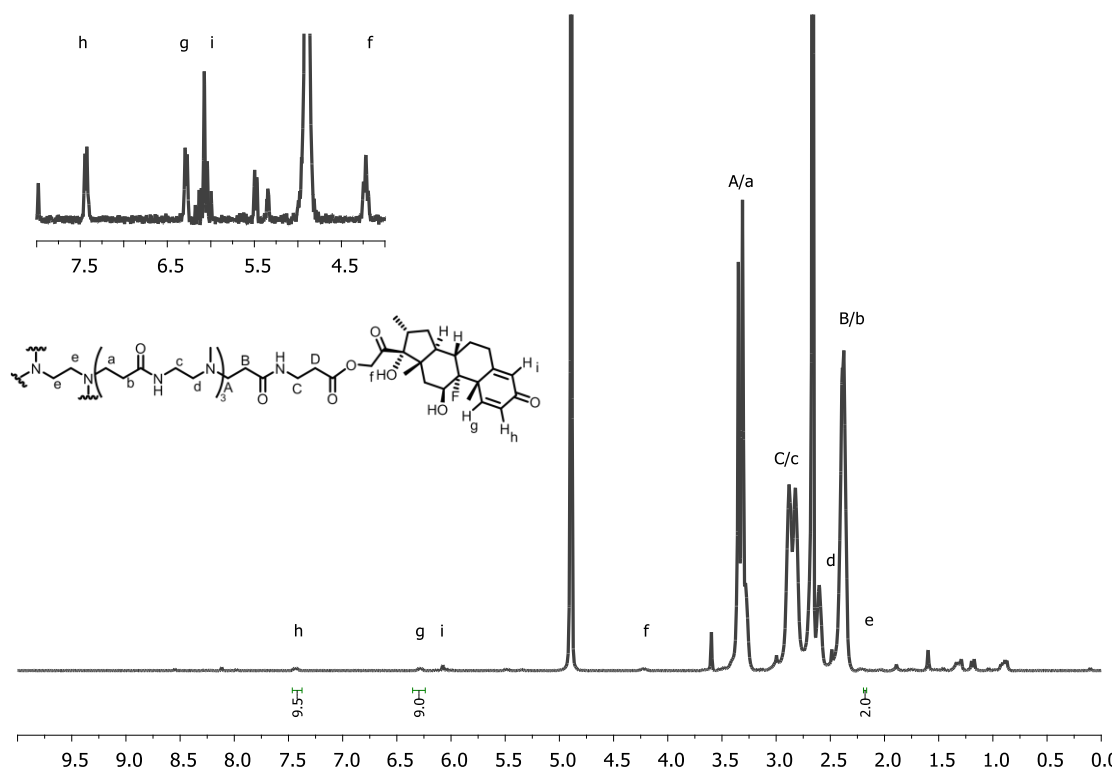


Figure 4.11. ^1H NMR spectrum for DEX-PAMAM G3.5 conjugate

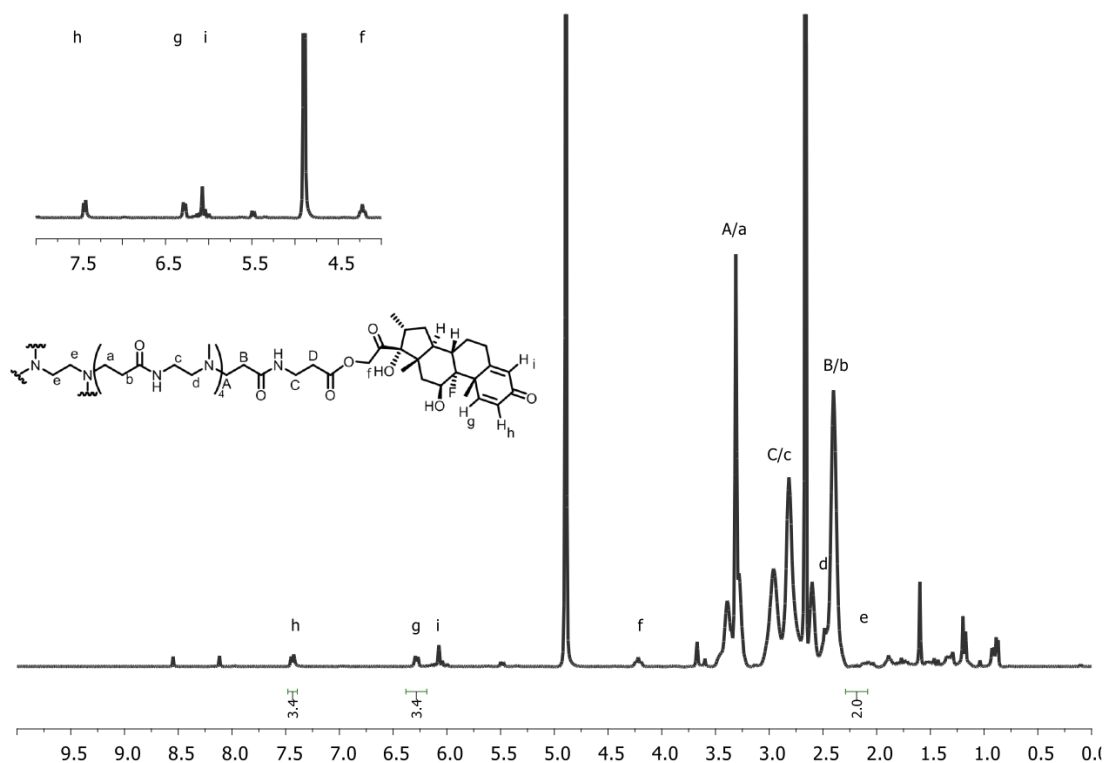


Figure 4.12. ^1H NMR spectrum for DEX-PAMAM G4.5 conjugate

4.2.3. Particle Size and Zeta Potential Measurements

Particle size and zeta potential measurements were made for both PAMAM dendrimers, DEX-PAMAM complex and DEX-PAMAM conjugate formulations. Results of the measurements were presented in Table 4.3.

Results indicated that DEX complexation increased particle size, while conjugation did not have a significant effect on particle size. Zeta potential measurements showed that PAMAM G3 and G4 and their formulations were charged positively, and the other PAMAM G3.5, G3-OH, G4.5 and G4-OH and their formulations were negatively charged as expected. Zeta potential results also showed that, zeta potential values, either positive or negative, were increased in presence of DEX.

Table 4.3. Particle size (PS) and zeta potential values for blank PAMAM dendrimers and DEX-PAMAM formulations (n=3)

	Mean PS (nm)	SD	Mean Zeta Potential (mV)	SD
PAMAM G3	49.72	0.15	0.48	0.21
PAMAM G 3.5	183.70	43.61	-16.70	2.09
PAMAM G3-OH	68.25	2.34	-13.60	2.19
PAMAM G4	78.16	7.07	0.96	0.80
PAMAM G 4.5	143.67	19.64	-45.90	8.81
PAMAM G4-OH	84.82	9.61	-8.75	0.48
G3 Complex	217.17	42.06	9.72	0.11
G 3.5 Complex	230.87	35.75	-75.53	4.17
G3-OH Complex	174.23	19.35	-34.80	0.82
G4 Complex	423.17	18.97	23.77	0.50
G4.5 Complex	131.20	19.43	-52.23	2.54
G4-OH Complex	124.83	0.68	-32.43	1.99
G 3.5 Conjugate	131.83	4.09	-54.93	1.32
G 4.5 Conjugate	145.07	9.21	-57.80	2.74

4.2.4. Loading Efficiency of The DEX-PAMAM Complex Formulations

Known amounts of DEX-PAMAM complex formulations were dissolved and DEX amount was quantified by HPLC. Calculations were made based on the amounts added for preparation. DEX loading efficiency percentages and DEX amounts in 1 mg of complex formulations were given in Table 4.4.

Table 4.4. Loading efficiency values for DEX-PAMAM complex formulations

Formulation	Loading Efficiency (%)	SD	DEX amount in 1 mg complex (μg)
G3.5 complex	1.74	0.19	2.09
G3 complex	8.09	0.22	6.97
G3-OH complex	1.42	0.22	2.87
G4.5 complex	15.17	1.70	5.30
G4 complex	16.0	0.75	8.15
G4-OH complex	6.68	0.08	6.48

4.3. In Vitro Release Studies for DEX-PAMAM Complex Formulations

Release profiles of the DEX-PAMAM complex formulations were investigated in order to evaluate the DEX amount that will be released following a topical or subconjunctival application of the formulations. Since the formulations will be expected to clear from the eye in an hour approximately (119,141), it is desired that DEX should be released immediately to penetrate to the back of the eye. Thus a 3 hours long release study has been performed in PBS at 37°C and the results were presented in Figure 4.13 (n = 3, mean \pm SD). The results showed that G3.5 complex has the highest release rate and at least 50% of DEX was released in 3 hours. G3.5 and G4.5 complex formulations, which has “-COOH” as an ending group in their structures, showed the highest release rate.

Release profiles were compared statistically by calculating the difference factor (f_1) and the similarity factor (f_2). f_1 and f_2 were calculated and presented in Table 4.5. Release profiles of all formulations were found statistically different, except that the formulations prepared with different generations of the same PAMAM type. G3 and G4 complexes, G3.5 and G4.5 complexes and G3-OH and G4-OH complexes showed statistically similar release profiles.

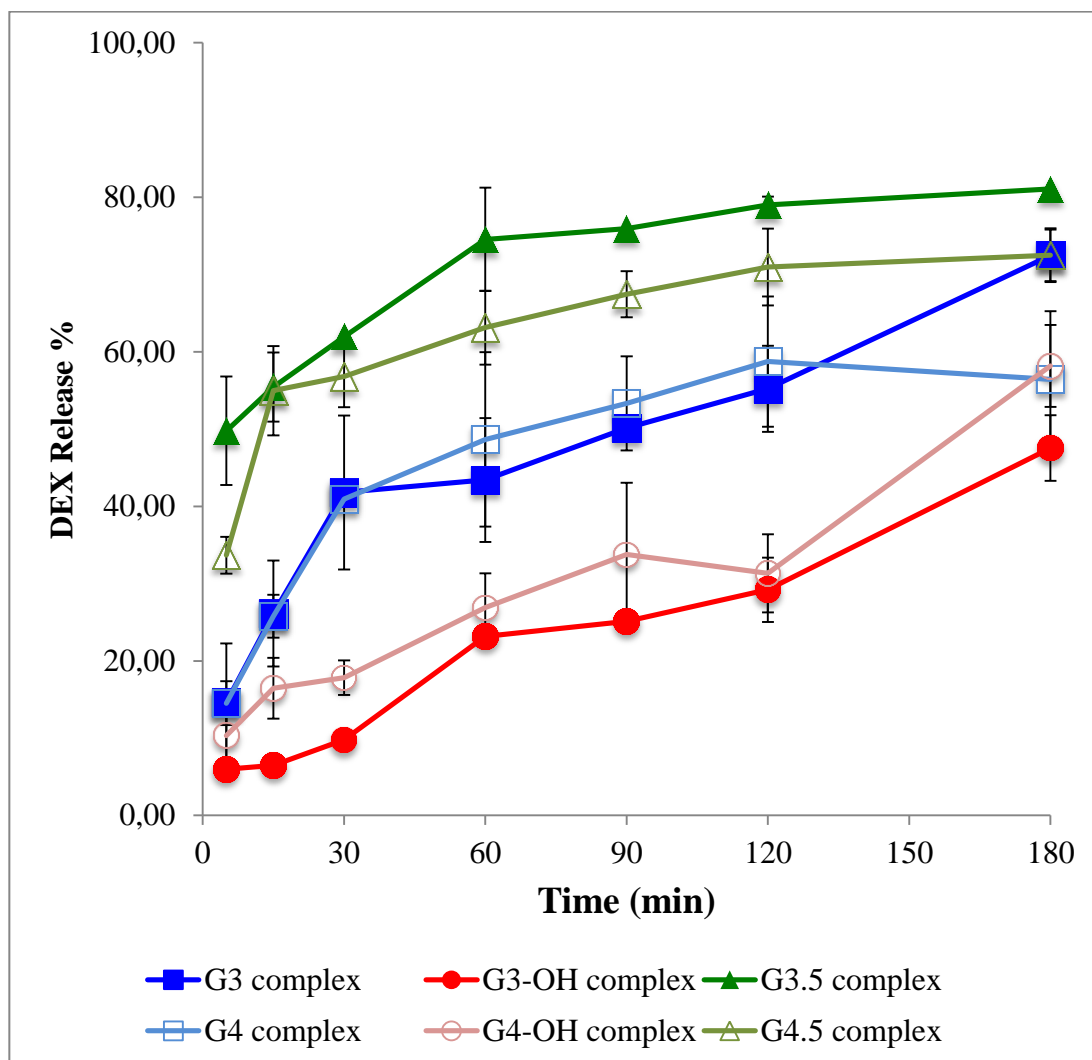


Figure 4.13. DEX release from DEX-PAMAM complex formulations

Table 4.5. f_1/f_2 statistics for DEX release from DEX-PAMAM complex formulations

	G3	G3-OH	G3.5	G4	G4-OH	G4.5
G3		51.5/31.6	57.3/29.1	1.77/58.6	35.9/38.6	38.1/36.8
G3-OH	51.5/31.6		224.4/16.1	50.6/31.6	24.4/56.2	64.9/20.0
G3.5	57.3/29.1	224.4/16.1		60.1/29.2	145.3/19.3	13.9/51.1
G4	1.77/58.6	50.6/31.6	60.1/29.2		34.7/37.3	40.6/37.1
G4-OH	35.9/38.6	24.4/56.2	145.3/19.3	34.7/37.3		115.4/23.9
G4.5	38.1/36.8	64.9/20.0	13.9/51.1	40.6/37.1	115.4/23.9	

4.4. Hydrolysis Studies of DEX-PAMAM Conjugates

Results of the hydrolysis study in presence of cornea and SCRPE tissues showed that, enzymatic degradation of DEX-PAMAM conjugates was very slow. Less than 8% of DEX was released in 6 days, and the results indicated that the hydrolysis did not affected by the type of the tissue. It was also observed that DEX-PAMAM G3.5 conjugates has a higher hydrolysis rate, which is expected since this formulation has higher molar DEX ratio (1:10) than DEX-PAMAM G4.5 conjugate (1:4).

Released DEX rate by hydrolysis was plotted against time and the results were given in Figure 4.14.

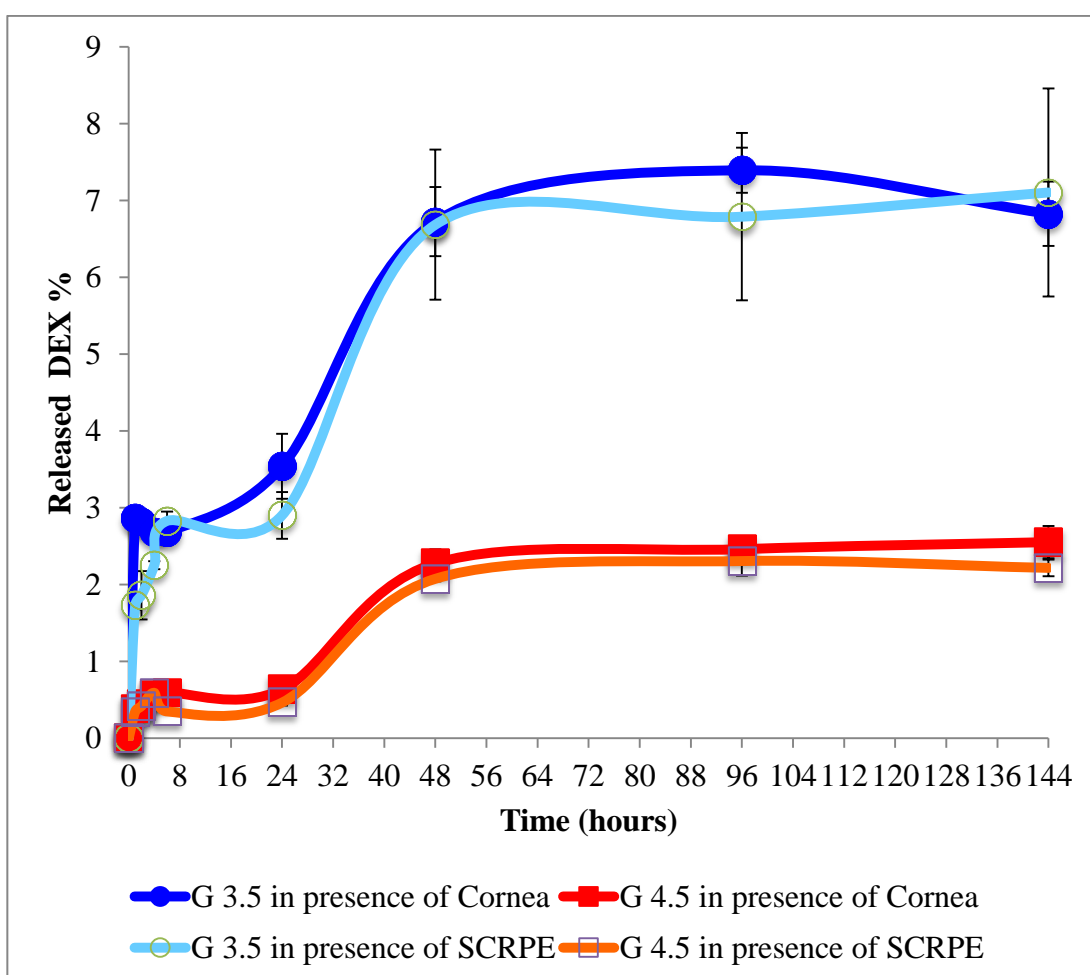


Figure 4.14. DEX release from DEX-PAMAM conjugates in presence of cornea and SCRPE tissues

4.5. Cell Culture Studies

4.5.1. MTT Cytotoxicity Assay

MTT assay results showed that DEX itself has a slight cytotoxicity against ARPE 19 cells and the blank dendrimers has no effect on cell viability. The difference between blank and DEX loaded dendrimers were found statistically significant. Results of the MTT assay were given in Figure 4.15.

Results of the MTT assay indicated that cationic dendrimers PAMAM G3 and PAMAM G4 have lower cytotoxicity than some of the anionic dendrimers but that difference is only significant between PAMAM G3.5 and PAMAM G4. Conjugate formulations were also found significantly more cytotoxic than complex formulations and plain dendrimers. No significant difference was observed between the dendrimer types for conjugate formulations ($p < 0.05$).

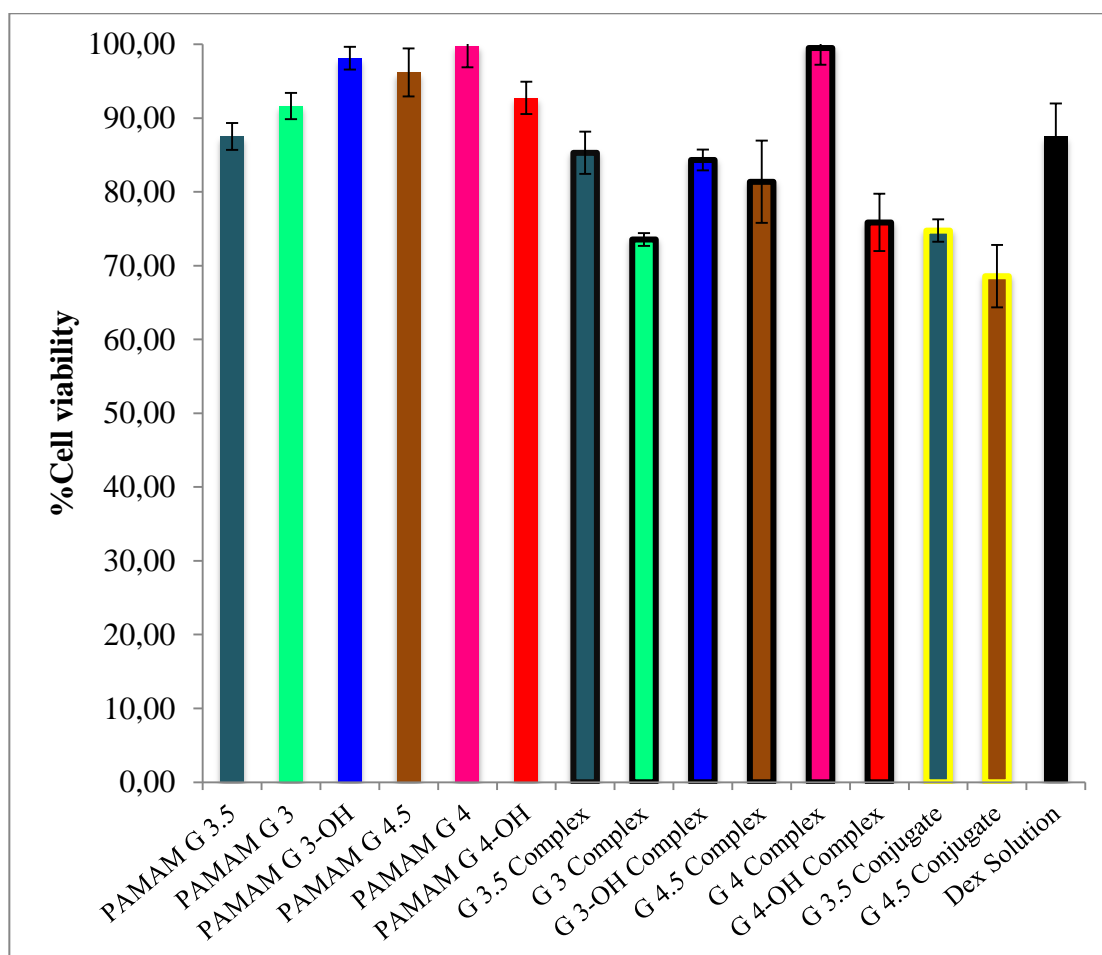


Figure 4.15. % Cell viability results following the MTT assay

4.5.2. In Vitro Permeability Studies

Cell monolayer integrity was tested by measuring transepithelial resistance prior to perform permeability studies. It was observed that the TEER values of the cells slightly reduced during the experiment, but this change were found to be insignificant. The change in the transepithelial resistance is given in Figure 4.16. The apical to basolateral permeation of DEX, DEX-PAMAM conjugates and DEX-PAMAM complex formulations, was presented in the Table 4.6. Results indicated that G4.5 complex had the highest permeation, where all of the DEX-PAMAM complex formulations showed higher permeation comparing to DEX solution and DEX-PAMAM conjugates and the differences between all groups were found statistically significant ($p < 0.05$). DEX penetration from DEX-PAMAM conjugates were found low since there were no free DEX to quantify due to hydrolyze dependent slow release rate of DEX from conjugates. Apparent permeability coefficients were also given as a histogram in Figure 4.17.

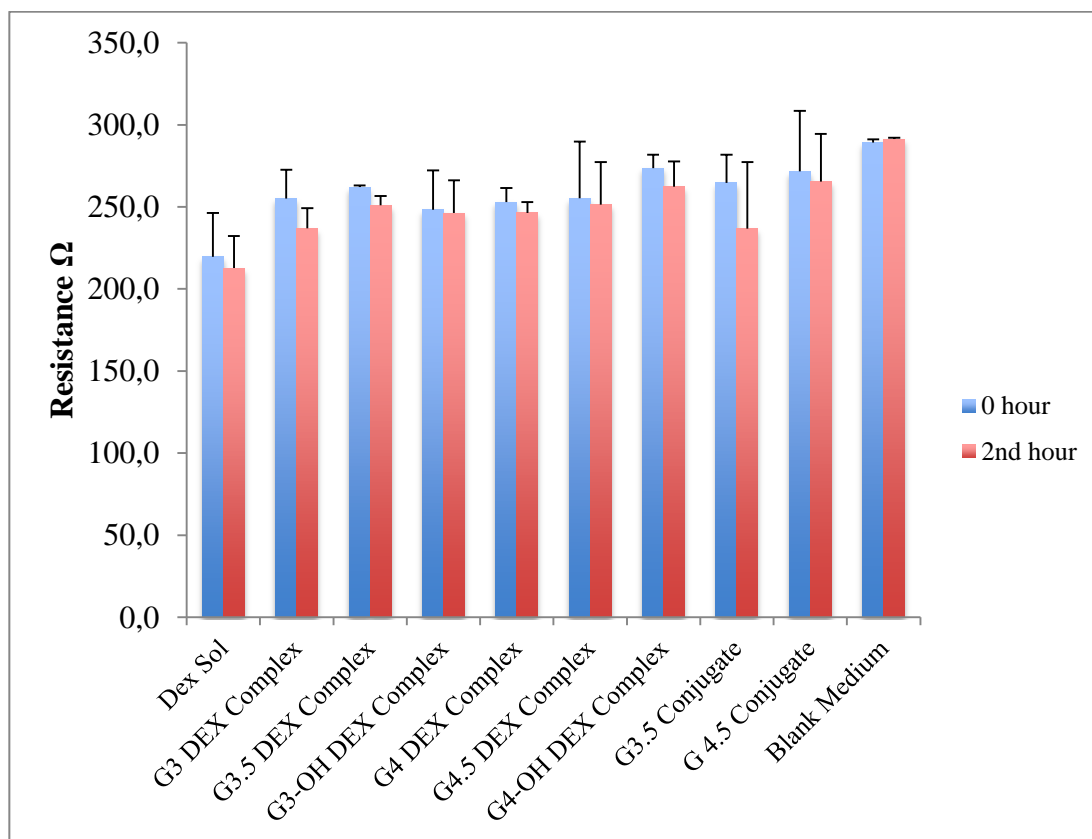


Figure 4.16. Transepithelial electrical resistance change during permeability study

Table 4.6. Apical to basolateral permeation of DEX from DEX-PAMAM formulations

Formulation	Apical (%)	Basolateral (%)	P_{app} (cm/s)
DEX Solution	95.64	0.94	0.579075E-06
G 3 Complex	88.50	4.41	2.70809E-06
G3.5 Complex	86.34	3.01	1.85146E-06
G3-OH Complex	95.15	1.40	0.86258E-06
G4 Complex	88.76	3.78	2.32427E-06
G4.5 Complex	85.45	15.15	9.30766E-06
G4-OH Complex	89.85	12.92	7.94206E-06
G3.5 Conjugate	46.59	0.22	0.137086E-06
G4.5 Conjugate	53.51	0.86	0.527889E-06

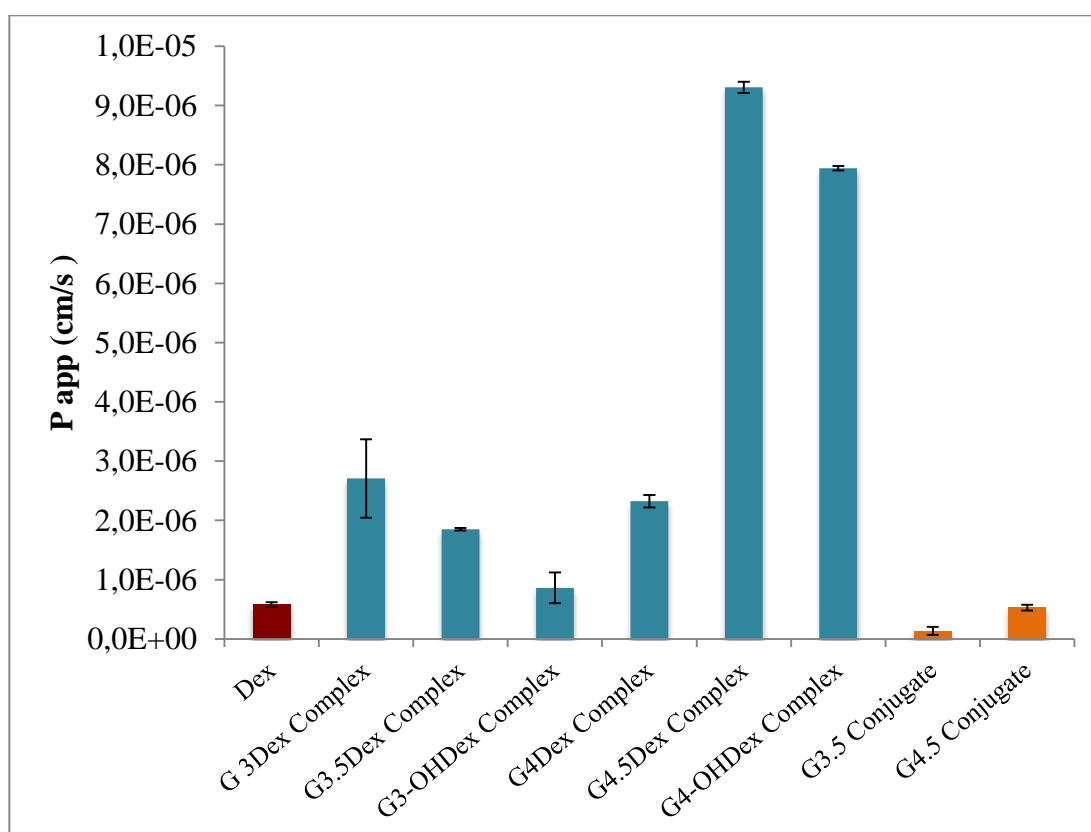


Figure 4.17. Apparent permeability coefficients (P_{app}) for DEX and DEX:PAMAM formulations

4.6. Ex Vivo Transport Studies

Results of ex vivo transport studies, for DEX-PAMAM complex formulations, across cornea was presented in Figure 4.18 and across SCRPE was presented in Figure 4.19 respectively. The results indicated that both cationic dendrimer complexes (PAMAM G3 and PAMAM G4) have a lower transport level against long odds. The results also indicated that all anionic dendrimer complexes (G3.5, G4.5, G3-OH and G4-OH) have higher drug transport level than DEX solution and the cationic dendrimers. Dendrimers with $-COOH$ ending groups (G3.5 and G4.5 complexes) showed slightly higher transport profile than $-OH$ ending ones (G3-OH and 4-OH complexes) and generation 3 dendrimers has higher DEX transport than generation 4 dendrimers. SCRPE transport has been found higher than corneal transport as expected.

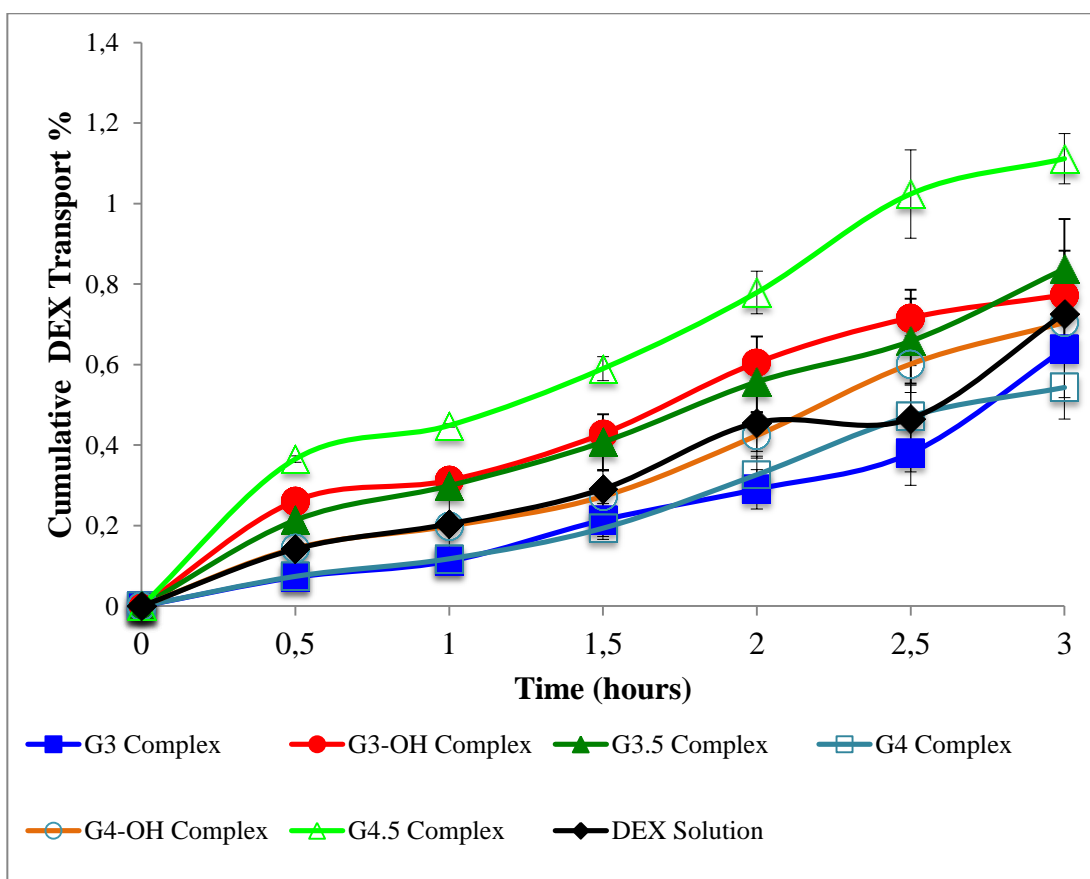


Figure 4.18. DEX transport levels across cornea from DEX-PAMAM complex formulations

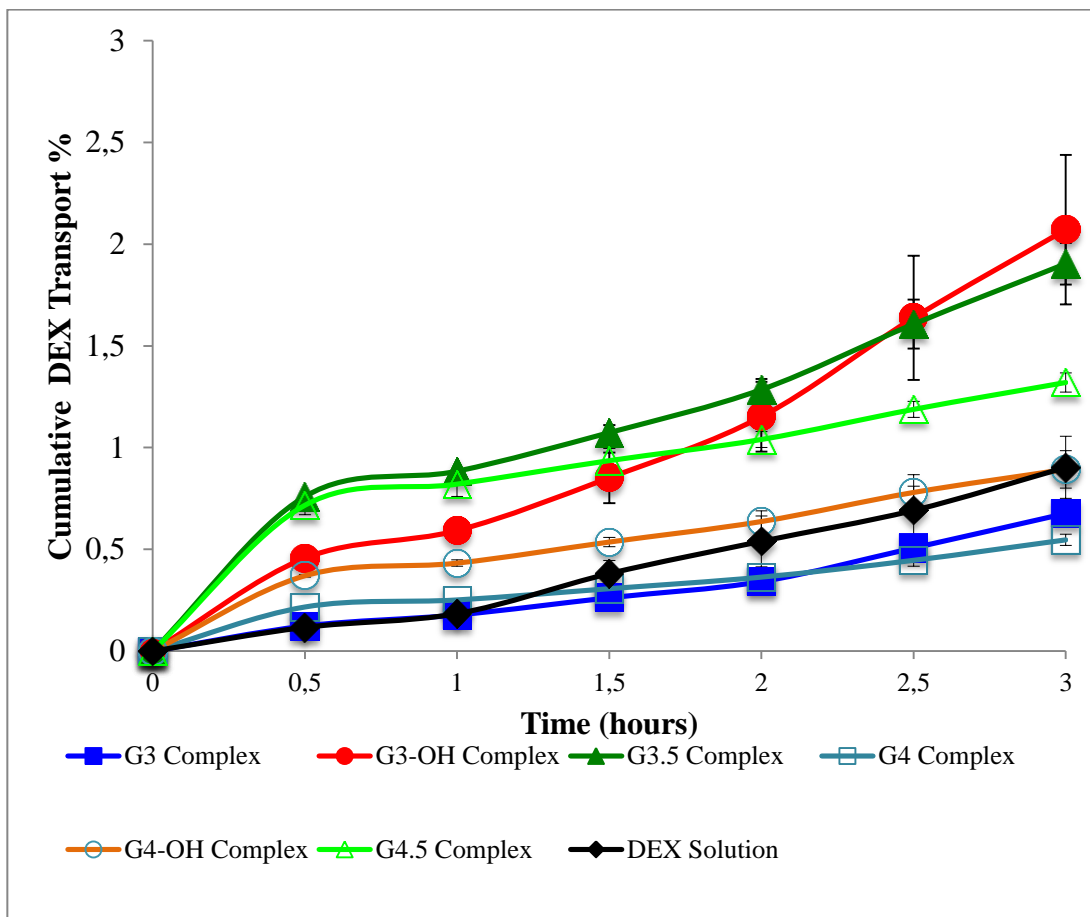


Figure 4.19. DEX transport levels across SCRPE from DEX-PAMAM complex formulations

Apparent permeability co-efficients (P_{app} , cm/s) were calculated and statistically compared using One Way ANOVA and Tukey HSD test for post-hoc analysis. Results were presented in Table 4.7. In terms of corneal transport, DEX permeation from DEX-PAMAM G4.5 complex was found significantly higher than the other formulations ($p < 0.05$). DEX transport from DEX-PAMAM G3.5 and G3-OH complex across SCRPE were found to be significantly higher than DEX solution and cationic dendrimer complexes, which were DEX-PAMAM G3 and G4. DEX-PAMAM G3 and G4 complex formulations showed statistically similar permeability both across cornea and SCRPE, which indicates that the transport was affected by the charge of the dendrimer.

Table 4.7. Apparent permeability co-efficients (Papp, cm/s) for ex vivo transport studies

Formulation	Papp (cm/s) Across Cornea	Papp (cm/s) Across SCRPE
G3 Complex	4.20 E ⁻⁰⁷	4.59 E ⁻⁰⁷
G3-OH Complex	5.46 E ⁻⁰⁷	14.8 E ⁻⁰⁷
G3.5 Complex	5.91 E ⁻⁰⁷	12.1 E ⁻⁰⁷
G4 Complex	4.08 E ⁻⁰⁷	3.43 E ⁻⁰⁷
G4-OH Complex	4.89 E ⁻⁰⁷	5.74 E ⁻⁰⁷
G4.5 Complex	7.72 E ⁻⁰⁷	7.94 E ⁻⁰⁷
DEX Solution	4.77 E ⁻⁰⁷	5.34 E ⁻⁰⁷

4.7. In Vivo Studies for DEX-PAMAM Complex Formulations

4.7.1. Ocular Distribution of DEX-PAMAM Complex Formulations

Following Topical Application

Tissue distribution of DEX following topical application of complex formulations, were given in Figure 4.20 to Figure 4.26. The results of the study showed that all DEX-PAMAM complex formulations have reached higher DEX concentrations in ocular tissues compared to plain DEX suspension. Anionic dendrimeric formulations have higher DEX concentrations in tissues, compatible with the ex vivo transport study results. All the differences in DEX amount in the tissues, except aqueous humor, have been found statistically significant ($p < 0.05$).

Drug cumulation was observed in conjunctiva, sclera and cornea tissues, which is expected since these tissues are the main barriers in ocular drug delivery. Significant increase in vitreous and retina-CRPE drug levels was achieved with DEX-PAMAM G3.5 and 4.5 complexes. It was also observed that cationic dendrimeric formulations showed more affinity to cornea than the other formulations.

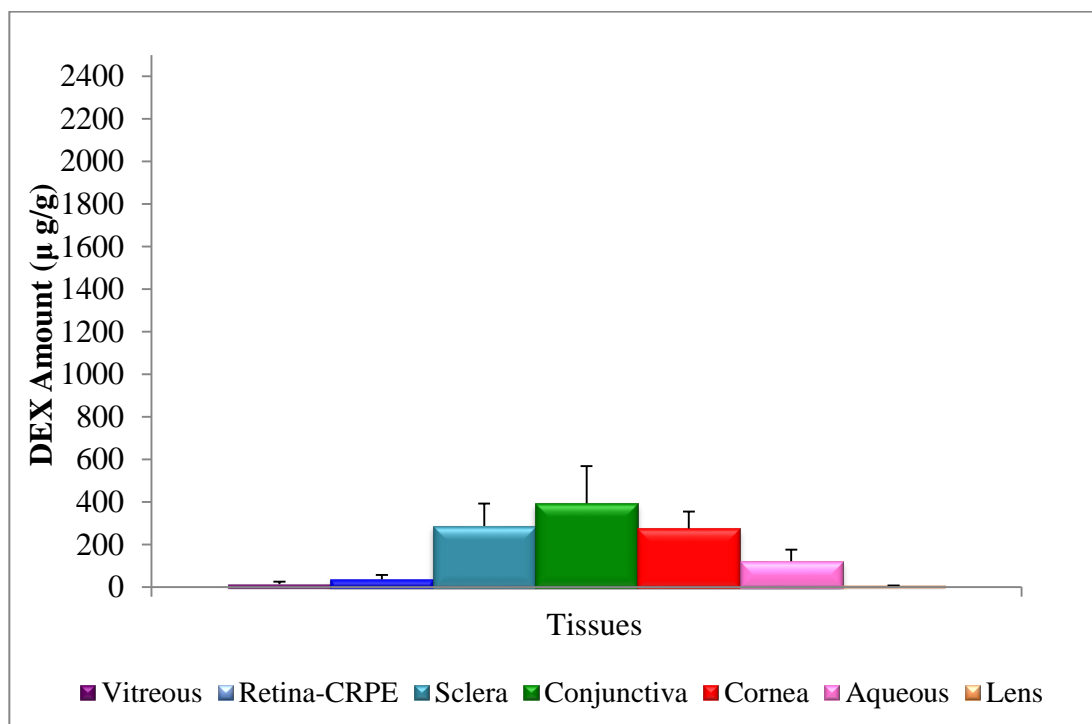


Figure 4.20. DEX ocular tissue distribution following topical application of DEX suspension

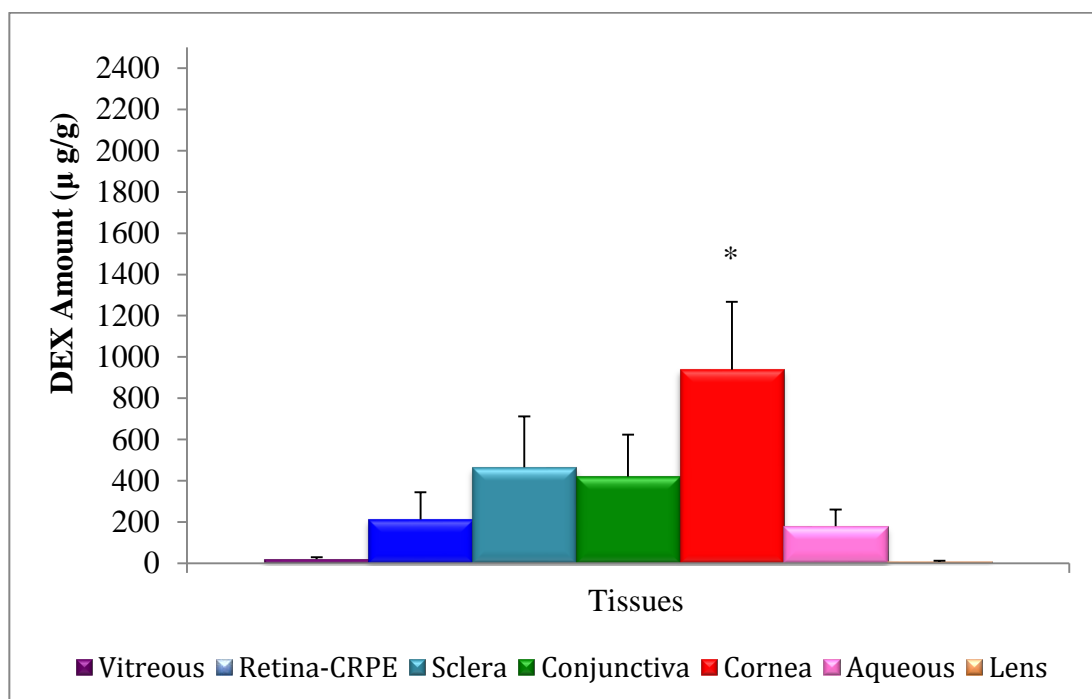


Figure 4.21. DEX ocular tissue distribution following topical application of DEX-PAMAM G3 complex (* Significantly different than DEX suspension, $p < 0.05$)

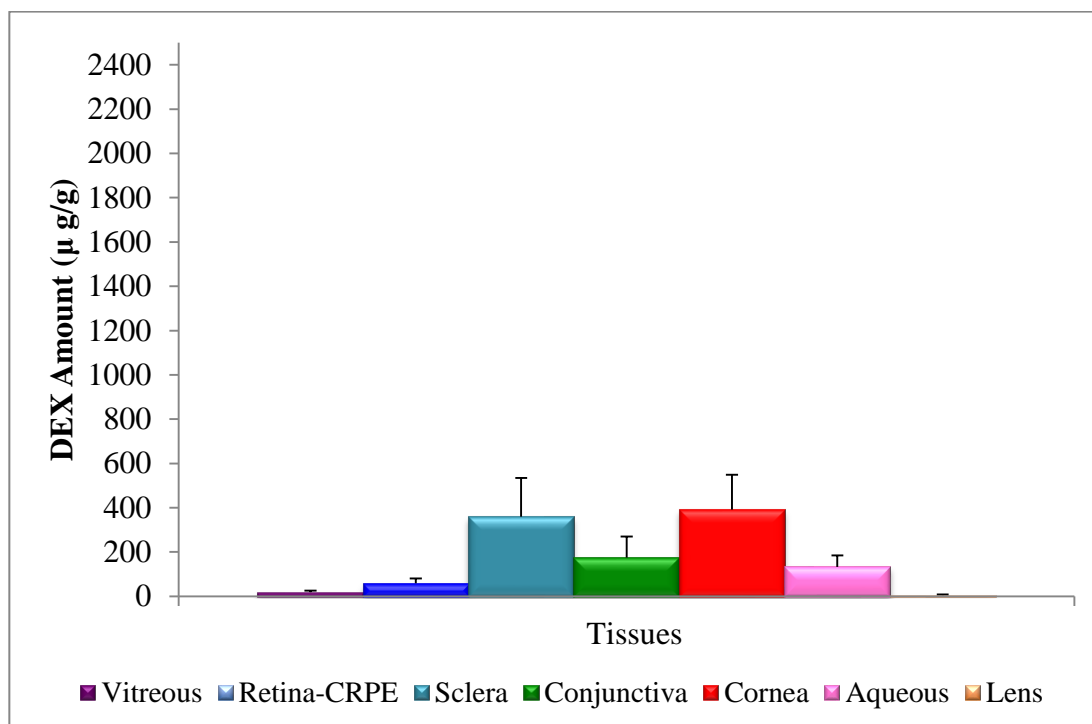


Figure 4.22. DEX ocular tissue distribution following topical application of DEX-PAMAM G4 complex (* Significantly different than DEX suspension, $p < 0.05$)

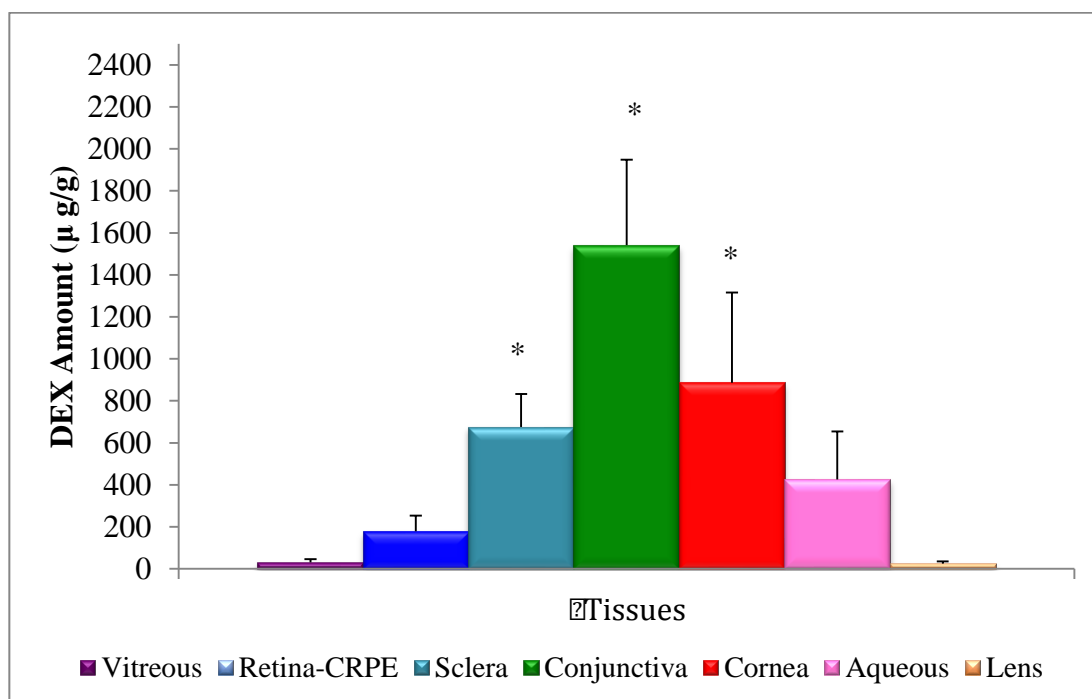


Figure 4.23. DEX ocular tissue distribution following topical application of DEX-PAMAM G3-OH complex (* Significantly different than DEX suspension, $p < 0.05$)

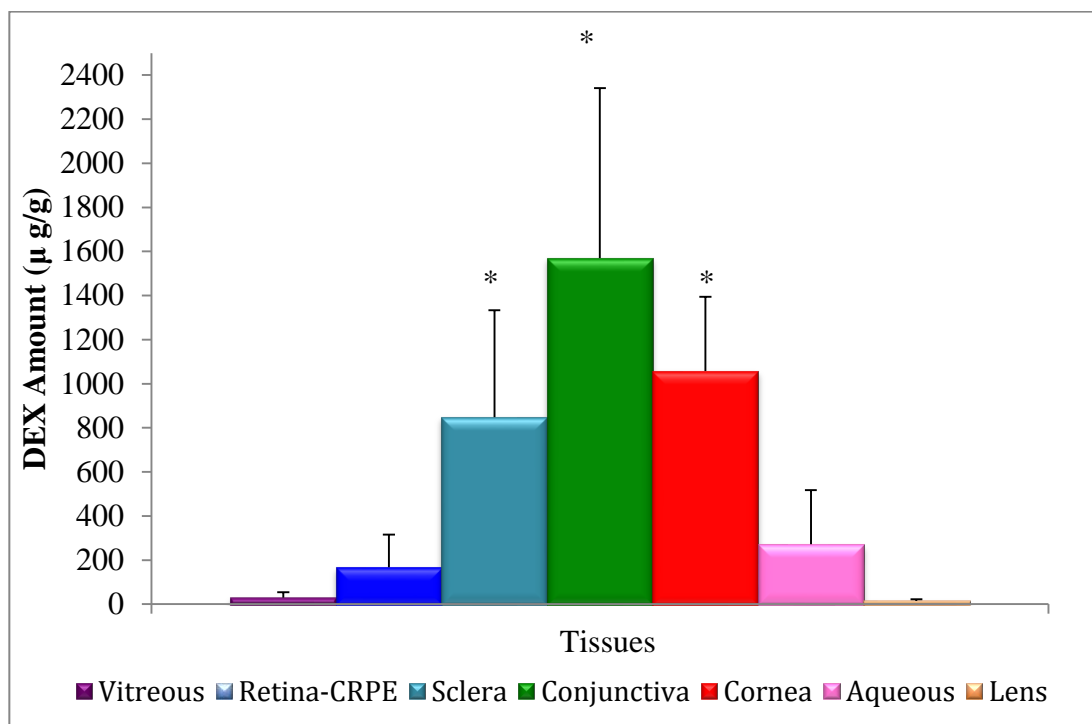


Figure 4.24. DEX ocular tissue distribution following topical application of DEX-PAMAM G4-OH complex (* Significantly different than DEX suspension, $p < 0.05$)

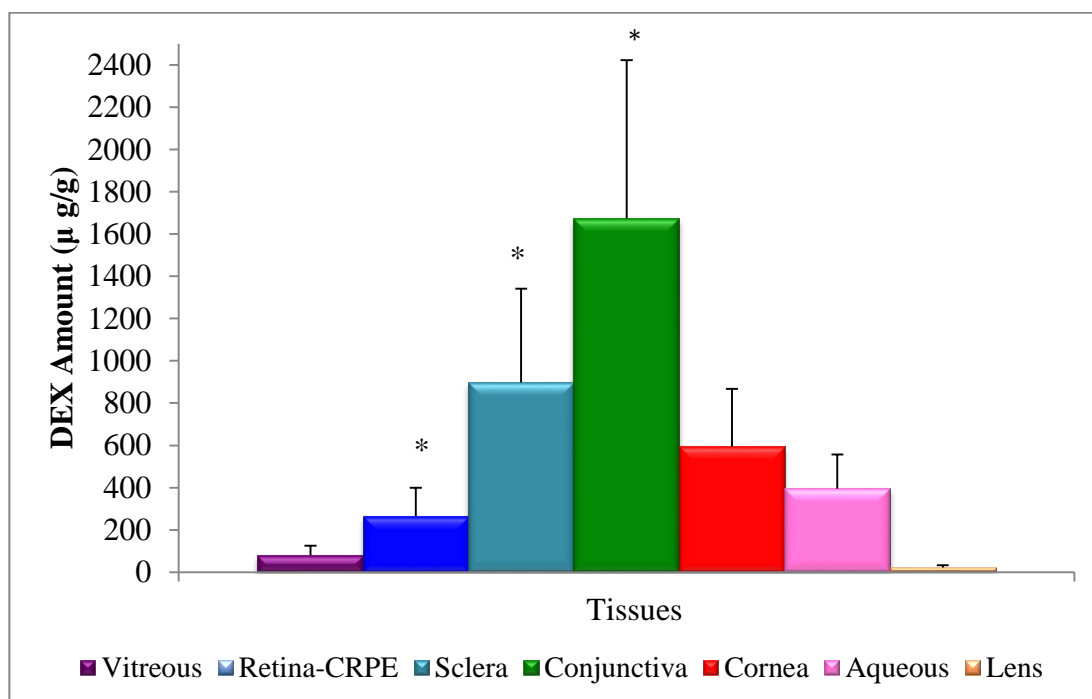


Figure 4.25. DEX ocular tissue distribution following topical application of DEX-PAMAM G3.5 complex (* Significantly different than DEX suspension, $p < 0.05$)

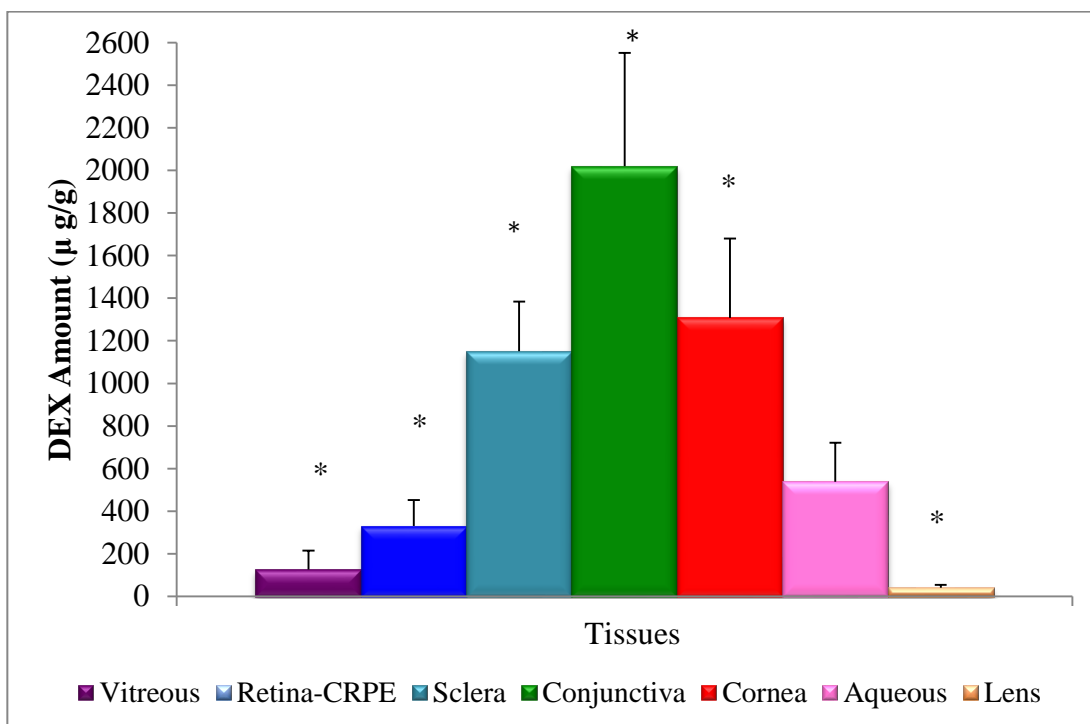


Figure 4.26. DEX ocular tissue distribution following topical application of DEX-PAMAM G4.5 complex (* Significantly different than DEX suspension, $p < 0.05$)

4.7.2. Ocular Distribution of DEX-PAMAM Complex Formulations Following Subconjunctival Application

Tissue distributions of DEX, following subconjunctival application of selected DEX-PAMAM complex formulations and DEX suspension were given in Figure 4.27 to Figure 4.29. The results of the study showed that DEX suspension stays at the injection site and the tissue distribution is poor. On the other hand, both anionic and cationic PAMAM dendrimer formulations (G4 and G4.5 complexes) enhanced tissue distribution of DEX where cationic formulation showed higher retinal tissue drug levels. The difference in retina-CRPE and sclera DEX levels, have been found statistically significant ($p < 0.05$) than DEX suspension. The difference between DEX-PAMAM G4 and 4.5 were found insignificant.

Drug cumulation was observed in conjunctiva and sclera tissues as expected due to the location of the injections.

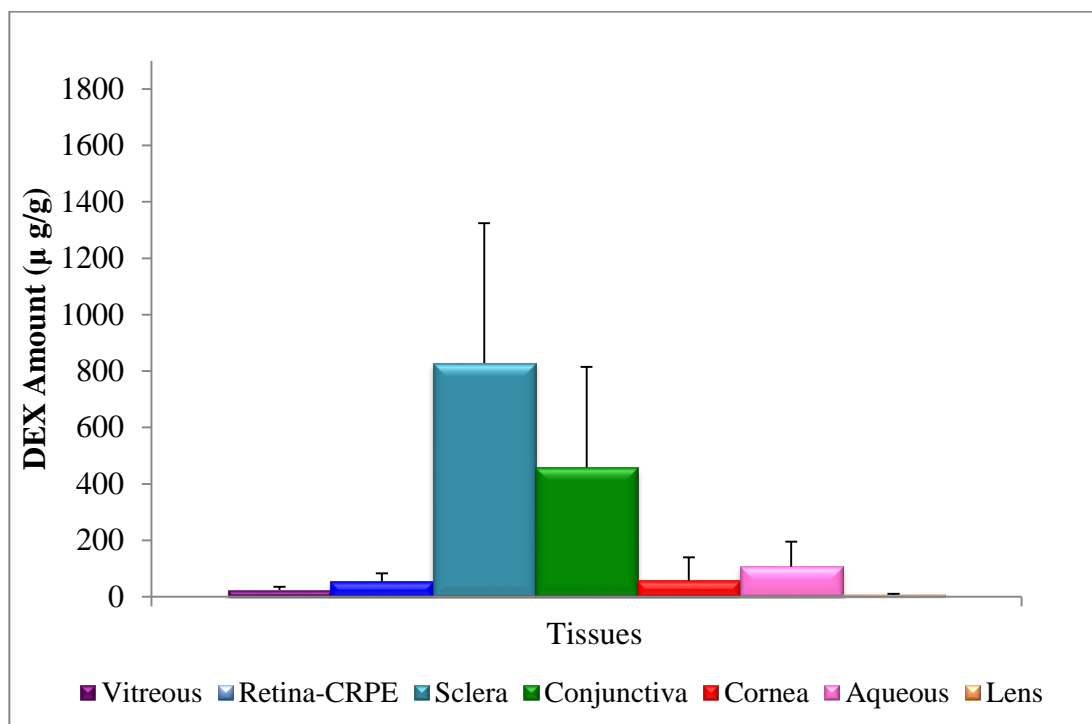


Figure 4.27. DEX ocular tissue distribution following subconjunctival application of DEX suspension

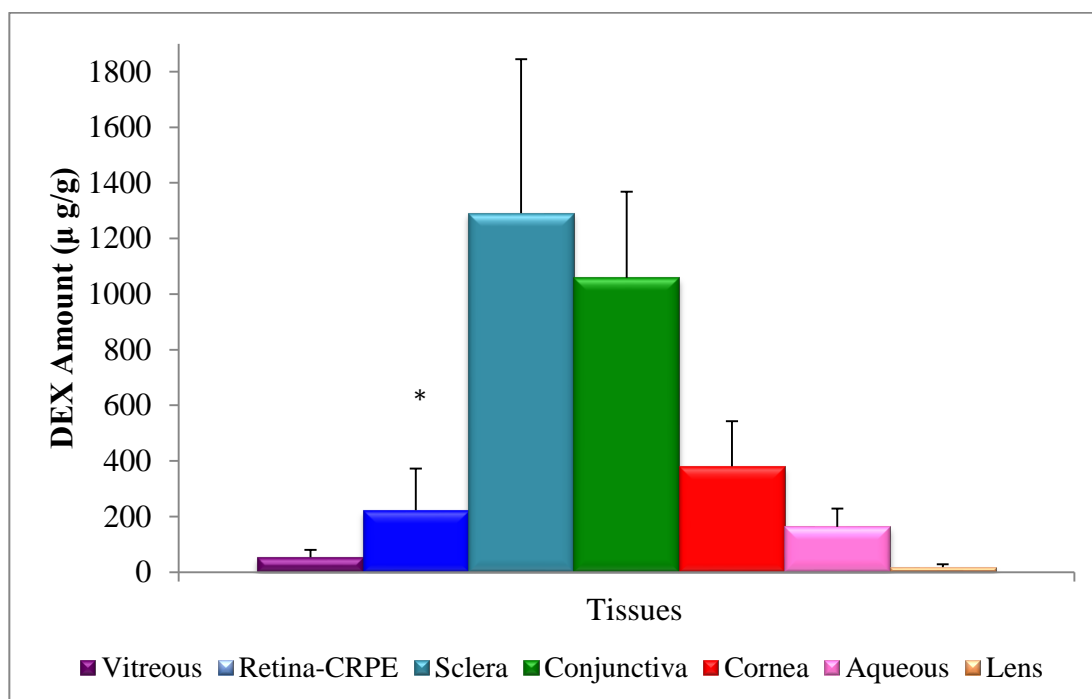


Figure 4.28. DEX ocular tissue distribution following subconjunctival application of DEX-PAMAM G4 complex (* Significantly different than DEX suspension, $p < 0.05$)

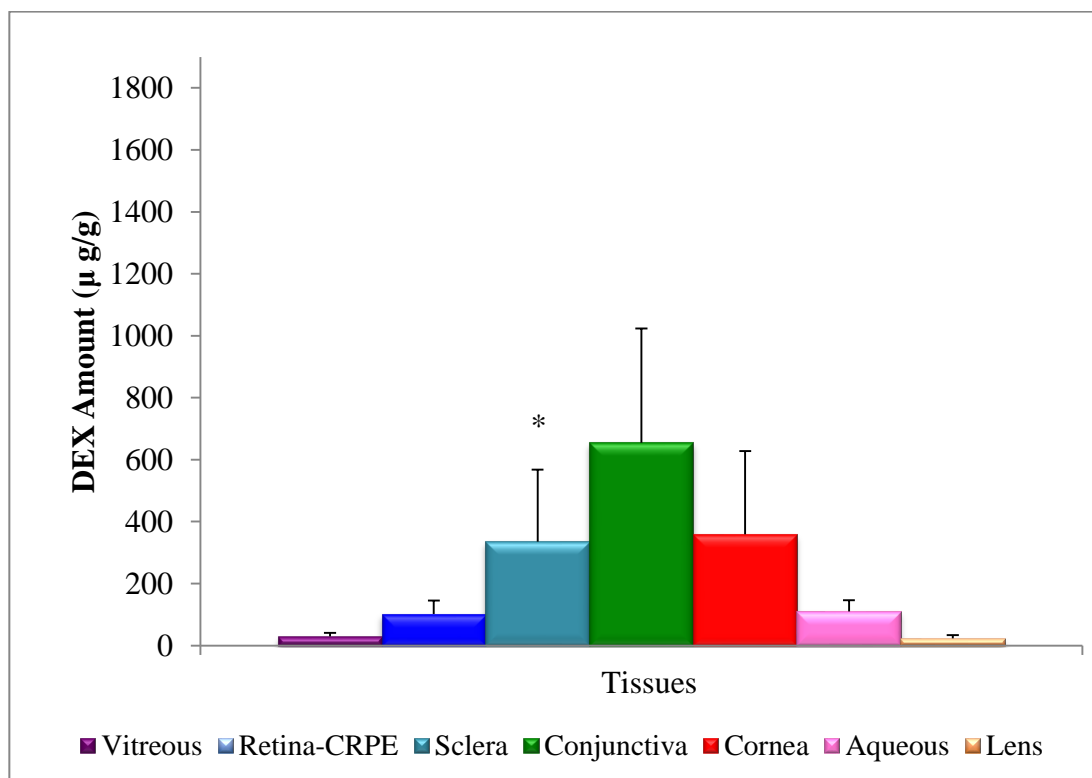


Figure 4.29. DEX ocular tissue distribution following subconjunctival application of DEX-PAMAM G4.5 complex (* Significantly different than DEX suspension, $p < 0.05$)

4.8. In Vivo Studies for DEX-PAMAM Conjugate Formulations

4.8.1. Fluorotron Analysis for DEX-PAMAM Conjugate Formulations Following Subconjunctival Application

Fluorotron analysis results indicated that fluorescence levels following subconjunctival injection of DEX-PAMAM G3.5 and G4.5 conjugates were similar between two formulations and the initial fluorescence level was found very close to the background measurements. These results showed that conjugate levels in vitreous and retina following subconjunctival injection were not high enough to compare the formulations or the length of duration in the eye.

Fluorescence signals in the rats' eyes following subconjunctival application of DEX-PAMAM conjugates were given in Figure 4.30 and Figure 4.31. Vitreous (data point 45) and retina-choroid (data points 23-25) fluorescence levels were also calculated based on the fluorotron results and presented in Figure 4.32 and Figure 4.33.

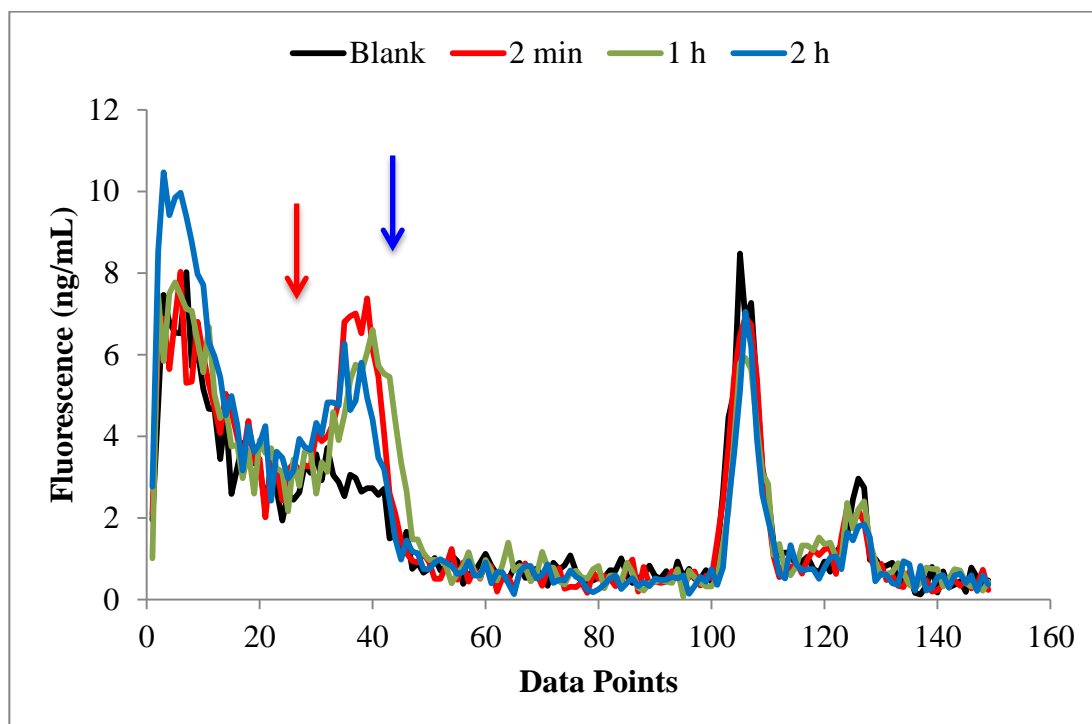


Figure 4.30. Fluorescence signals following subconjunctival application of DEX-PAMAM G3.5 conjugate (Red arrow = retina-choroid, Blue arrow = vitreous)

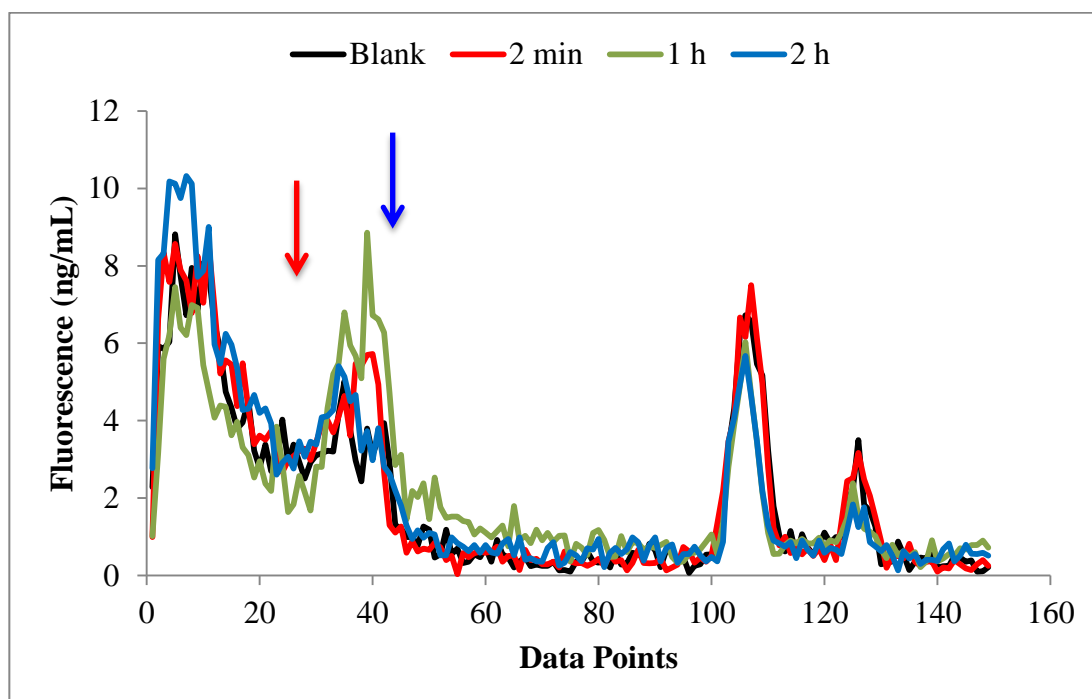


Figure 4.31. Fluorescence signals following subconjunctival application of DEX-PAMAM G4.5 conjugate (Red arrow = retina-choroid, Blue arrow = vitreous)

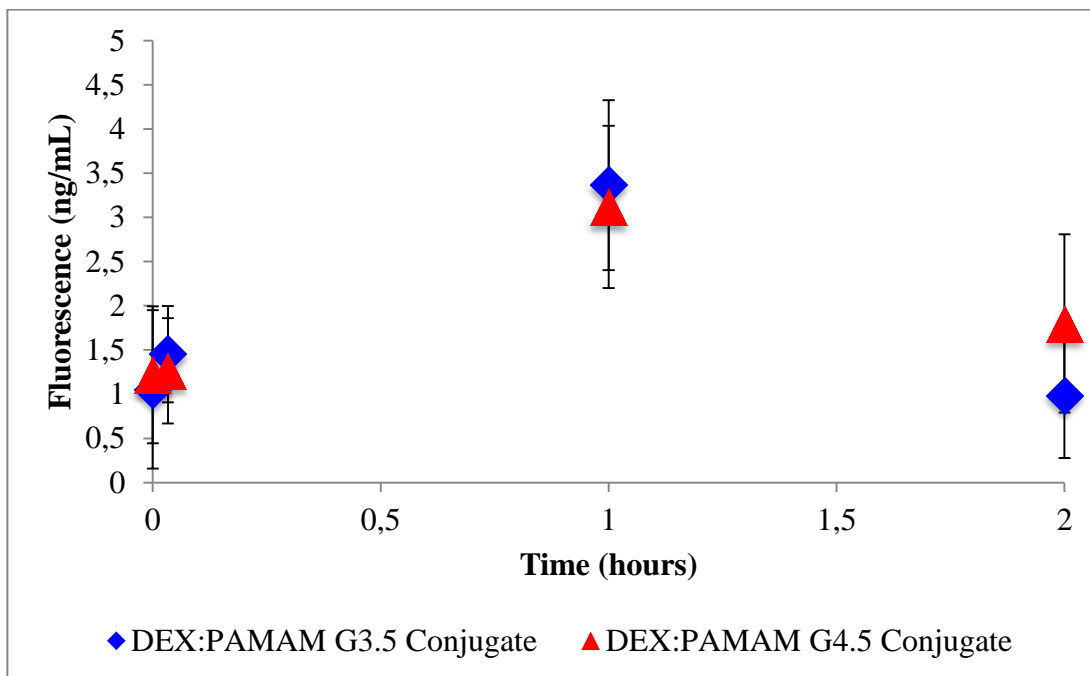


Figure 4.32. Fluorescence levels in vitreous following subconjunctival injection of DEX-PAMAM conjugates

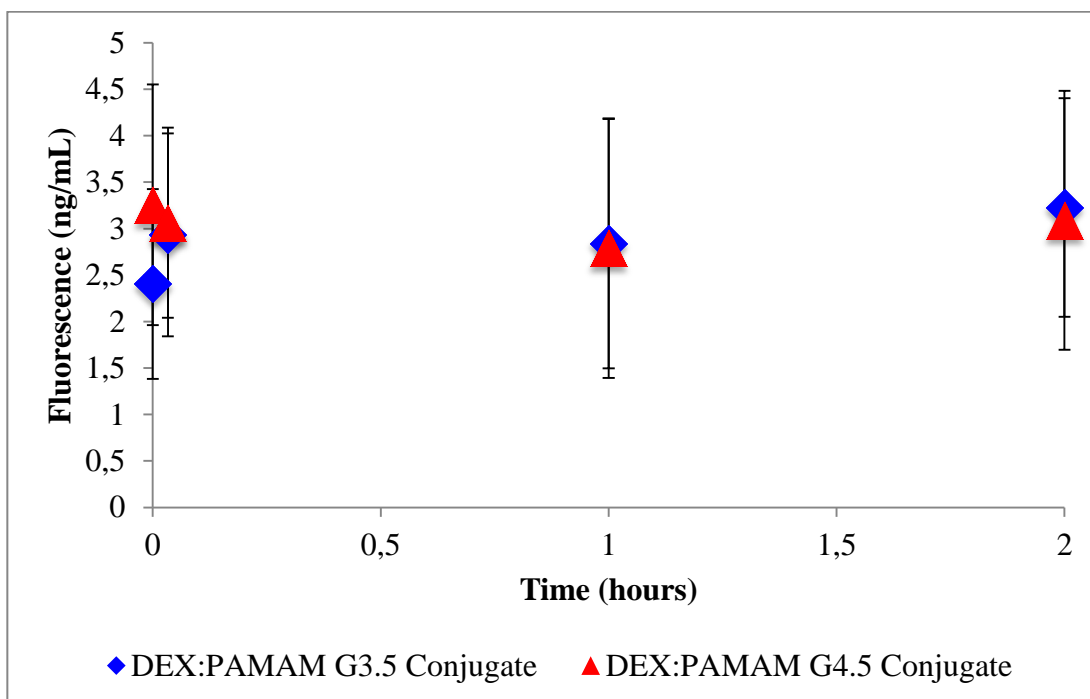


Figure 4.33. Fluorescence levels in retina-choroid following subconjunctival injection of DEX-PAMAM conjugates

4.8.2. Ocular Distribution of DEX-PAMAM Conjugate Formulations Following Subconjunctival Application

Tissue distribution of DEX-PAMAM conjugates following subconjunctival application of conjugate formulations, were given in Figure 4.34 and Figure 4.35. The results of ocular tissue distribution study following subconjunctival application showed that less than 10% of the injected formulations left in the tissues 2 hours after the injection. But the distribution of the formulations was enhanced when compared to DEX suspension (Figure 4.27) and there were significant amount of signal in retina and vitreous, even the majority of the signal was in the sclera and choroid, which were closer to the injection site.

The difference in conjugate levels in the tissues was found statistically insignificant for the two DEX-PAMAM conjugate formulations. The only significant difference was found in cornea levels where DEX-PAMAM G4.5 cornea levels were found higher than DEX-PAMAM G3.5 conjugate ($p < 0.05$).

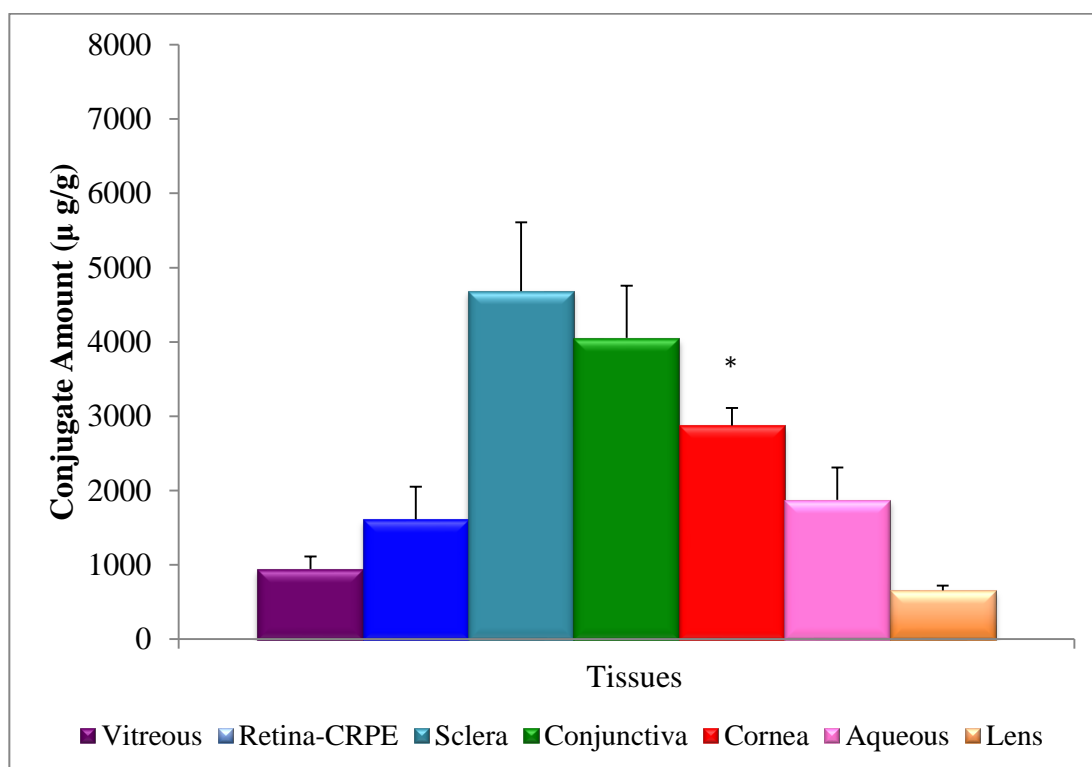


Figure 4.34. DEX-PAMAM G3.5 conjugate tissue distribution 2 hours after subconjunctival application (* Significantly different from G4.5 conjugate, $p < 0.05$)

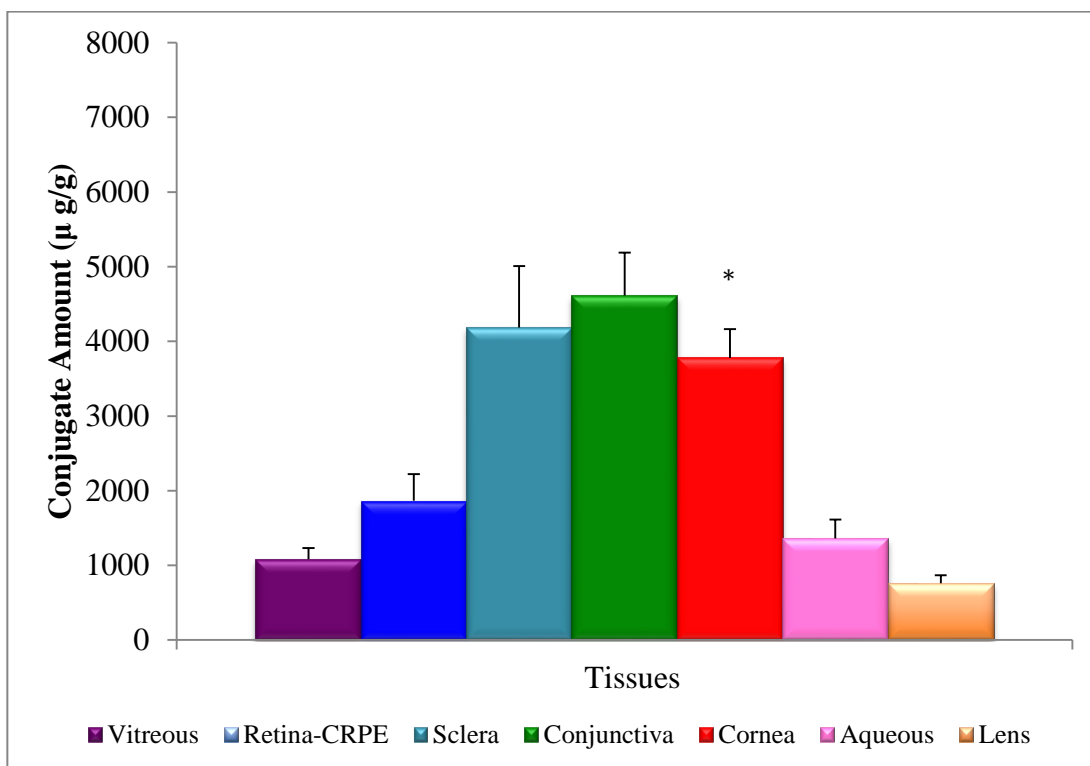


Figure 4.35. DEX-PAMAM G4.5 conjugate tissue distribution 2 hours after subconjunctival application (* Significantly different from G3.5 conjugate, $p < 0.05$)

4.8.3. Fluorotron Analysis for DEX-PAMAM Conjugate Formulations Following Intravitreal Application

Fluorescence signals in the rats' eyes following intravitreal application of DEX-PAMAM conjugates were given in Figure 4.36 and Figure 4.37. Vitreous (data point 45) and retina-choroid (data points 23-25) fluorescence levels were also calculated based on the fluorotron results and presented in Figure 4.38 and Figure 4.39.

Both initial and following time point measurements indicated that PAMAM G3.5 conjugate has higher fluorescent levels following 1 mg/mL intravitreal conjugate injection. The results of this study also showed that both formulations were present in vitreous for 24 hours with a decreasing level.

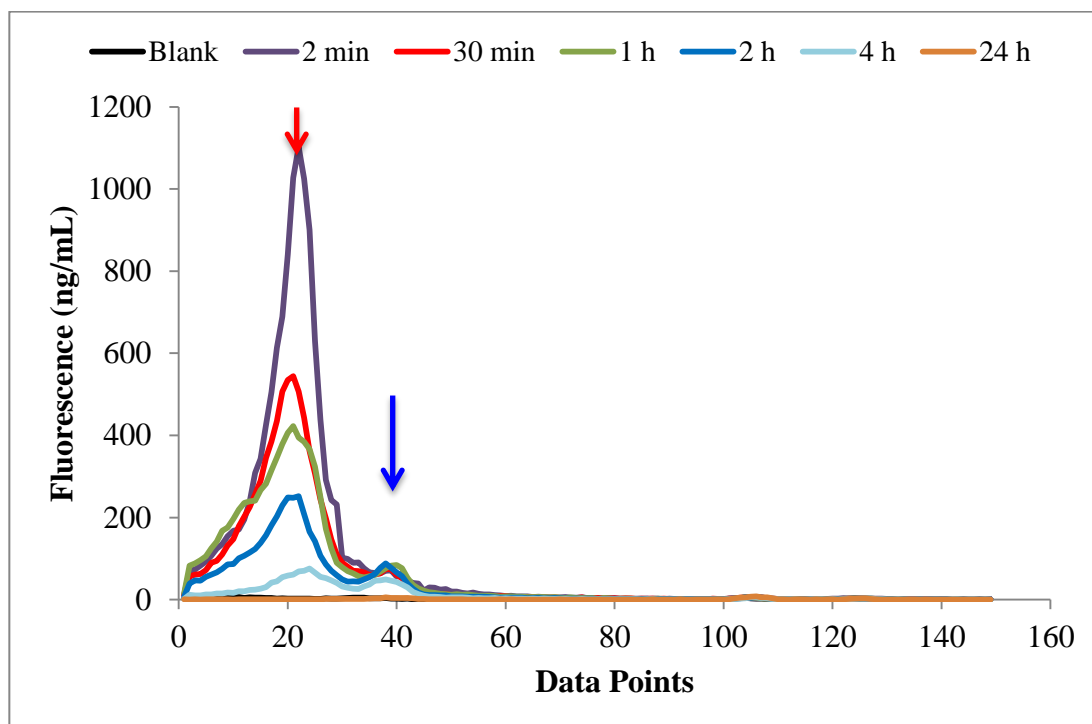


Figure 4.36. Fluorescence signals following intravitreal application of DEX-PAMAM G3.5 conjugate (Red arrow = retina-choroid, Blue arrow = vitreous)

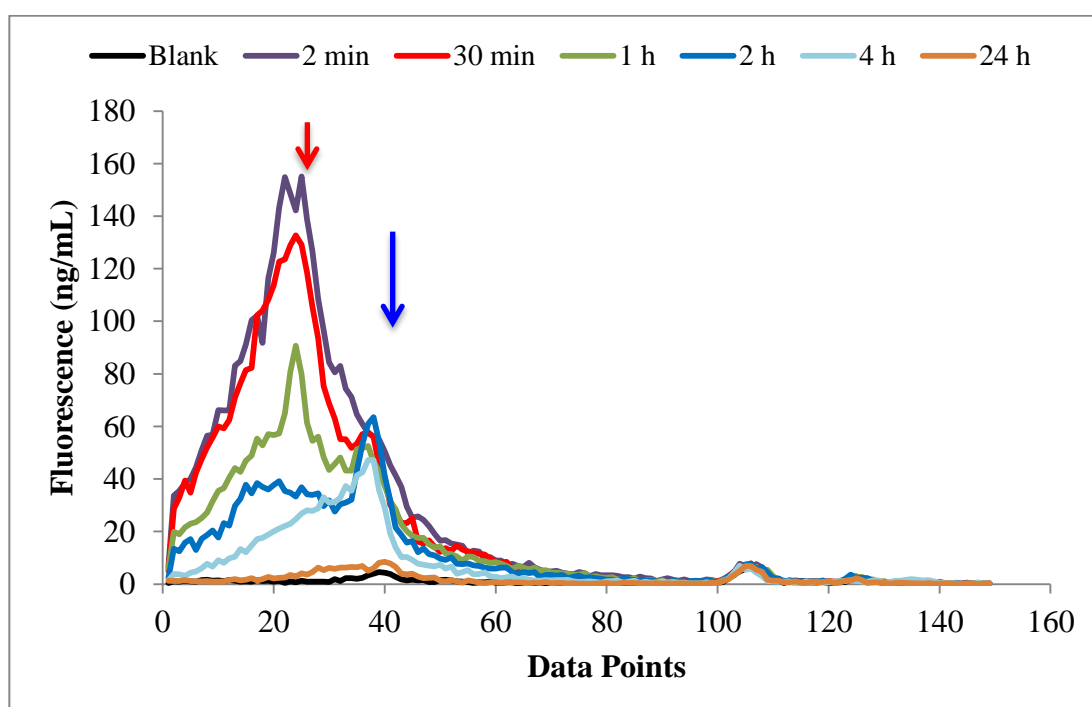


Figure 4.37. Fluorescence signals following intravitreal application of DEX-PAMAM G4.5 conjugate (Red arrow = retina-choroid, Blue arrow = vitreous)

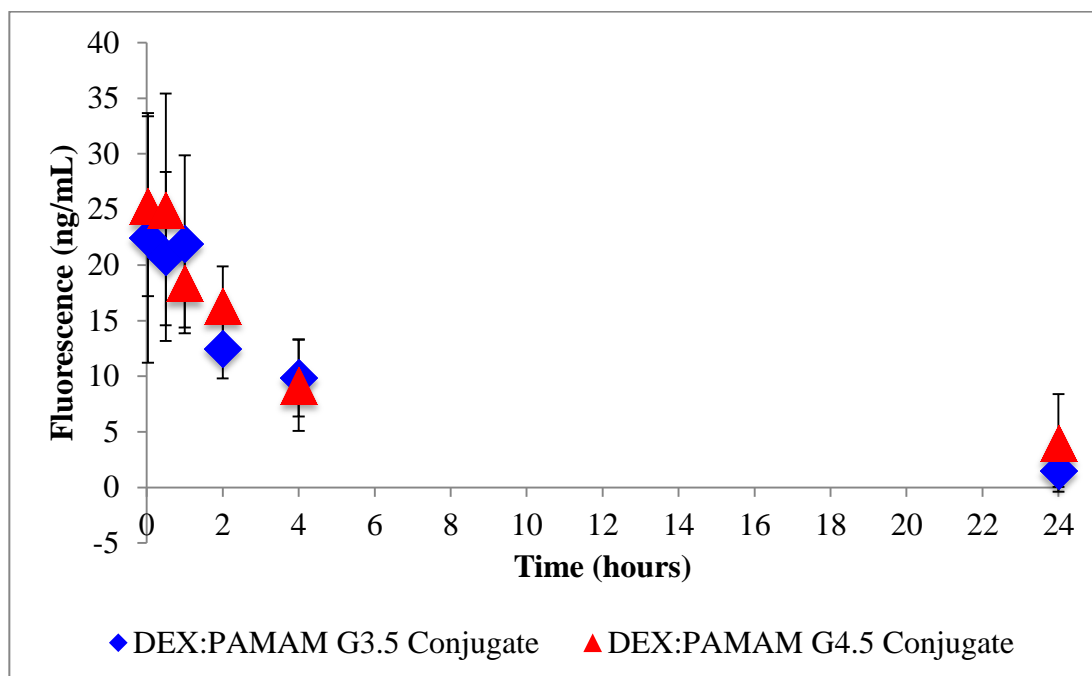


Figure 4.38. Fluorescence levels in vitreous following intravitreal injection of DEX-PAMAM conjugates

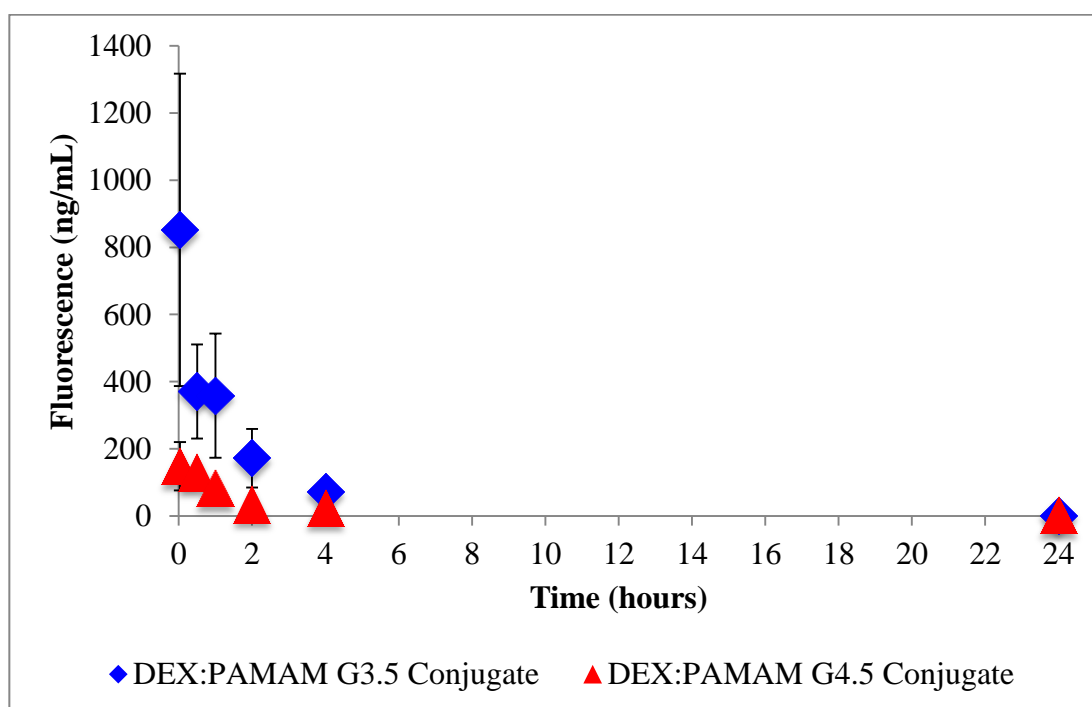


Figure 4.39. Fluorescence levels in retina-choroid following intravitreal injection of DEX-PAMAM conjugates

4.8.4. Ocular Distribution of DEX-PAMAM Conjugate Formulations Following Intravitreal Application

Tissue distribution of DEX-PAMAM conjugates following intravitreal application of the formulations, were given in Figure 4.40 and Figure 4.41. The results of ocular tissue distribution study following intravitreal application showed that, 24 hours after injection tissue conjugate levels in total ocular tissues were around 33% for DEX-PAMAM G3.5 conjugates while it was around 15% for DEX-PAMAM G4.5 conjugates. It was also observed that vitreous and retina-CRPE tissues had higher amount of conjugate levels comparing to the other ocular tissues, which is a goal to be achieved in retinal drug delivery.

Lower vitreous level of DEX-PAMAM G4.5 indicated that this formulation is getting cleared faster than DEX-PAMAM G3.5 conjugates, since the drug has been injected into the vitreous. The difference between the formulations was found significant except for sclera and aqueous humor tissues respectively ($p < 0.05$).

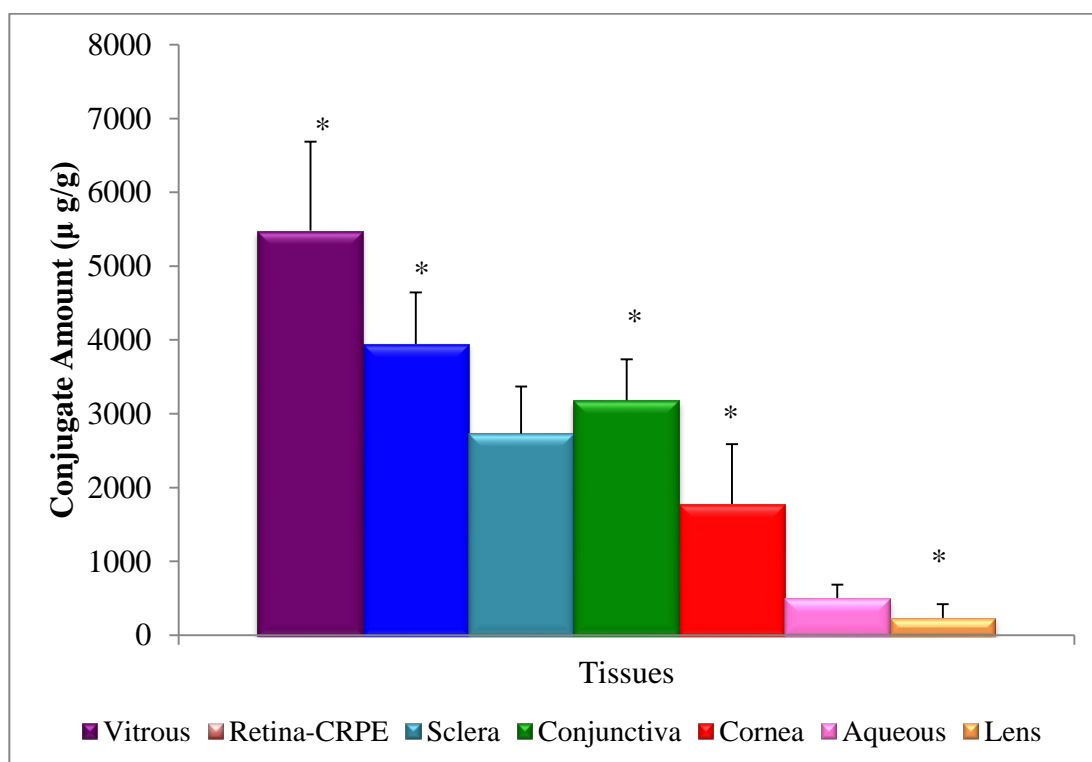


Figure 4.40. DEX-PAMAM G3.5 conjugate tissue distribution 24 hours after intravitreal application (* Significantly different from G4.5 conjugate, $p < 0.05$)

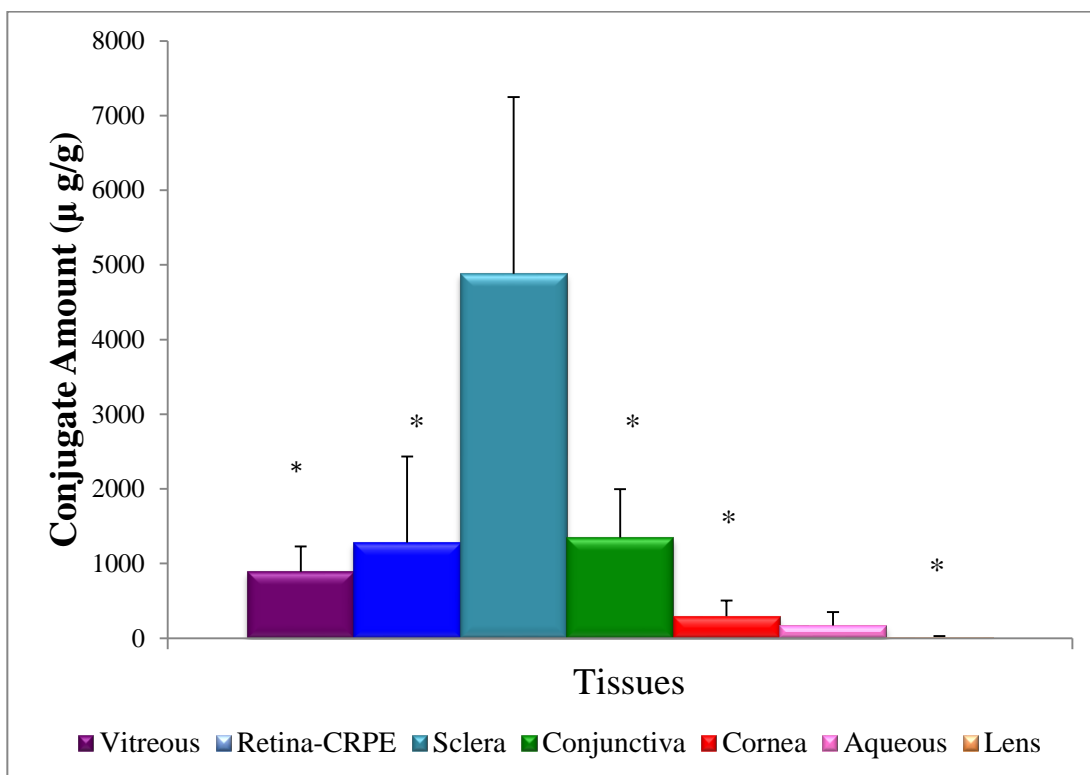


Figure 4.41. DEX-PAMAM G4.5 conjugate tissue distribution 24 hours after intravitreal application (* Significantly different from G3.5 conjugate, $p < 0.05$)

4.8.5. Fluorotron Analysis for Blank PAMAM Dendrimers Following Intravitreal Application

In this study plain PAMAM dendrimers without DEX conjugation were tested to compare the effect of the charge and generation of dendrimers. Comparison of the dendrimer formulations showed that cationic dendrimers (PAMAM G3 and PAMAM G4) has higher tissue levels, and stayed longer than anionic dendrimers (PAMAM G3.5 and PAMAM G4.5). 24 hours after injection, there is still some fluorescence for all dendrimers, even the majority of the dendrimer was cleared. Fluorescence levels in retina-choroid were found to be higher than vitreous, which indicates that dendrimers reached the target tissue.

Fluorescence signals in the rats' eyes following intravitreal application of PAMAM dendrimers were given in Figure 4.42 to Figure 4.45. Vitreous (data point 45) and retina-choroid (data points 23-25) fluorescence levels were also calculated based on the fluorotron results and presented in Figure 4.46 and Figure 4.47.

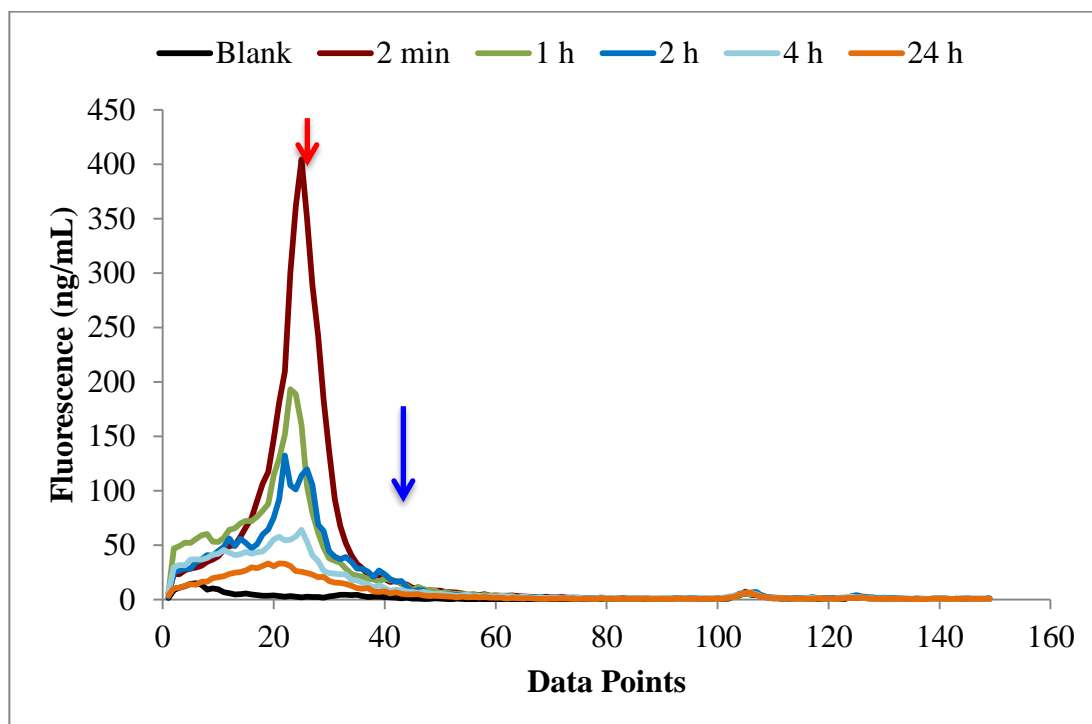


Figure 4.42. Fluorescence signals following intravitreal application of Alexa 488 labeled blank PAMAM G3 dendrimer (Red arrow = retina-choroid, Blue arrow = vitreous)

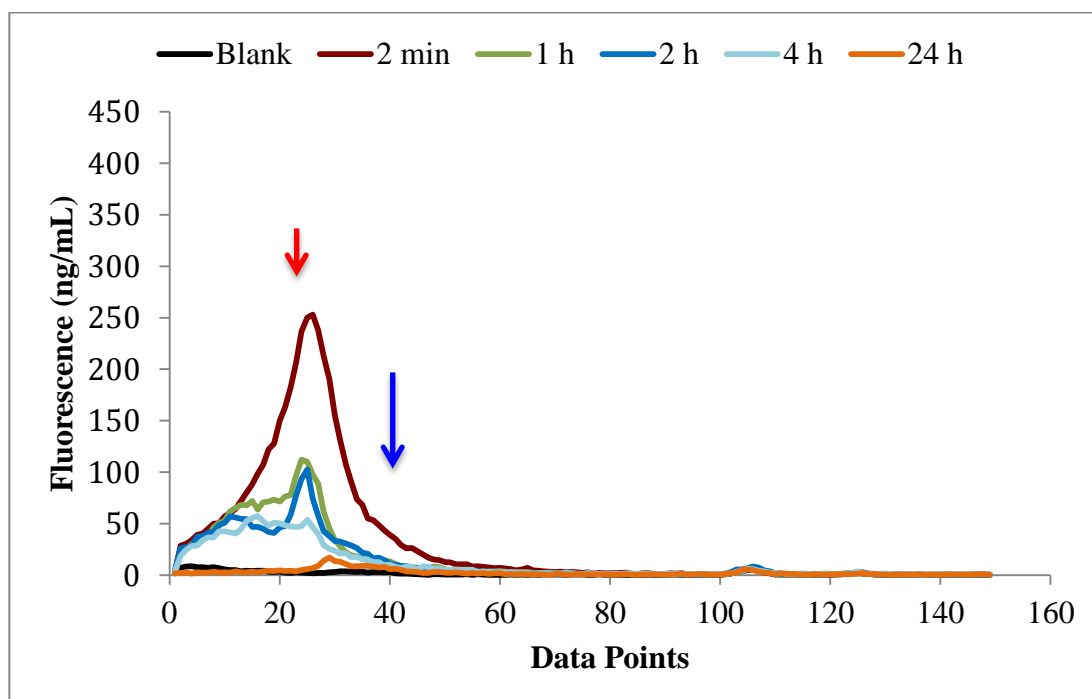


Figure 4.43. Fluorescence signals following intravitreal application of Alexa 488 labeled blank PAMAM G4 dendrimer (Red arrow = retina-choroid, Blue arrow = vitreous)

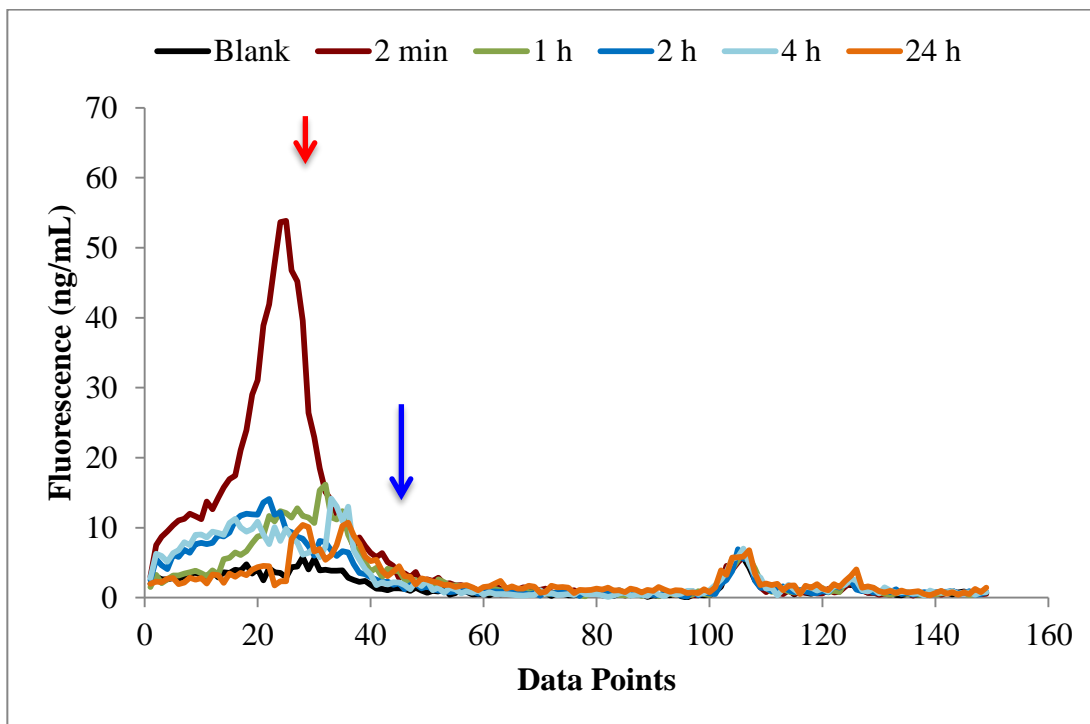


Figure 4.44. Fluorescence signals following intravitreal application of Alexa 488 labeled blank PAMAM G3.5 dendrimer (Red arrow = retina-choroid, Blue arrow = vitreous)

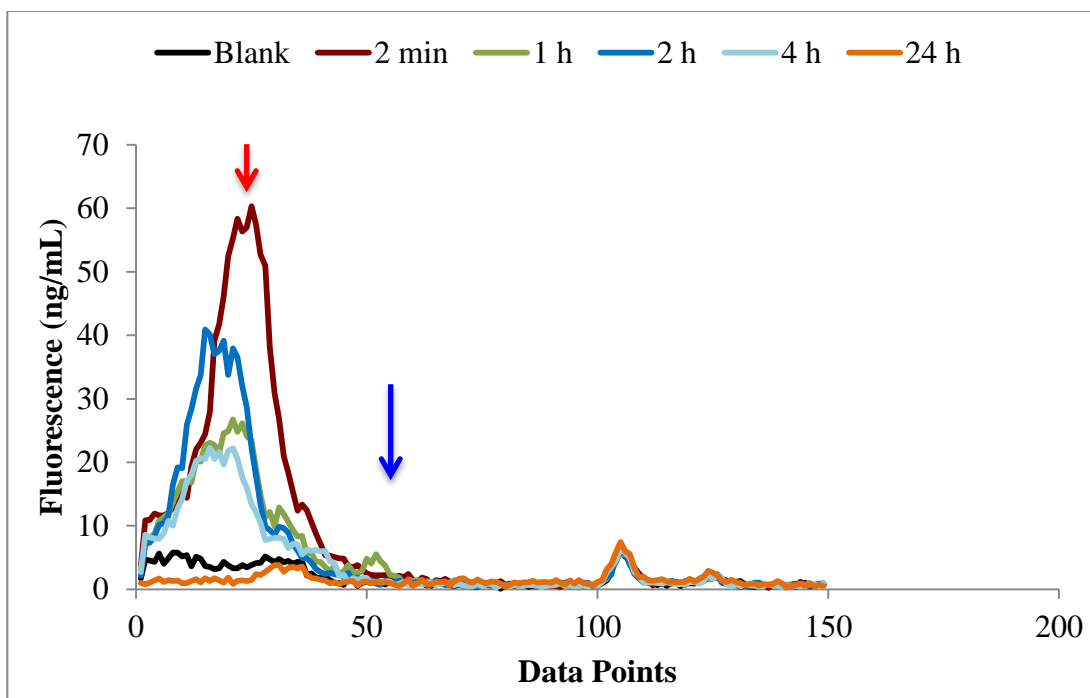


Figure 4.45. Fluorescence signals following intravitreal application of Alexa 488 labeled blank PAMAM G4.5 dendrimer (Red arrow = retina-choroid, Blue arrow = vitreous)

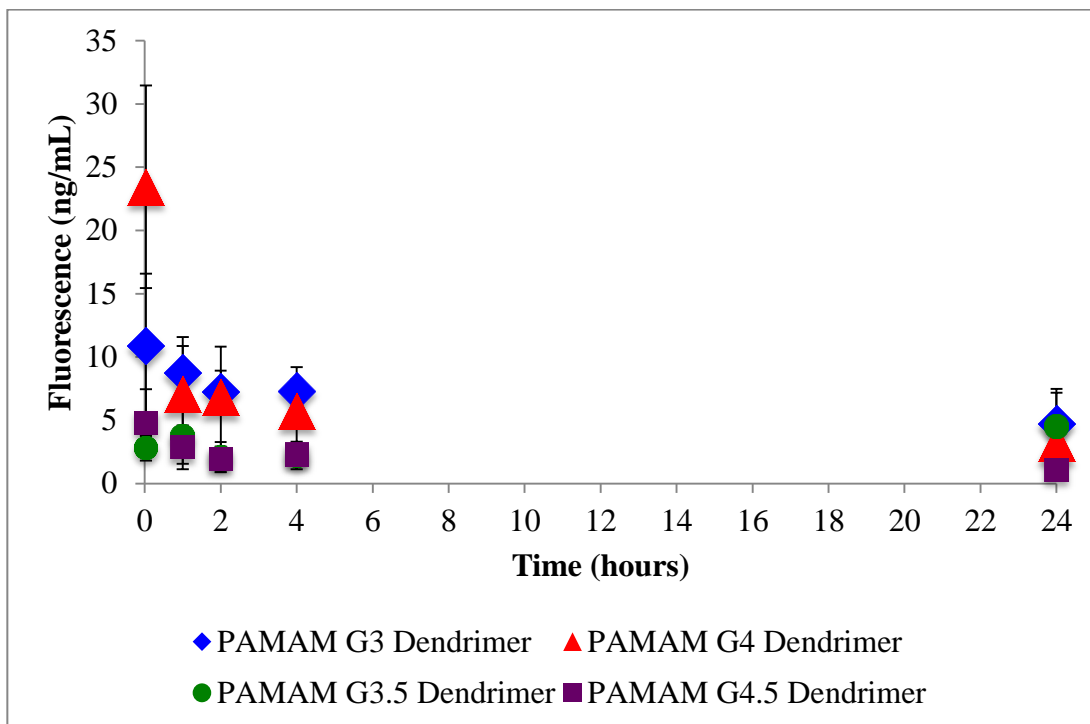


Figure 4.46. Fluorescence levels in vitreous following intravitreal injection of Alexa 488 labeled blank PAMAM dendrimers

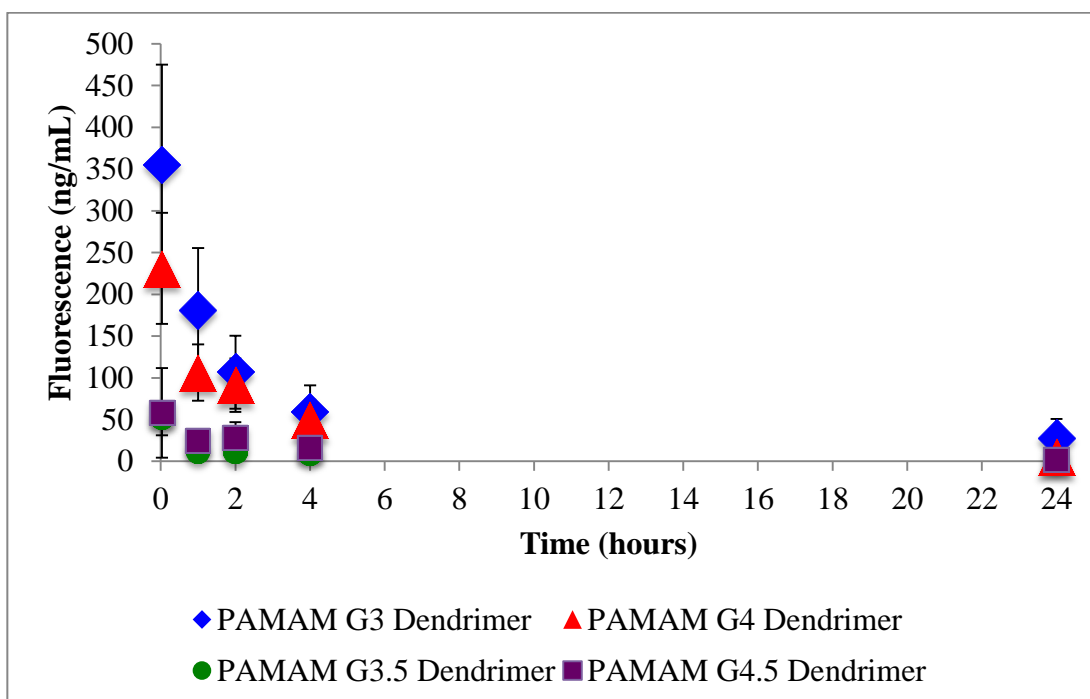


Figure 4.47. Fluorescence levels in retina-choroid following intravitreal injection of Alexa 488 labeled blank PAMAM dendrimers

4.8.6. Ocular Distribution of Blank PAMAM Dendrimers Formulations Following Intravitreal Application

Tissue distribution of PAMAM dendrimers following intravitreal application of Alexa 488 labeled dendrimers, were given in Figure 4.48 to Figure 4.51 as percentage of the injected dendrimer amounts. The results of ocular tissue distribution study following intravitreal application indicated that cationic dendrimers (PAMAM G3 and G4) distribute in the front of the eye and cumulates in lens and cornea tissues, while anionic dendrimers (PAMAM G3.5 and 4.5) stay mostly in the back of the eye or get cleared from the eye.

96.8% of PAMAM G3, 99.7% of PAMAM G4, 28.8% of PAMAM G3.5 and 44.6% of PAMAM G4.5 were found distributed in the ocular tissues 24 hours after the injection respectively. These results indicated that cationic dendrimers (PAMAM G3 and G4) were still in the ocular tissues 24 hours after the injection and anionic dendrimers (PAMAM G3.5 and 4.5) have been cleared faster. However it was also observed that cationic dendrimers were traveled to the front of the eye, which indicates that they have higher affinity for tissues like cornea, lens and aqueous rater than vitreous and retina.

The differences in all tissue dendrimer levels were found statistically significant, except sclera tissue levels ($p < 0.05$). All tissue dendrimer levels except sclera levels were found statistically higher for cationic dendrimers. According to these results, in further studies cationic dendrimers might be considered for extended stay rather than anionic ones.

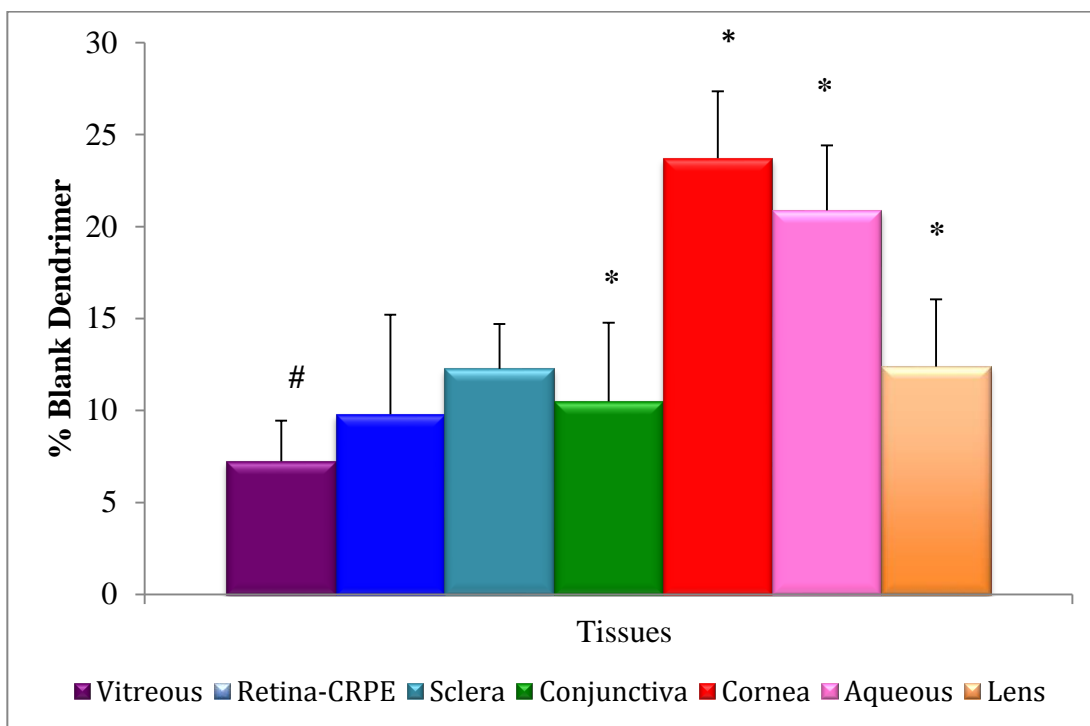


Figure 4.48. Blank PAMAM G3 dendrimer tissue distribution 24 hours after intravitreal application (* Significantly higher, # Significantly lower, $p < 0.05$)

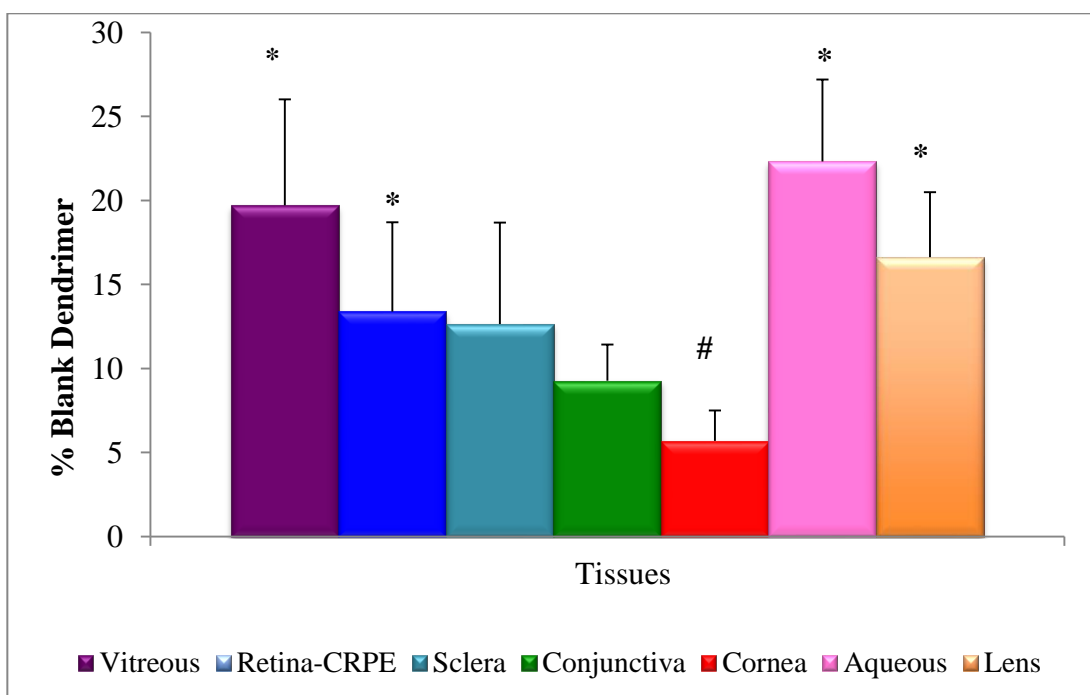


Figure 4.49. Blank PAMAM G4 dendrimer tissue distribution 24 hours after intravitreal application (* Significantly higher, # Significantly lower, $p < 0.05$)

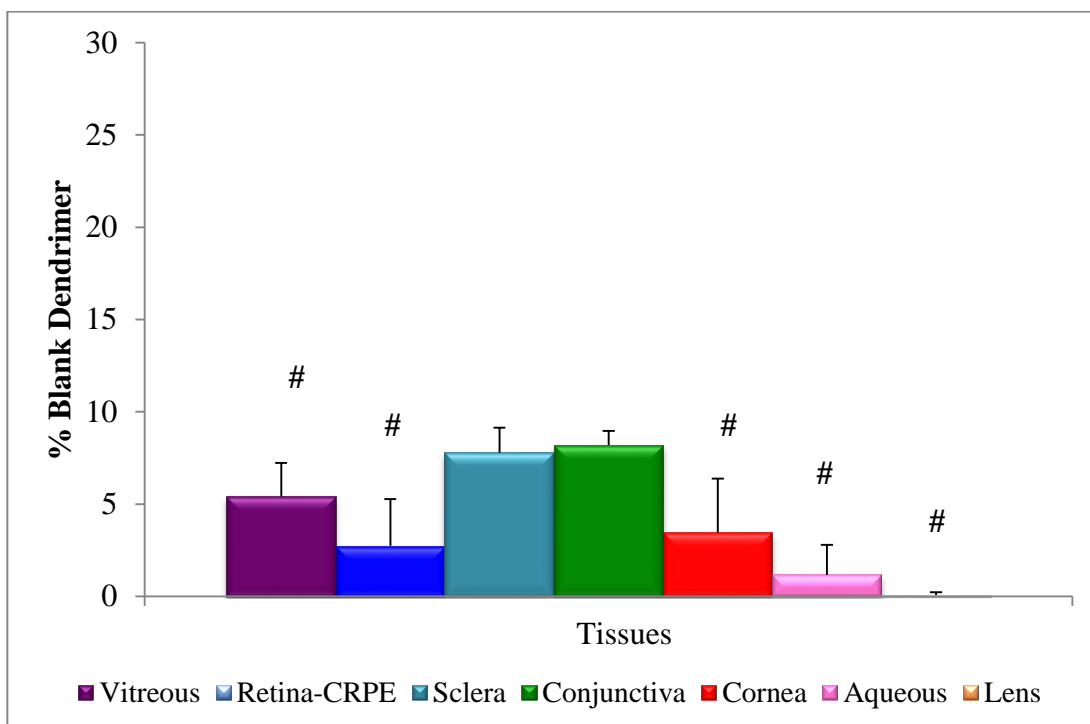


Figure 4.50. Blank PAMAM G3.5 dendrimer tissue distribution 24 hours after intravitreal application (* Significantly higher, # Significantly lower, $p < 0.05$)

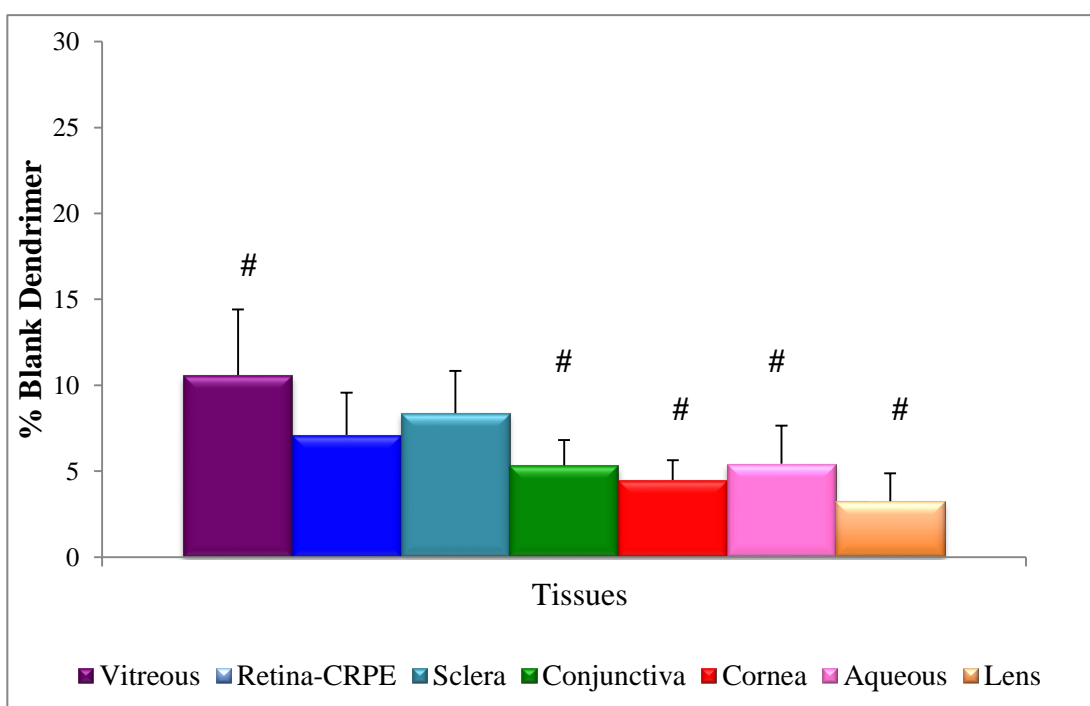


Figure 4.51. Blank PAMAM G4.5 dendrimer tissue distribution 24 hours after intravitreal application (* Significantly higher, # Significantly lower, $p < 0.05$)

5. DISCUSSION

Many attempts have been made to enhance retinal delivery of drugs, since the treatment of posterior segment diseases like diabetic retinopathy has been a challenge. Topical, systemic, intravitreal and periorbital routes have been tested to deliver pharmaceuticals to the back of the eye. However the tight junctions of blood retinal barrier (BRB) restrict the entry of systemically administered drugs into the retina. High vitreal drug concentrations are required in the treatment of posterior segment diseases and this can be made possible only with the invasive applications (147,148) .

Corticosteroids like dexamethasone, fluocinolone, prednisolone, and triamcinolone or their derivatives have been shown to be beneficial in treating several disorders of the eye, including diabetic retinopathy. Currently, there are several FDA approved corticosteroid products for the back of the eye such as; Ozurdex™ (biodegradable DEX implant; macular edema; 0.7 mg; Allergan), Retisert™ (non-biodegradable fluocinolone acetonide implant; uveitis; 0.59 mg; Bausch & Lomb), and Trivaris™ (Allergan)/Triesence™ (Alcon) (triamcinolone acetonide suspension; uveitis and topical corticosteroid responsive inflammatory conditions; 4 mg) (149) . However, high dose corticosteroid use is associated with risks including ocular hypertension, intraocular pressure and lens opacification and it is not possible to end the treatment when they're surgically implanted (150) . Thus it is still required to develop a formulation that can deliver required amount of drug to reach the therapeutic dose in the retina via a non-invasive/less invasive route or less frequent injections instead of surgical implantation.

Dendrimers can provide unique solutions to complex delivery problems for ocular drug delivery. PAMAM dendrimers have been widely employed in drug delivery for their solubility and permeability enhancer properties as well as their modifiable surface properties. Furthermore PAMAM dendrimers demonstrated physicochemical characteristics (pH, osmolality, viscosity), which are compatible with ocular dosage forms. Selection of functional group on the surface (amine, carboxylate and hydroxyl), size and molecular weight of the dendrimer were reported to be the important parameters to be considered in designing a delivery system (2,119) .

It was already reported that it's not possible to deliver DEX to back of the eye via topical application in a conventional dosage form (47) . Although DEX has been demonstrated in various formulation studies to enhance it's ocular bioavailability and biodistribution, which is initially poor due to it's hydrophobic structure, it has not been formulated in any dendrimeric systems yet. In this study the aim was to prepare DEX formulations using PAMAM dendrimers with various generations and surface functional groups to evaluate their in vitro and in vivo behaviors in order to develop a DEX-PAMAM system for diabetic retinopathy treatment. The goals of the formulations were either improving bioavailability and biodistribution of DEX or extending the residence time of DEX in vitreous and retina to reduce the application frequency. Two approaches, dendrimer complexation and dendrimer conjugation, have been followed using several PAMAM dendrimers and the results were compared in order to understand the effects of these dendrimers on retinal drug delivery of DEX.

5.1. Quantification of DEX and DEX-PAMAM Conjugates

A simple, rapid accurate HPLC method was modified from the literature and validated for DEX quantification (151,152) . The linearity of an analytical procedure is its ability to obtain test results that are directly proportional to the concentration of analyte in the sample. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example by calculation of a regression line by the method of least squares. The method was found linear in a range of 10-500 $\mu\text{g/mL}$, and used for DEX quantification for some of the in vitro study samples. Precision and accuracy results are usually expressed as the variance, standard deviation or coefficient of variation (CV) of a series of measurements. CVs were found less than 2% which were considered acceptable based on the ICH Q2A/Q2B guidelines (137) . The HPLC method developed in this study showed specificity and selectivity with linearity in the working range and good precision and accuracy, making it very suitable for quantification of DEX.

For quantification of the samples with lower DEX levels, a previously developed and validated LC/MS method was used (149) . DEX standard curve was

developed with a linear range from 0.5 ng/mL to 500 ng/mL, which was sensitive enough to quantify the DEX amount in in vivo and ex vivo study samples.

Since it was not possible to quantify the free DEX amount in DEX-PAMAM conjugate samples due to the low hydrolysis rate, it was planned to quantify the conjugate levels. For this purpose, conjugates were labeled using a fluorescent marker Alexa 488, which is a bright, green-fluorescent dye with excitation ideally suited to the 488 nm laser line. Fluorospectrofotometric analysis was performed (excitation: 495, emission: 519) to quantify the amount of the labeled conjugates in the tissues.

5.2. DEX-PAMAM Formulation Studies

Drugs can be associated with dendrimers via covalent conjugation to the surface, or via encapsulation of drugs within the structure. Each of these approaches has demonstrated different therapeutic benefits. The hydrophobic interior of dendrimers combined with their multiple sites for hydrogen bonding and ionic interactions make them novel polymeric systems for encapsulating especially hydrophobic drugs and improve their solubility and membrane permeability. On the other hand, it was reported that covalent conjugation of drugs significantly reduces initial burst release of drug and enables better control over the rate of drug release (111) .

Vandamme et.al reported that prolonged precorneal residence of pilocarpine and tropicamide, provided by complexation with PAMAM dendrimer solutions G1.5, G4 and dendrimer solution G4-OH. They also claimed that the results of the miotic and mydriatic activity tests on albino rabbits indicated that PAMAM dendrimer solutions improved the bioavailability of the drugs (119) . Iezzi et.al. have studied fluocinolone acetonide conjugation of hydroxyl-terminated PAMAM dendrimers as targeted, sustained intravitreal drug delivery vehicles to treat neuroinflammation in the outer retina. The intrinsic ability of intravitreally-delivered PAMAM dendrimers to localize within activated microglia is utilized to develop sustained intracellular delivery systems to attenuate neuroinflammation. Hydroxyl-terminated generation-4 PAMAM dendrimers are shown to localize in the outer retina of two rat models of retinal degeneration (128) .

In this study both complex and conjugate formulations of DEX was prepared, using various dendrimers with different generations and surface charges to improve its' retinal bioavailability.

DEX-PAMAM complex formulations were prepared using 6 different PAMAM dendrimers to increase ocular permeability of DEX to deliver it to retina, following topical or subconjunctival application. PAMAM dendrimers were chosen as two different generation with three different functional ending groups (-NH₂, -COOH and -OH) to compare the effect of size and charge of the dendrimers. The formulations were designed to release the drug immediately after application to increase the DEX amount that can reach to retina.

DEX-PAMAM conjugates were prepared with the purpose of obtaining sustained release of DEX to increase ocular residence time following subconjunctival or intravitreal injection, hence reduce the application frequency. Two generation of PAMAM dendrimers with -COOH functional group (PAMAM G3.5 and G4.5) were used for DEX conjugation because of the ability of -COOH group to form an ester bond with DEX.

5.3. Characterization of the Formulations

5.3.1. FTIR Analysis of DEX-PAMAM Complex Formulations

FTIR spectroscopy is a well established, non-destructive method for highly sensitive and selective determination and identification of chemical structures, whereas it offers accessibility to the bond interactions in these systems. FTIR spectroscopy works basically by exposing the sample to infrared radiation recording the wavelengths of radiation in the infrared region of the spectrum are absorbed by the sample. Each sample will have a characteristic set of absorption bands in its infrared spectrum (153) .

FTIR spectroscopy is mainly used for the routine analysis of the chemical transformations occurring at the surface of dendrimers. The ability of the dendrimers to form a complex with drugs depends on the nature of the surface groups of dendrimer and electrostatic interactions between the dendrimer and the drug. In determination of drug dendrimer interaction by IR spectroscopy the identification of the vibrational signature of the drug-dendrimer interactions is only possible by

comparison of the interacting systems to the spectra of the dendrimers and drug. Appearance/disappearance of characteristic peaks provides the proof of synthesis and complexation progress (154,155) .

Evidence of complex formation between PAMAM dendrimers and DEX was obtained from FTIR study, which investigates the functional groups of DEX involved in the complexation (Figure 4.4 to 4.9). DEX and PAMAM dendrimer spectrograms were compared with the DEX-PAMAM complex spectrograms. Characteristic DEX peaks such as C=O stretch peak at $1700\text{--}1720\text{ cm}^{-1}$ in the DEX spectrum disappeared in all of the spectra of DEX-PAMAM complex spectrums. The O–H stretching bands ($3200\text{--}3400\text{ cm}^{-1}$) are present in both DEX and dendrimer structures, thus disappearance of C–H stretch peak was not expected upon complexation but there were shifts observed for these bands, which might indicate the formation of hydrogen bonds between DEX and dendrimers. Disappearance of characteristic DEX peaks indicated that DEX added to the formulations was complexed with the dendrimers and there were no free DEX present.

5.3.2. $^1\text{H-NMR}$ Analysis of DEX-PAMAM Conjugate Formulations

NMR is a nuclei specific spectroscopy that uses a large magnet to probe the intrinsic spin properties of atomic nuclei. Like all spectroscopies, NMR uses a component of electromagnetic radiation to promote transitions between nuclear energy levels. NMR can be considered a leading nondestructive analytical tool for the analytical chemist in structural analysis of biomolecules. $^1\text{H-NMR}$ spectroscopy provides information about the numbers and environments of all the hydrogens in the structure (156) .

NMR spectroscopy is valuable technique in the characterization of dendrimers and it permits determination of the structure and dynamics of molecules in solution. Routine NMR analyses are especially useful during the step by step synthesis of dendrimers, even up to high generations. For organic dendrimers, $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ are useful in conjugation chemistry with shielding/deshielding effects shifts in peaks (157,158) .

Number of drug molecules attached to the dendrimers, were determined by $^1\text{H-NMR}$ (Varian 400 MHz) and $^1\text{H-NMR}$ spectrums were presented in Figure 4.10

to Figure 4.12. The final DEX number per PAMAM 3.5 dendrimer was calculated as 10 and per PAMAM 4.5 dendrimer was calculated as 4 respectively.

5.3.3. Particle Size and Zeta Potential

Dynamic light scattering that utilizes time variation of scattered light from suspended particles under Brownian motion to obtain their hydrodynamic size distribution is the most commonly used technique in particle size measurements. The electrical double layer plays a very important role in stability, permeability and toxicity of dendrimeric systems. The electrical double layer might be a result of ion adsorption, surface particle dissociation or both (159). Particles with zeta potentials of greater than +30 mV or less than -30 mV are considered strongly cationic and strongly anionic, respectively. Since most cellular membranes are negatively charged, zeta potential can have an effect on membrane permeability, and toxicity associated with cell wall disruption (160).

Kompella et.al have previously investigated the ocular distribution of subconjunctivally administered fluorescent polystyrene particles with various size (20 nm, 200 nm and 2 μ m) in Sprague-Dawley Rats. It was reported that the particles with 20 nm particle size, were rapidly eliminated from the injection site and were not exist in periocular tissue after 60 days of administration whereas 200 nm and larger particles remained at the site of administration for at least two months (161). In another study it was demonstrated that following the intravitreal injection, polylactide (PLA) nanoparticles with approximately 300 nm particle size, exhibited trans-retinal movement and localized in RPE cells four months after the administration (162). Based on the literature information, nanoparticulate systems bigger than 100 nm are expected to stay longer in the ocular injection site. Thus longer retention time is predicted for dendrimers with higher generations based on their bigger particle sizes.

Results of the particle size measurements indicated that DEX complexation increased particle size 3-4 times, whereas conjugation did not have a significant effect on particle size. Both complex and conjugate formulations sizes changes in a range approximately between 125-250 nm with the exception of PAMAM G4-DEX

complex which was measured as 423 nm. High particle size of this formulation might be a result of aggregation.

Zeta potential measurements indicated that cationic PAMAM G3 and G4 dendrimers and their formulations were positively charged, and anionic dendrimers PAMAM G3.5, G3-OH, G4.5 and G4-OH and their formulations were negatively charged as expected. Zeta potential results also showed that, zeta potential values, either positive or negative, were increased in presence of DEX which itself is a slightly anionic molecule. That result might also support the FTIR results, which indicated that there was no free DEX on the surface of complexes.

5.4. In Vitro Release Studies for DEX-PAMAM Complex Formulations

DEX release study was performed for 3 hours at 37°C in PBS and equal amount of the fresh medium was replaced with samples at sampling time points. The results of the in vitro release study in PBS, indicated that all DEX-PAMAM complex formulations has a fast release profile as it was expected. Rapid release is desired for complex formulations, since they were planned to apply either topically or subconjunctivally to across ocular barriers and deliver the drug to the vitreous and retina tissues. The release study has ended after 3 hours of sampling, since their ocular residence time will be shorter (119) . The results showed that G3.5 complex has the highest release rate and at least 50% of DEX was released from all formulation in 3 hours. The high release percentages of DEX from complex formulations also indicate that DEX solubility was enhanced with dendrimer complexation. Patri et.al. reported that the release rate of methotrexate from the dendrimer complex with PAMAM G5–Folic acid showed a similar pattern, with 50% of the drug being released from the dialyzer in PBS in 2.5h (110) .

G3.5 and G4.5 complex formulations, which has “–COOH” as an ending group in their structures, showed the highest release rate. This might be a result of the surface charges, where G3.5 and G4.5 complex formulations also presented the highest anionic zeta potential values. The difference factors (f_1) and the similarity factors (f_2) were calculated to compare release profiles. Based on the f statistics, G3 and G4 complexes, G3.5 and G4.5 complexes and G3-OH and G4-OH complexes

showed statistically similar release profiles, which indicates that DEX release is dependent on the surface group of the dendrimer.

5.5. Hydrolysis Study of DEX-PAMAM Conjugates

The fate of drug release from a dendrimer conjugate mostly depends on the nature of the linking bond or spacer between the drug and dendrimer. Both ester and amide bonds might be cleavable by enzymes. However, ester cleavage is generally more facile than amide cleavage in terms of drug release (90) . Stability and release properties of PAMAM conjugates have been investigated in several studies. The amide-linked Naproxen – PAMAM G0 conjugates exhibited stability in plasma and liver homogenate, whereas the ester-linked conjugates released the drug immediately (163) . In another study results were similar and it was reported that higher release profiles of ibuprofen linked through ester bond to PAMAM G4 dendrimers over the ibuprofen linked by amide bond (143) . Venlafaxine linked directly to the PAMAM G 2.5 dendrimers via a hydrolysable ester linkage and 50% of the drug was released within 18 hours (164) . Quinidine conjugated to the anionic PAMAM G 2.5 and cationic PAMAM G3; it was reported that PAMAM dendrimer conjugates via ester bond using a glycine spacer, released the drug completely within 24 hours (165) . Malik et.al. reported the coupling of cisplatin to G3.5 PAMAM dendrimers via an ester linkage. Despite the high aqueous solubility and stability of these conjugates, they were reported to fail producing the desired anticancer activity due to limited drug release, on the contrary of the other conjugation studies (166) .

Results of the hydrolysis study (Figure 4.14) in presence of cornea and SCRPE tissues showed that, enzymatic degradation of DEX-PAMAM conjugates was very slow. Following 6 days of incubation, less than 8% of DEX was released from the conjugates and the results indicated that the hydrolysis did not effected by the type of the tissue. This might be a result of the enzyme saturation or a steric hindrance between the enzyme and the bond. Thus using a cleavable linkage during conjugation process would have resulted in better DEX release from conjugates. It was also observed that DEX-PAMAM G3.5 conjugate, which has higher molar DEX ratio (1:10), showed higher release rate than PAMAM G4.5 (1:4), as it was expected.

5.6. Cell Culture Studies

5.6.1. MTT Cytotoxicity Assay

It was reported that dendrimer cytotoxicity might restrict biopharmaceutical application of dendrimers as drug delivery vehicles. Thus evaluation of the cytotoxicity is an important parameter in especially ocular drug delivery. PAMAM and PPI dendrimers having terminal amine groups are cationic in nature and display significant differences in toxicity compared to anionic and neutral dendrimers. Toxicity profiles of these cationic dendrimers are extensively governed by terminal amine functionality, which increases with dendrimer generation (167,168) . Lower generation dendrimers with anionic or neutral polar surface groups were reported to have lower toxicity as compared to higher generation dendrimers with neutral and cationic surface groups. Masking or modifying the surface amine groups of dendrimers by surface engineering was one of the most popular approaches to minimize toxicity (72,73) .

Dendrimer interactions with biological cells were investigated to examine the role of dendrimer generation (i.e. size, surface charge density), on human corneal epithelium cell (ARPE 19) growth particularly with respect to evidence of cytotoxicity.

MTT assay results showed that DEX itself has a slight cytotoxicity and the blank dendrimers has no effect on cell viability. The difference between blank and DEX loaded dendrimers were found statistically significant ($p < 0.05$). These results indicated that presence of DEX is important in terms of cytotoxicity, whereas blank dendrimeric carriers have not presented any toxicity themselves. The results of the MTT assay also indicated that the cationic dendrimers (with amine end groups) PAMAM G3 and PAMAM G4 did not induce more cytotoxicity than dendrimers with peripheral carboxyl or hydroxyl functional groups was surprising, because cationic macromolecules including cationic dendrimers were reported to induce toxicity due to the chemical nature of polycations. However, Vandamme and coworkers also reported that cationic dendrimers that they have studied showed less cytotoxicity and irritation in comparison with anionic or neutral dendrimers (119) . Fisher et.al. have tested cytotoxicity of cationic PAMAM dendrimers on L929 mouse fibroblasts for up to 24 hours and cationic PAMAMs were reported to induce only a

moderate decrease in cell viability and were considered to be highly compatible polycations (169) .

Cell viability for all type of plain dendrimers were found higher than 87%, which can be concluded they have no significant toxicity on human corneal epithelium cells at a concentration of 1 mg/mL, when incubated for 24 hours.

5.6.2. In Vitro Permeability Studies

Dendrimers have shown enormous potential as drug delivery systems because they can cross the cell barriers by both paracellular and transcellular pathways (94) .

It was reported that PAMAM dendrimer biopermeability is dependent on a combination of structural features such as their size, molecular weight, geometry and number of surface groups (170) . Both cationic and anionic PAMAM dendrimers have reported that they co-localize within the endosomal and lysosomal markers by opening tight junctions, and that endocytosis was responsible for their internalization and intracellular trafficking across Caco-2 cell line (171) . Additional study by Tajarobi et al. reported that drug permeability in presence of PAMAM dendrimers across Madin-Darby Canine Kidney (MDCK) cells was dependent on size of the dendrimer and their interactions with the cells (172) .

Results of the permeability study which were performed on ARPE 19 cell line (Table 4.6 and Figure 4.17) indicated that G4.5 complex had the highest DEX permeation, whereas all of the DEX-PAMAM complex formulations showed higher drug permeation comparing to DEX solution and the differences between all groups were found statistically significant ($p < 0.05$). Higher generation dendrimers (PAMAM G4.5, G4-OH and G4) showed higher DEX permeation rates than lower generations (PAMAM G3.5, G3-OH and G3). Kitchens et al. (173) carried out extensive studies on cationic, neutral and anionic dendrimers. It was found that permeability was enhanced with an increase in the number of anionic surface groups in the PAMAM- COOH series. These results were compatible with our study where it was found PAMAM G4.5 shows significantly higher permeation than PAMAM G3.5.

It was also observed that drug permeability for DEX complexes of PAMAM G4, which is a cationic dendrimer, showed significantly lower permeation than

anionic PAMAM G4.5 and G4-OH complexes, against all odds. On the contrary, Kitchens et.al has reported that cationic, amine terminated PAMAM G2 exhibited greater permeability than neutral and hydroxyl functionalized generation 2 PAMAMs (173) . The difference between these two studies might be a result of the difference in cell lines, which were ARPE 19 and Caco-2, as well as the difference of the formulations. DEX permeation from DEX-PAMAM conjugates was found significantly lower that is probably a result of low hydrolysis of DEX from the conjugates due to the absence of free DEX.

5.7. Ex Vivo Transport Studies

Ex vivo transport studies across rabbit cornea and SCRPE tissues were performed for PAMAM-DEX complexes (G3, G3.5, G3-OH, G4, G4.5 and G4-OH) in comparison with DEX solution. DEX-PAMAM complex formulations were designed to enhance solubility of DEX and improve its ocular permeation across cornea or SCRPE following topical or subconjunctival application. The duration of the transport experiment (3 hours) was well within the length of tissue stability reported in literature (174) .

Following topical application, drug has to cross through corneal barrier in order to reach the back of the eye. On the other hand, following subconjunctival application, sclera, choroid and retinal pigment epithelium (SCRPE) were the barriers that drug needs to pass through to reach retina. DEX transport across SCRPE has been found higher than corneal transport as expected, because cornea has more intact tight junctions when compared to SCRPE (175) . It was reported that RPE to be a major barrier for the transport of hydrophilic substances, but for lipophilic materials the choroid-RPE and sclera were approximately equivalent barriers (176) .

Results of the ex vivo transport studies, for DEX-PAMAM complex formulations, across cornea was presented in Figure 4.18 and across SCRPE was presented in Figure 4.19. In the light of the data it was found that surface charge is a significant factor for transport across cornea and SCRPE. The results indicated that both cationic dendrimer complexes (PAMAM G3 and PAMAM G4) have a lower DEX transport level than anionic ones (PAMAM G3.5, PAMAM G3-OH, PAMAM G4.5 and PAMAM G4-OH). Although these results are compatible with in vitro

permeability results that were performed on ARPE 19 cell line. There are very few studies in the literature that evaluated the polymeric system permeation through cornea or SCRPE depend on their surface charge. It was reported that sclera has been more permeable to negatively charged molecules than positively charged molecules and this can be attributed to the presence of negatively charged proteoglycans in this tissue (177) . Based on this information, higher permeation of anionic dendrimers could be explained by the repulsive forces between anionic dendrimers and negatively charged corneal and scleral barriers.

The results showed that all anionic dendrimer complexes have higher DEX transport level when compared to DEX solution and the cationic dendrimers. DEX transport from complex formulations of dendrimers with –COOH ending groups (PAMAM G3.5 and G4.5) were slightly higher than –OH ending ones (PAMAM G3-OH and G4-OH). This might be related to the zeta potentials measurements, where –COOH ending complex formulations have presented higher anionic charges. Furthermore, generation 3 dendrimers has higher transport than generation 4 dendrimers across both cornea and SCRPE.

Apparent permeability co-efficients (Papp, cm/s) were calculated and statistically compared using One Way ANOVA and Tukey HSD test for post-hoc analysis. The values measured for drug permeability through cornea and SCRPE, compare favorably with the values found for a variety of drug molecules that were reported in the literature (174,178,179) . In terms of corneal transport, DEX-PAMAM G4.5 complex permeation was found significantly higher than the other formulations ($p < 0.05$). This might be concluded that DEX-PAMAM G4.5 complex would be a good candidate for topical application to deliver DEX to retina. This hypothesis was also confirmed with in vivo biodistribution studies following topical application.

Overall, ex-vivo transport studies indicated that both corneal and SCRPE transport of DEX can be enhanced with PAMAM dendrimer complexation. Transport is highly affected by charge and surface group of the dendrimers as well as the generation.

5.8. In Vivo Studies for DEX-PAMAM Complex Formulations

Bessonova and coworkers have investigated ocular tissue distribution of DEX, following topical application conventional drug carriers such as an ointment or a suspension. The concentration of dexamethasone was determined in third eyelid, cornea, aqueous humor, iris, lens, vitreous body, and retina-choroid. 3 h after topical administration the highest concentrations of DEX were measured in the anterior structures of the eye (47) . As it was reported in this study, it's not possible to deliver DEX to back of the eye via topical application in a conventional dosage form.

For diabetic macular edema treatment, Tanito et.al have developed DEX – cyclodextrin microparticle eye drops in order to deliver DEX to retina following topical application. It was reported that DEX - cyclodextrin microparticles significantly reduced retinal thickness and improved visual acuity in diabetic macular edema. Even the ocular DEX distribution and DEX levels in retina have not been evaluated, the increased response was encouraging that retinal diseases could be treated with topical eye drops (180) .

DEX-PAMAM complex formulations were designed to enhance DEX transport across ocular barriers. Ex-vivo transport studies was performed on rabbit eye tissues and the results have supported that dendrimer complexation improves DEX transport across both cornea and SCRPE. Thus, DEX-PAMAM complex formulations were topically and subconjunctivally applied to Sprague Dawley rats and the ocular tissue distribution of DEX was investigated.

The results of the study showed that all DEX-PAMAM complex formulations have reached higher DEX concentrations in ocular tissues compared to plain DEX suspension. Anionic dendrimeric formulations have higher DEX concentrations in tissues, compatible with the ex vivo transport study results. All the differences in DEX amount in the tissues, except aqueous humor, have been found statistically significant ($p < 0.05$).

Drug cumulation was observed in conjunctiva, sclera and cornea tissues, which is expected since these tissues are the main barriers in ocular drug delivery. Significant increase in vitreous and retina-CRPE drug levels was achieved with DEX-PAMAM G3.5 and 4.5 complexes.

In a recent study with drug suspensions showed that an increase in corticosteroid solubility, enhances in vivo transscleral retinal transport (149) . Dendrimer complexation is known with its' solubility enhancer effect, and ex-vivo transport studies indicated that DEX transport across SCRPE is increased with DEX-PAMAM complexation. Two of the formulations were selected to apply rats subconjunctivally in order to evaluate tissue distribution of DEX. Anionic DEX-PAMAM G4.5 complex was chosen based on the successful results in ex vivo transport results and in vivo topical application studies. Cationic DEX-PAMAM G4 was also tested to compare the effects of surface charge. Both anionic and cationic PAMAM dendrimer formulations (G4 and G4.5) were able to enhance tissue distribution and the difference in retina-CRPE and sclera DEX levels following subconjunctival application when compared to DEX suspension. On the other hand the difference between DEX-PAMAM G4 and 4.5 were found insignificant.

In the light of this data, it can be concluded that DEX-PAMAM complex formulations enhance DEX delivery to retina following both topical and subconjunctival application. Especially DEX-PAMAM G3.5 and G4.5 complex formulations might be suggested as strong candidates for topical DEX delivery to the back of the eye, since they have provided higher DEX tissue levels. Therapeutic efficiency of these increased drug levels might be evaluated with further studies.

5.9. In Vivo Studies for DEX-PAMAM Conjugate Formulations

There is compelling evidence that sustained drug delivery to the retina can be achieved by administering drugs through the subconjunctival route as well as intravitreal route. Several formulation strategies have been developed to achieve sustained drug delivery for to the retina, including microparticles, nanoparticles, liposomes, implants which can possibly prolong drug release. However there is another factor affecting the therapeutic efficiency of these systems that is retention time of the drug in the target tissue. For sustained retinal drug delivery, it's hypothesized that systems, which are retained in the periocular space, would be more appropriate to release the drug over a prolonged period for subsequent delivery to the posterior segment (181) .

The half-life in the vitreous is another important factor that can determine the therapeutic efficacy. Following intravitreal injection, the drug is eliminated either by the anterior route or posterior route. The anterior elimination route involves drug diffusion across the vitreous into the aqueous humor followed by elimination through aqueous turnover and uveal blood flow. The posterior elimination pathway involves drug permeation across the blood–retinal barrier and requires optimum passive permeability or active transport mechanisms. As a result, hydrophilicity and large molecular weight tend to increase the half-life of the compounds in the vitreous humor (41) .

In vitro enzymatic hydrolysis studies indicated that DEX release from DEX-PAMAM conjugate formulations were less than 8%, following 6 days of incubation with either cornea or SCRPE tissues. This release rate was even slower than expected and should be modified by using a cleavable linker in the process of conjugation. On the other hand, achieving a sustained delivery system also depends on the clearance time of the delivery system. Thus, retina-choroid and vitreous levels of Alexa 488 labeled conjugates were investigated following both subconjunctival and intravitreal applications using an ocular photometry system. Alexa 488 labeled anionic and cationic dendrimers without DEX conjugation were also evaluated following intravitreal application to investigate the effects of generation and surface charge of dendrimer on clearance.

Fluorotron Master, an ocular fluorophotometry system, delivers a blue excitation light through the optics of the system to the eye and the resulting emitted fluorescent light gets collected via the same optical system. A measurement area called as the focal diamond occurs at the point where the excitation and emission lights intersect. Levels of fluorescence were measured within this focal diamond, and the focal diamond is automatically moved along the axis of the eye in the posterior to anterior direction (182) .

Alexa 488 concentrations in the eye were plotted against distance data points separated by 0.25 mm on an optical axis. Tyagi et al. has developed a fluorophotometric method in rat eyes using the same instrument and data points 23, 45, and 77 were assigned as concentrations in retina-choroid, vitreous, and, anterior

chamber respectively (42) . Thus data points 23-25 and 45 were used to plot the fluorescence levels in retina-choroid and vitreous.

With this study it was demonstrated that fluorescence levels can be monitored noninvasively in different ocular tissues after subconjunctival, and intravitreal injections in rats using ocular fluorophotometry. However, one of the limitations of ocular fluorophotometry is that this technique cannot be used for drug molecules that are not fluorescent labeled.

Fluorotron analysis results indicated that fluorescence levels following subconjunctival injection of DEX-PAMAM conjugates were similar between two formulations and the initial fluorescence level was found very close to the background measurements. These results showed that conjugate levels in vitreous and retina following subconjunctival injection were not high enough to compare the formulations or the length of duration in the eye. Fluorotron results of intravitreal application study showed that both formulations were present in vitreous for 24 hours with a decreasing level. Furthermore, it was observed that retina-choroid dendrimer levels are higher than vitreous, which indicates that dendrimers are travelling to retina from injection site before getting cleared from the eyes. Based on these results, it can be concluded that for both application routes, the clearance is too fast to achieve a sustained DEX delivery even if the release profiles would be optimized using a linker.

Plain PAMAM dendrimers without DEX conjugation were also tested to compare the effect of the charge and generation of dendrimers. Comparison of the dendrimer formulations showed that cationic dendrimers (PAMAM G3 and PAMAM G4) has higher tissue levels, and stayed longer in the vitreous and retina-choroid tissues than anionic dendrimers (PAMAM G3.5 and PAMAM G4.5) which can be explained with Hyaluronan, a negatively charged glycosaminoglycan that present in the vitreous (21) . 24 hours after injection, there is still some fluorescence for all dendrimer types, even the majority of the dendrimer was cleared. However the clearance is still too fast for both cationic and anionic dendrimers to provide a controlled release injectable system.

The extent of delivery to retina-choroid after subconjunctival injection was found to be lower compared to intravitreal route, possibly due to multiple clearance

pathways. This was expected since following subconjunctival injection the drug may encounter several elimination pathways including episcleral and conjunctival vasculature prior to entering the choroid (183) .

Ocular tissue distributions of PAMAM dendrimers (PAMAM G3, G4, G3.5 and G4.5) and DEX-PAMAM conjugate formulations were also investigated following both subconjunctival and intravitreal injections. The results of ocular tissue distribution study following subconjunctival application was compatible with the fluorotron study and showed that less than 10% of the injected formulations left in the tissues 2 hours after the injection. But the distribution of the formulations was enhanced when compared to DEX suspension and there were significant amount of signal in retina and vitreous, which indicates the transport across SCRPE was enhanced. Ocular tissue levels following intravitreal application of formulations showed that, 24 hours after injection tissue conjugate levels in total ocular tissues were around 33% for DEX-PAMAM G3.5 conjugates whereas it was around 15% for DEX-PAMAM G4.5 conjugates. These results were also compatible with the clearance results obtained by fluorotron analysis. Following intravitreal application, it was also observed that vitreous and retina-CRPE tissues had higher amount of conjugate levels comparing to the other tissues, which is expected since the injection site was vitreous and there were no barriers present to across.

96.8% of PAMAM G3, 99.7% of PAMAM G4, 28.8% of PAMAM G3.5 and 44.6% of PAMAM G4.5 dendrimers were found distributed in the ocular tissues 24 hours after the injection respectively. These results indicated that cationic dendrimers were still in the ocular tissues 24 hours after the injection and anionic dendrimers have been cleared faster. However it was also observed that cationic dendrimers were traveled to the front of the eye, which indicates that they have higher affinity for tissues like cornea, lens and aqueous rater than vitreous and retina.

6. CONCLUSION

Drug delivery to the posterior segment of the eye including choroid, retina, and vitreous is still a challenging task. Diabetic retinopathy is one of the major posterior segment diseases and dexamethasone is a corticosteroid drug commonly employed for diabetic retinopathy treatment.

The purpose of this study was to develop a DEX delivery system using PAMAM dendrimers, in order to improve patient compliance while treating diabetic retinopathy. Three different approaches were investigated to reduce the discomfort and side effects of the treatment:

- Topical application with improved ocular bioavailability
- Subconjunctival injection which is a less invasive and less destructive route when compared to intravitreal application
- Intravitreal injection with reduced frequency

Various PAMAM dendrimers with anionic and cationic functional groups were used to prepare DEX complex or conjugate formulations to investigate their effects on retinal delivery of DEX to achieve any of these goals.

The results of the study showed that DEX-PAMAM conjugates which were designed to prolong the drug presence in retina, were not able to maintain the required drug level in the target tissue. Conjugates have been cleared in 24 hours following the intravitreal injection, before they release the DEX in their structure. Furthermore their enzymatic hydrolysis should be modified to be able to control release rate, by adding a cleavable linker between dendrimer and the drug before any further investigation of these formulations.

On the other hand DEX-PAMAM complex formulations that were expected to improve DEX solubility and ocular permeability, were managed to increase DEX amount reaches to retina following both topical or subconjunctival applications. Especially DEX-PAMAM G3.5 and G4.5 complexes (dendrimers with -COOH functional groups) were able to enhance in vitro permeability and ex vivo transport of DEX, as well as in vivo ocular distribution.

In the light of the obtained data, it can be concluded that DEX-PAMAM complex formulations prepared with anionic PAMAM G3.5 and G4.5 dendrimers are able to enhance ocular bioavailability of DEX. They are promising delivery systems for DEX delivery to retina following topical application, which is the safest and easiest application route.

For future studies, it might be a good approach to perform *in vivo* efficacy studies on animals with diabetic retinopathy to evaluate if these formulations can provide the required dose of DEX for therapeutic efficiency. Required DEX dose should be adjusted by optimization of the formulations and to achieve a therapeutic response, prior to efficacy studies. Following efficacy studies, stability of the promising formulations and the effect of the sterilization procedure on these formulations should also be investigated in order to claim clinical relevance.

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- 36th CRS Annual Meeting, 18-22 July 2009 – Copenhagen / Denmark, Poster presentation.
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Review Article

Dendrimeric Systems and Their Applications in Ocular Drug Delivery

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Ophthalmic drug delivery is one of the most attractive and challenging research area for pharmaceutical scientists and ophthalmologists. Absorption of an ophthalmic drug in conventional dosage forms is seriously limited by physiological conditions. The use of nonionic or ionic biodegradable polymers in aqueous solutions and colloidal dosage forms such as liposomes, nanoparticles, nanocapsules, microspheres, microcapsules, microemulsions, and dendrimers has been studied to overcome the problems mentioned above. Dendrimers are a new class of polymeric materials. The unique nanostructured architecture of dendrimers has been studied to examine their role in delivery of therapeutics and imaging agents. Dendrimers can enhance drug's water solubility, bioavailability, and biocompatibility and can be applied for different routes of drug administration successfully. Permeability enhancer properties of dendrimers were also reported. The use of dendrimers can also reduce toxicity versus activity and following an appropriate application route they allow the delivery of the drug to the targeted site and provide desired pharmacokinetic parameters. Therefore, dendrimeric drug delivery systems are of interest in ocular drug delivery. In this review, the limitations related to eye's unique structure, the advantages of dendrimers, and the potential applications of dendrimeric systems to ophthalmology including imaging, drug, peptide, and gene delivery will be discussed.

1. Introduction

Drug delivery to the eye is still one of the most challenging tasks for pharmaceutical scientists. The eye is characterized with its complex structure with high resistance to drugs as well as other foreign substances. The anterior and posterior segments of the eye function both independently upon an ocular application [1]. Thus, ocular drug delivery can be classified into anterior and posterior segments.

Conventional drug delivery systems are not effective enough to meet the requirements in the treatment of ocular diseases [2]. However, "more than 90% of the marketed ophthalmic formulations" are in the form of eye drops, and most of them target the "anterior segment eye diseases" [3]. Poor bioavailability of drugs from ocular dosage forms is mainly due to the "precorneal loss factors" including solution drainage, lacrimation, tear dynamics, tear dilution, tear turnover, conjunctival absorption, transient residence time in the cul-de-sac, and low permeability of the corneal

epithelial membrane which are the major challenges to anterior segment drug delivery following topical administration.

Treatment of "posterior segment diseases" still remains as an unsolved issue. Most of the ophthalmic diseases affect neural retina, choroid, and vitreous. For example, glaucoma, diabetic retinopathy, age-related macular degeneration (AMD), and various forms of retinitis pigmentosa are damaging the posterior eye segment, which may cause impaired vision and even blindness [4]. Delivery of drugs to the posterior segment is more challenging than to the anterior segment, due to the acellular nature of the vitreous body and the longer diffusion distance [5]. Thus, posterior eye segment diseases have become an important therapeutic target with unmet medical needs. The major goal in the treatment of posterior segment diseases is the delivery of therapeutic doses of drugs to the tissues while reducing the effects. Systems developed to achieve this goal range from simple solutions to novel drug delivery systems, such as nanoparticles, liposomes, micelles, dendrimers, iontophoresis, and gene delivery systems [5–7].

Dendrimers are “tree-like,” nanostructured polymers that have been interesting in terms of ocular drug delivery. They are attractive systems for drug delivery due to their nanosize range, ability to display multiple surface groups that allows for targeting, and easy preparation and functionalization [8]. Ongoing studies in developing improved ocular dendrimeric systems may not only serve to enhance the drug delivery to the ocular surface, but also may provide effective delivery of therapeutic agents to intraocular tissues, such as the retina or choroid, using noninvasive delivery methods.

2. Challenges in Ocular Drug Delivery

Eye has a unique physical structure with protective barriers, which offers many challenges to the effective delivery of drugs to the eye. The eyeball is divided into 2 segments: the anterior segment containing the cornea, crystalline lens, iris, ciliary body, and fluid-filled aqueous humor and the posterior segment that includes the sclera, choroid vessels, retina, macula, optic nerve, and fluid-filled vitreous humor [2]. This organ is well protected with various specialized cellular modifications that give rise to various barriers that partially isolate the eye from the rest of the body, which can be a challenge for drug delivery [9]. These special processes/barriers are as follows.

- (i) The “inner and outer blood–retinal barriers” separate the retina and the vitreous from the systemic circulation, and because of the absence of the cellular components in vitreous body, it reduces convection of molecules [10].
- (ii) The inner limiting membrane controls the exchange and entry of particles from the vitreous to the retina.
- (iii) The “blood-aqueous barrier” limits the transport of molecules from the blood to the inner part of the eye [11].
- (iv) Intact structure of corneal epithelium with desmosomes and tight junctions offers resistance to the passage of most drugs due to the presence of layers: hydrophobic epithelium, hydrophilic stroma, and hydrophobic endothelium [12].
- (v) The tear film forms a mucoaqueous barrier that continuously washes away the particles at the anterior surface of the eye [9].

The anatomical and physiological barriers mentioned above are a challenge to ocular drug delivery. Solubility, lipophilicity, molecular size and shape, charge, and degree of ionization of the drug also affect the penetration rate to the eye [13]. Drug delivery systems’ biocompatibility is also relevant when ocular delivery is concerned. The specific challenge of designing an ocular therapeutic system is to achieve an efficient concentration of the drug at the active site for the duration to provide the therapeutic efficacy [14, 15]. The requirements of an ideal topical formulation to the eye must be fulfilled as follows: the formulation must be well tolerated and easy to administer; avoid systemic absorption and increase drug retention time in the eye.

Various ophthalmic formulations have been developed to improve ocular penetration, reduce toxicity, and improve tolerability [16, 17].

Typically “less than 5% of the topically applied drug” penetrates the cornea and reaches intraocular tissues, while most of the instilled dose is often absorbed systemically via the nasolacrimal duct and conjunctiva [3]. The eye drops are easy to instill but only a very small amount of the instilled dose is absorbed into the target tissues. It becomes necessary to apply large doses of drugs frequently to achieve the effective therapeutic dose which leads to an increase in both local and systemic side effects [18]. Since the penetration to cornea is often poor, either systemic or intravitreal administration (injection or implant) is required in order to treat posterior segment diseases. Due to strong blood-ocular tissue barrier, systemic administration requires large doses, while intravitreal injections and implants are very invasive and are associated with a high degree of retinal damage risk, such as retinal detachment and endophthalmitis [19]. Thus, there has been growing attention to transscleral route in order to deliver drug to the back of the eye [20]. Sclera is more permeable than cornea and even it is highly permeable to the large molecules of even protein size. However, it is more complicated to deliver the drug to retina, because in case of transscleral application the drug must pass across the choroid and retina pigment epithelium (RPE) [21].

The major goal is to develop suitable drug delivery systems with improved bioavailability of drugs, increased retention time, reduced side effects, cellular targeting, better patient compliance, and providing extended therapeutic effects [22]. Currently, nanocarrier-based ocular drug delivery systems including dendrimers appear to be the most promising way to meet the requirements of an ideal ocular drug delivery system.

3. Dendrimer Structure, Synthesis, and Properties

Dendrimers are monodisperse macromolecules with several reactive end groups that surround a small molecule and form an internal cavity. Their tree-like branched architecture displaying a variety of controlled terminal groups is in particular very promising for biomedical applications [23]. Especially low generation dendrimers can encapsulate hydrophobic drug molecules into their internal cavities. Because of this unique structure, dendrimers are able to solubilize poorly water-soluble drugs [24]. In addition to the extraordinary structural control, another outstanding feature of dendrimers is their actual mimicry of globular proteins. They are referred to as “artificial proteins,” based on their systematic, electrophoretic, dimensional length scaling and other biomimetic properties [25, 26].

Elements are added to dendrimer structure by a chemical reaction series and build a branching spherical structure from a starting atom such as nitrogen. The central core molecule should have at least two reactive functional groups and the repeated branches are organized in a series of “radically concentric layers” called “generations” [27]. A schematic

representation of a generation 2 dendrimer is given in Figure 1. Dendrimers are generally prepared using either a divergent method or a convergent one [28]. In the *divergent method*, dendrimer grows outwards from a multifunctional core molecule. On the other hand, in the *convergent approach*, the dendrimer is constructed stepwise, starting from the end groups and progressing inwards. When the branched polymeric arms (dendrons) grew enough, they are attached to a multifunctional core molecule [29]. A schematic representation of divergent and convergent methods, is given in Figure 2. Other approaches have been developed based on the divergent and convergent methods such as *double exponential growth*, *lego chemistry*, and *click chemistry*. Preparation of monomers from a single starting material for both divergent and convergent methods is possible using double exponential growth approach. Then two result products are reacted to give a trimer, which can be used to repeat the growth again [30]. In lego chemistry strategy, phosphorus dendrimers are prepared from highly functionalized cores and branched monomers. After several variations in general synthetic scheme, a scheme is developed that allows multiplications of the number of terminal surface groups from “48 to 250” in one step [31].

Compared to other polymers, dendrimers have so many advantages such as their nanosize ranging from 1 to 100 nm with lower polydispersity index that allows them to avoid RES uptake. Furthermore, targeting anywhere in the body is also possible, thanks to the multiple functional groups on their surface which makes it possible to attach vector devices [32, 33]. Dendrimers have the ability to encapsulate drug molecules into their internal cavities which leads to enhanced solubility, permeability, and retention effect depending on their molecular weight. It was reported that drug absorption is increased with dendrimers association in the cationic > uncharged > anionic order, where cationic dendrimers show permeation enhancement due to their ability of interacting lipid bilayers. Smaller generation dendrimers also have an enhancer effect on permeability since they have a better ability to move between cells [34].

Dendrimer cytotoxicity is related to the core chemistry; the nature of the dendrimer surface is the most influencing factor, because the interaction between surface cationic charge of dendrimers and negatively charged biological membranes is the main reason of toxicity. Lower generation dendrimers with anionic or neutral polar surface groups were reported to have higher biocompatibility as compared to higher generation dendrimers with neutral apolar and cationic surface groups. It was reported that following repeated intravenous use or topical ocular application, dendrimers with cationic end groups are often toxic, whereas anionic dendrimers are not. Thus, in order to reduce toxicity, it is necessary to modify the surface amine groups of these dendrimers with neutral or anionic moieties [35–37]. Recently, several studies have been published to report that ocular dendrimeric formulations were developed without cytotoxicity or irritation [38, 39]. For ocular drug delivery, it is very important to make sure that the dendrimers are safe because serious side effects may occur due to cytotoxicity at the ocular tissues. Safe dendrimer formulations for ocular drug delivery should have properties such as

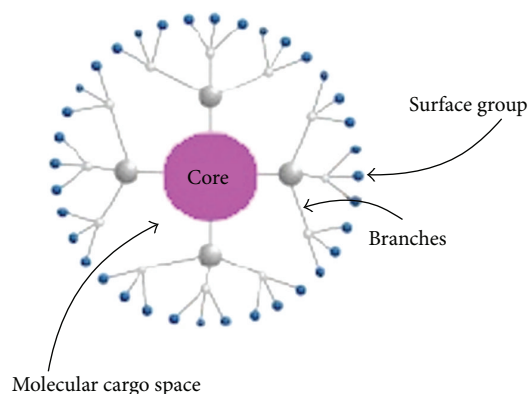


FIGURE 1: Schematic representation of a generation 2 dendrimer.

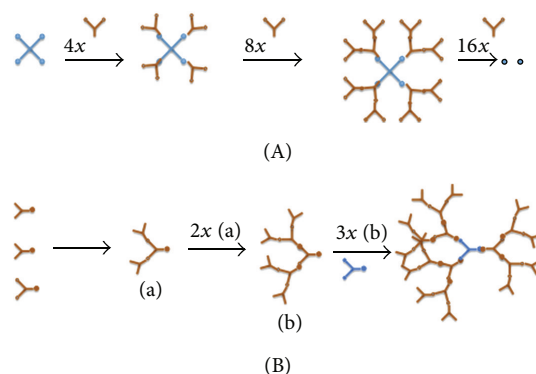


FIGURE 2: Schematic representation of divergent and convergent methods: (A) the divergent growth method (B) the convergent growth method.

biocompatibility and low immunogenicity; thus they should be carefully designed and evaluated. Furthermore, in order to overcome the potential toxicity of the dendrimers, it is very important for ophthalmologists to participate and contribute to the scientific process alongside with chemists, formulation scientists and engineers.

4. Types of Dendrimers

The first “dendrimer family” that was synthesized, characterized, and commercialized was poly(amidoamine) (PAMAM) dendrimers (Figure 3(a)) which were synthesized by the “divergent” method [25]. The structure of PAMAM dendrimers starts from an ammonia (NH_3) or ethylenediamine ($\text{C}_2\text{H}_8\text{N}_2$) molecule as a core that binds to the amine groups of branches (R-NH_2) and amide ($-\text{CONH}_2$). Dendrimers growth reaches a critical point where the branching arms limit their development into higher generations due to steric effect that starts with G7. This effect decreases the synthetic yields of generations between G7 and G10 and prohibits the synthesis of any larger dendrimers [40, 41]. PAMAM dendrimers have a size range between 1.1 and 12.4 nm as their generations grow through 1–10 [42]. These dimensions have been compared to proteins (3–8 nm), linear polymer-drug conjugates (5–20 nm), and viruses (25–240 nm). Overall,

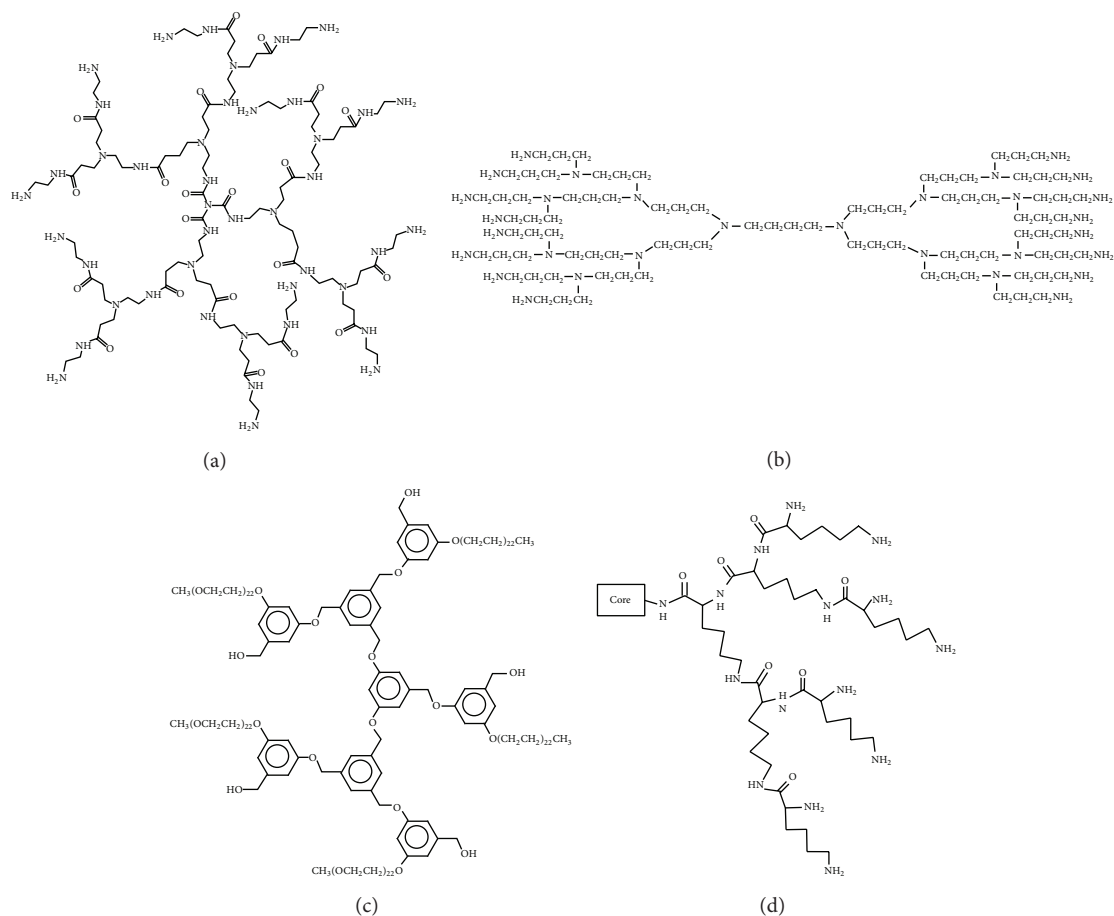


FIGURE 3: Structures of various types of dendrimers: (a) PAMAM dendrimer, (b) polypropyleneimine dendrimer, (c) polyaryl ether dendrimer, and (d) biodegradable polylysine dendron.

PAMAM dendrimers are considered as ideal carriers for drug delivery due to their high aqueous solubility, large variety of surface groups, and unique architecture. For medical applications, the most widely studied PAMAM dendrimers have been the derivatives with an $-NH_2$ surface, a $-COOH$ surface, and an $-OH$ surface [43].

Poly(amidoamine) organosilicon (PAMAMOS) dendrimers are silicon containing first commercial dendrimers which are inverted unimolecular micelles that contain exterior hydrophobic organosilicon and interiorly hydrophilic, nucleophilic polyamidoamine [44].

Polypropyleneimine (PPI) dendrimers (Figure 3(b)) have been investigated for their medical applications, but it has been shown that the presence of multiple cationic amine groups causes significant toxicity. These dendrimers are generally poly-alkyl amines with primary amine end groups and they are commercially available up to generation 5 [45]. Polyaryl ether dendrimers (Figure 3(c)) also have been evaluated for drug delivery, but it was found that it is required to use solubilizing groups at the periphery of them due to their poor water solubility [46].

In addition, biodegradable dendrimers have been designed such as those based on polylysine (Figure 3(d)), poly(disulfide amine), polyether, or polyester and after

suitable surface modifications they have been developed as promising antiviral, antibacterial, chemotherapeutic, and vaccine carrier candidates. Glycodendrimers, that include carbohydrates in their architecture, also have great potential as drug carriers. Most of the glycodendrimers have saccharide residues on their outer surface, but glycodendrimers with a sugar central core have also been described [47]. Amino acid-based dendrimers, peptide dendrimers, hydrophobic dendrimers, and asymmetric dendrimers were also investigated for a variety of pharmaceutical applications [30, 48].

Surface engineered dendrimers were developed as a strategy for abatement of dendrimer toxicity. Functionalization helps the dendrimers gain some beneficial properties for their use as a drug delivery system as well as reducing the inherent toxicity [37]. One of the most popular modifications of dendrimer surface is PEGylation which offers so many advantages in addition to cytotoxicity reduction such as improved bioavailability/oral delivery application related to improved biodistribution and pharmacokinetics, enhanced solubility, increase in drug loading, sustained and controlled delivery of drugs, better transfection efficiency, and tumor localization [49]. Acetylation is another surface engineering approach to reduce toxicity based on modification of surface amino groups with acetyl groups [50].

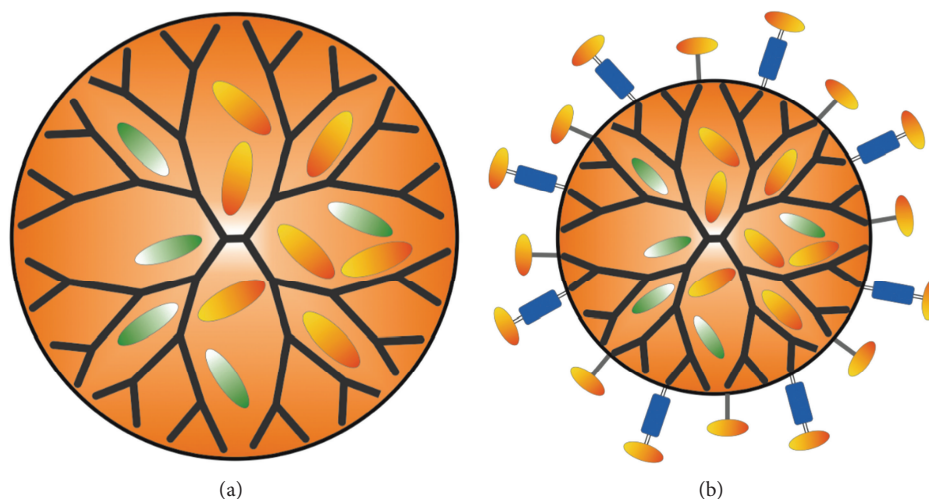


FIGURE 4: Schematic representation of drug encapsulated (a) and drug conjugated dendrimers (b).

There are already several dendrimer-based FDA-approved products in the market. For example, Stratus CS Acute Care (Dade Behring), containing dendrimer-linked monoclonal antibody, was launched for “cardiac diagnostic testing,” while another product based on modified “Tomalia-type PAMAM” dendrimers, SuperFect (Qiagen), is a well-known gene transfection agent available for a wide range of cell lines [51, 52]. In addition, VivaGel, which is a formulation of “polyanionic lysine G4 dendrimers” with an anionic surface of “naphthalenedisulfonate (SPL7013) in a Carbopol gel” that shows antiviral activity against HIV and HSV for the treatment, has already been taken into clinical trials by Starpharma, according to FDA requirements. It is currently in Phase III clinical trials and it is also the subject of a license agreement with Durex condoms for use as a condom coating [53, 54].

5. Interactions between Dendrimers and Drug Molecules

The dendrimer end group functionality can be modified to obtain molecules with novel biological properties such as cooperative receptor-ligand interactions, which will help dendrimers to interact with poorly soluble drugs. Dendrimers are able to increase the cellular uptake, bioavailability, and therapeutic efficacy, and they can also be used to optimize the biodistribution and to reduce the systemic toxicity, clearance, and degradation rate drugs [55].

There are two methods of dendrimer drug delivery: either lipophilic drugs can be encapsulated within the hydrophobic dendrimer cavity to make them water soluble or drugs can be covalently attached onto the dendrimer surface. Encapsulation traps the drug inside the dendrimer using the interaction between drug and the dendrimer or the steric bulk of the exterior of the dendrimer. The nature of drug encapsulation may be either a simple physical entrapment or can involve nonbonding-specific interactions within the dendrimer. On the other hand, the drug is attached to the exterior of

the dendrimer in dendrimer/drug conjugates. Conjugates are usually prodrugs that are either inactive or have decreased activity. The covalent conjugation of the drugs was mainly used for targeting and achieving the higher drug payload, whereas the noncovalent interactions have resulted in higher solubility of insoluble drugs [23, 56]. A basic schematic representation of drug encapsulated and drug conjugated dendrimers is given in Figure 4. The unique architectural feature of dendrimers and dendrons makes them also ideal for the construction of cross-linked covalent gels, and for the self-assembled noncovalent gels [57].

Drug dendrimer interactions are affected by the structure, generation, concentration, and surface engineering of the dendrimers. For example, PAMAM and PPI have a slightly different dendritic framework. This difference in the internal branches makes PPI dendrimers relatively more hydrophobic compared to PAMAM dendrimers and that results in different solubilizing power [58]. Furthermore, modification of dendrimer surface can improve the therapeutic efficacy of drugs in terms of targeting and reduced toxicity.

5.1. Encapsulation of Drugs within Dendritic Structure. The acid-base reaction between the dendrimers and the guest molecules such as drugs with coulomb attractions pulls the guest molecules inside the dendrimer structure, whereas the hydrogen bonding keeps them together.

Jansen and coworkers reported the first encapsulation of a dye inside a dendrimer in 1994, the so-called “dendritic box” [59]. Guest molecules could be entrapped in the dendritic cavities during the synthetic process, with the help of a shell preventing diffusion from the structures, even after prolonged heating, sonication, or solvent extraction [60, 61]. Following encapsulation of dyes to dendrimers, anticancer drug encapsulation was the focus of the research. Kojima et al. encapsulated the anticancer drugs methotrexate and doxorubicin using G3 and G4 ethylenediamine-based poly(amidoamine) (PAMAM) dendrimers with poly(ethyleneglycol) monomethyl ether (M-PEG) grafts [62]. The same group also attached methotrexate

and folic acid to the exterior of the dendritic structure and targeted the tumor cells using drug-dendrimer conjugates [63].

Dendrimers with an apolar core and polar shell have been referred to as “unimolecular micelles,” whereas the dendritic structure is independent of dendrimer concentration unlike conventional micelles [64]. However, this approach has a disadvantage that it is difficult to control the release of drugs from the dendrimer core. Poly(ethylene glycol) (PEG) has been used to modify dendrimers by conjugating PEG to the dendrimer surface to form a unimolecular micelle by providing a hydrophilic shell around the dendritic core. PEGylated dendrimers are of particular interest in drug delivery because they are biocompatible and highly water soluble and they have ability to modify the biodistribution of carrier systems [65, 66].

Zimmerman et al. synthesized “cored dendrimers” with modified dendritic architecture to encapsulate the drug. Following the synthesis, the core was removed via cleavage of ester bonds, while the rest of the structure remained the same as a consequence of robust ether linkages [67, 68].

“Dendrimeric block copolymers” have been synthesized with linear hydrophilic blocks and a hydrophobic dendritic block and their ability to complex “poorly water soluble” molecules have been investigated. A series of “G1–G5 PAMAM-block-PEG-block-PAMAM triblock copolymers” were synthesized by Kim et al. and studied as potential polymeric gene carrier [69].

Dendrimer-mediated complexation has advantages in terms of stability, release control, high drug loading, and lower toxicity of entrapped drugs. However, the noncovalent complexation often leads to lower drug encapsulation and complex stability compared to covalent conjugation [70].

5.2. Dendrimer Drug Conjugation. The outer surfaces of dendrimers have been investigated as potential interaction sites with drugs to increase the loading capacity. The number of available surface groups for drug interactions increases in twofolds with each higher generation of dendrimer. Drugs can be conjugated to dendritic systems through ester, amide, or some other linkage depending on dendritic surface, which can be hydrolysed by endosomal or lysosomal enzymes, inside the cell [55, 56]. There are several ionizable groups on the surface of dendrimers, where ionizable drugs can attach electrostatically. The main used points of attachment to conjugate drugs to dendrimers are amides, esters, disulfides, hydrazones, thiol-maleimide, and sulfinyl [43]. Many reports on drug loaded-dendrimers have shown that the release of the free drug can be enhanced by a suitable linker choice, especially, the linker/spacer length and flexibility. Some of the linkers are pH-sensitive and have proven to enhance intracellular release of the free drug [71].

Patri et al. compared the covalently conjugated drug and noncovalent inclusion complex in terms of release kinetics and efficacy, using generation 5 PAMAM dendrimers for targeting methotrexate. This study indicated that the inclusion complex releases the drug immediately and drug is active *in vitro*, while covalently conjugated drug is better suited for

specifically targeted drug delivery [72]. A greater control over drug release can be achieved by the covalent drug attachment using biodegradable linkages than electrostatic drugs-dendrimer complexes. However, the major disadvantage of the conjugation is the possibility of less active drug release or too slow drug liberation potential to be effective *in vivo* [73].

5.3. Dendritic Gels. Pharmaceutical applications of hydrogels have attracted attention because of their architectural properties, drug loading capacity, and controlled drug release capability. Hydrogels are hydrophilic and “three-dimensional polymeric networks” have found application in drug delivery due to their high water absorbing capacity [74]. “*In situ* forming gels” have been investigated for a variety of applications including ocular, oral, nasal, vaginal, rectal, and injectable. [75, 76]. Dendrimers and dendrons’ can be prepared with controlled molecular sizes due to their dendritic structure and their nature is between traditional gel polymers and the organic compounds with low molecular weight used in the “self assembled supramolecular gels” [57].

A “polymer network” is usually obtained with the use of a crosslinker during polymerization. Synthesis of hydrogels has been a function of the multivalent crosslinker behavior of dendritic molecules [77]. It has been shown that the dendritic branching has an important role in the self-assembly control. Furthermore, the repeated use of its key structural motifs can lead to multiple interactions between branched units and strengthen the noncovalent interactions responsible for the “self-assembly process” [78].

6. Ocular Applications of Dendrimeric Drug Delivery Systems

Dendrimers have been investigated for ophthalmic drug delivery since it offers a number of advantages as a carrier system. It has been reported that dendrimers were used for several purposes such as drug delivery, gene delivery, antioxidant delivery, peptide delivery, biomedical imaging, and genetic testing in ophthalmology [79]. A list of the ocular applications of dendrimers is given in Table 1.

Dendrimers are able to transport into and out of the cells. PAMAM dendrimers can have different cell entry pathways, depending on the functional groups on the surface. Anionic PAMAM dendrimers are endocytosed primarily through a caveolin-mediated process, whereas neutral and cationic dendrimers are internalized in cells following a clathrin-mediated process. These pathways can be highly beneficial in the case of crossing the epithelial and retinal barriers in the cornea and retina [80, 81].

Different ocular application routes have been successfully used for dendrimeric drug delivery and better water solubility, permeability, bioavailability, and biocompatibility have been reported. Vandamme and Brobeck have evaluated several series of poly(amidoamine) (PAMAM) dendrimers for controlled ocular drug delivery and residence time of pilocarpine nitrate and tropicamide was found to be longer for the anionic dendrimer solutions. Results of a “miotic activity test” on albino rabbits showed that these PAMAM

TABLE 1: Ocular applications of dendrimers and dendrimeric delivery systems.

Drug	Dendrimer type	Administration	Treatment	Outcomes	References
Piropine nitrate and tropicamide	PAMAM G1.5-4	Topical	Myosis and mydriasis	Increased corneal residence and prolonged reduction of IOP	[82]
Carteolol	Phosphorus containing dendrimers	Topical	Glaucoma	Increased corneal residence and reduced toxicity and IOP	[83]
Gatifloxacin	Dendrimeric polyguanidylated translocators	Topical	Conjunctivitis and intraocular infections	Enhanced corneal transport and increased antimicrobial activity	[84]
Glucosamine and glucosamine 6-sulfate	PAMAM G3.5-COOH	Subconjunctival injection	Antiangiogenic in glaucoma surgery	Reduced inflammation and no scar formation	[38]
Carboplatin	PAMAM G3.5-COOH (dendrimeric nanoparticles)	Subconjunctival injection	Retinoblastoma	Increased half life and bioavailability Reduced drug toxicity and tumor mass	[39]
VEGF-ODN	Lipophilic amino-acid dendrimer	Intravitreal injection	CNV	Prolonged suppression of VEGF and neovascularization	[85]
—	Polypropyleneimine octaamine G2	Corneal scaffold	Corneal tissue engineering	Enhanced human corneal epithelial cell growth	[86]
—	Surface modified-COOH ending dendrimers	Corneal scaffold	Corneal tissue engineering	Promoted adhesion and proliferation of human corneal epithelial cells	[87]
—	Modified G1, G2, and G3 dendrimers	Topical (corneal hydrogel adhesive)	Corneal wounds	Wound sealing and no scar formation	[88]
Photosensitizer	G3 aryl ether dendrimer zinc porphyrin	Intravenous injection-photodynamic therapy	CNV	Accumulation in neovascularized area	[89]
Concanavalin A	Porphyrin glycodendrimers	Topical-Photodynamic therapy	Intraocular tumors and retinoblastoma	Enhanced targeting and reduced toxicity	[90]
—	Porphyrin dendrimers	Topical-Photodynamic therapy	AMD and CNV	Selective accumulation in inflammatory cells and prolonged retention time	[91, 92]
DNA	Phthalocyanine dendrimers	Topical	AMD and CNV	Accumulation in photo-irradiated areas and increased transgene expression	[93]
—	Anionic and cationic carbosilane dendrimers	Topical	Tolerance	Hydrogen bonding between mucin and PAMAM-enhanced retention time	[94]
Puerarin	PAMAM	Topical	Ocular hypertension and cataract	Increased bioavailability	[95]
Fluocinolone acetonide	PAMAM G4-OH	Intravitreal injection	Retinal neuroinflammation	Reduced inflammation	[96]
Brimonidine and timolol maleate	PAMAM hydrogel (G3)	Topical	Glaucoma	Increased uptake	[97]
Brimonidine and timolol maleate	Hybrid PAMAM dendrimer hydrogel/PLGA nanoparticle	Topical	Glaucoma	Increased uptake	[98]
—	Lys _x Cys _y dendritic polymers- <i>in situ</i> gel	Topical	Cataract incisions	Wound sealing	[99]

formulations improved pilocarpine nitrate bioavailability compared to the control and also prolonged the reduction of intraocular pressure (IOP), indicating increased precorneal residence time [82].

Durairaj et al. investigated dendrimeric polyguanidylated translocators (DPTs), which are a class of dendrimers with tritoyl branches and surface guanidine groups as potential ophthalmic carriers for gatifloxacin, a “fourth generation fluoroquinolone” approved for conjunctivitis treatment. The results have indicated that the DPT forms stable gatifloxacin complexes and enhances solubility, permeability, anti-MRSA activity, and *in vivo* delivery of gatifloxacin and seems like a potential delivery system allowing once a day dosing [84].

“Phosphorus containing dendrimers,” with one quaternary ammonium salt as core and several carboxylic acid terminal groups have been synthesized from generation 0 to generation 2 by Spataro et al. These dendrimers have been tested *in vivo* in a rabbit model to evaluate ocular carteolol delivery and an increase of the carteolol amount in the aqueous humour is observed. No irritation was observed, even several hours after cationic dendrimers were applied [83].

Shaunak et al. have synthesized water soluble conjugates of D(+)-glucosamine and D(+)-glucosamine 6-sulfate with anionic PAMAM (G3.5) dendrimers to obtain synergistic “immunomodulatory and antiangiogenic effect.” When dendrimer glucosamine and dendrimer glucosamine 6-sulfate conjugates were used together in a clinically relevant scar tissue formation rabbit model after glaucoma filtration surgery, it has been shown that the long-term success of the surgery has increased from 30% to 80%. Furthermore, neither microbial infections nor clinical, biochemical, or hematological toxicity was observed in all animals [38].

Intraocular tumors such as retinoblastoma present a high risk of complications with high metastatic potential. One of the limited studies that have been done for drug delivery to intraocular tumors has explored the use of PAMAM dendrimers with carboxyl end groups (G3.5-COOH) for extended half life and sustained delivery of carboplatin with lowered therapeutic toxicity. Carboplatin-loaded PAMAM dendrimer complexes were explored in a transgenic murine retinoblastoma model, following subconjunctival administration. It was reported that the carboplatin-loaded dendrimer nanoparticles not only crossed the sclera, but were also retained for an extended period of time in the tumor vasculature, providing a sustained treatment effect [39].

In another research, biocompatible conjugates of lipophilic amino-acid dendrimers have been developed with collagen scaffolds to obtain better physical and mechanical properties and adhesion ability. Dendrimers-based approach was used for antivascular endothelial growth factor oligonucleotide (VEGF-ODN) delivery and successfully tested in a rat model to treat choroidal neovascularization (CNV). The results indicated that dendrimer/ODN-1 complexes significantly suppressed VEGF expression in cell level studies around 40 to 60%. Examinations of injected rat eyes also showed that no significant toxicity and damage were caused by the complex injections [85].

The repair of wounds such as corneal wounds that arise from surgical procedures has a significant importance for clinical aspects and research. Therefore, Duan and coworkers have generated highly crosslinked collagen using G2 polypropyleneimine octaamine dendrimers to use it as a tissue-engineering corneal scaffold. The optical transparencies of the dendrimer crosslinked collagen, EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride), and glutaraldehyde crosslinked collagen thermal gels were compared and the transparency of dendrimer crosslinked collagen was found to be significantly higher. Results have shown that dendrimer crosslinked collagen gels improved “human corneal epithelial cell growth” and adhesion without cell toxicity [86]. The same group conjugated the “surface modified dendrimers” with cell adhesion peptides to be used as corneal tissue engineering scaffolds and the material has been incorporated into both bulk structures of the gels and onto the gel surface. Dendrimer amine groups were modified using carboxyl group and it was found that the surface modification promoted human corneal epithelial cell adhesion and proliferation [87].

Grinstaff has developed a series of dendrimeric adhesives, to repair corneal wounds, composed of generations 1, 2, and 3 (G1, G2, and G3) combined with PEG, glycerol, and succinic acid. The polymer was modified to contain terminal methacrylate groups, $([G1]-PGLSA-MA)_2-PEG$. Two strategies have been explored to form the ocular adhesives: a photocrosslinking reaction and a peptide ligation reaction to couple the individual dendrimers together. It was reported that both hydrogels were adhesive, elastic, soft, transparent, and hydrophilic. The *in vivo* studies in chicken eyes have indicated that the photocrosslinkable $([G1]-PGLSA-MA)_2-PEG$ adhesive completely sealed on postoperative day. The histological studies have also demonstrated that wounds sealed using these adhesive gels appeared to be more complete after 28 days as compared to sutured wounds. The advantage of photo-cross-linked gels is the light-induced ability of the polymer to crosslink and adhere to the tissue; however, there is a potential risk of ocular damage when using light [88].

Photodynamic therapy is a potentially efficient treatment for retinoblastoma along with the other various solid tumours. Makky et al. have designed a photosensitizer, porphyrin-based glycodendrimers with the mannose-specific ligand protein Concanavalin A conjugated on to their surface, to specifically target the tumor cells in the retina. It was reported that the mannosylated dendrimers demonstrated specific interactions with the receptors in the lipid bilayer and accumulation in malignant ocular tissue was enhanced [90]. Dendrimers have also been explored as drug carriers and photosensitizers for exudative age-related macular degeneration (AMD) and CNV treatment. Nishiyama et al. investigated porphyrin-based dendrimers for their efficacy in treating retinal tumors and exudative AMD associated with CNV. The formulations showed selective accumulation within 24 h in the CNV lesions when injected into a CNV rat model [91, 92]. The same group developed phthalocyanine core-based dendrimer photosensitizers, which can be used to compact and deliver therapeutic

genes with a targeting approach. Transgene expression was monitored only in the irradiated areas upon subconjunctival injection of the dendrimer formulation and followed by laser irradiation [93].

Sugisaki et al. investigated the accumulation of dendrimer porphyrin (DP), DP encapsulated polymeric micelles, and the efficacy of photodynamic therapy (PDT) using a corneal neovascularization model in mice. In this study a 3rd generation “aryl ether dendrimer zinc porphyrin” with carboxyl ending groups and polymeric micelles composed of the DP and PEG-poly(L-lysine) was used for PDT as a photosensitizer formulation. The results showed that following administration, in 1 hour to 24 hours, both DP and DP-micelle were accumulated in the neovascularized area [89].

Bravo-Osuna and coworkers investigated the *in vitro* and *in vivo* tolerance of carbosilane dendrimers (G1 and G3, anionic and cationic) for topical ophthalmic administration. Formulations were applied to New Zealand albino rabbits and it was reported that animals did not present either discomfort or clinical signs after the administration of dendrimer solutions. Nonionic interactions via hydrogen bonding between the PAMAM dendrimer surface moieties and mucins were observed. MTT test results also showed that anionic dendrimers were nontoxic for both conjunctival and corneal cells [94].

In a recent study, Puerarin-dendrimer complexes were prepared using PAMAM dendrimers (G3.5, G4, G4.5, and G5) and their physicochemical properties, *in vitro* release, corneal permeation, and ocular residence times were determined. Valia-Chien evaluated the corneal permeation and ocular residence time in rabbits using diffusion cells with excised corneas. It was reported that puerarin-dendrimer complexes exhibited longer residence time in rabbit eyes than puerarin eye drops, without damage to the corneal epithelium or endothelium. Also results of the *in vitro* release studies showed that puerarin release was much more slower from complexes than the free puerarin in PBS. However, corneal permeation studies suggested that there was no significant difference between puerarin-dendrimer complexes and puerarin eye drops on drug permeability coefficient [95].

Targeted drug therapy for retinal neuroinflammation was explored using “G4.0 hydroxyl-terminated PAMAM dendrimer-drug conjugate nanodevices” by Iezzi et al. Fluocinolone acetonide was conjugated to the dendrimers and *in vivo* efficacy was assessed for over a 4-week period, using the “Royal College of Surgeons rat retinal degeneration model.” It was reported that upon intravitreal administration PAMAM dendrimers were selectively localized within “activated outer retinal microglia” in two retinal degeneration rat models and the dendrimers were detected in the target cells, even 35 days after administration [96].

A PAMAM dendrimer hydrogel has been developed by Holden and coworkers that is made from “ultraviolet-cured PAMAM dendrimer” linked with PEG-acrylate chains for the delivery of two antiglaucoma drugs which were brimonidine (0.1% w/v) and timolol maleate (0.5% w/v). Dendrimeric hydrogel was obtained by crosslinking of the reactive acrylate groups, triggered by UV light. It was reported that the dendrimeric hydrogel was mucoadhesive and nontoxic to

epithelial cells of human cornea. Higher uptake from “human corneal epithelial cells” and significantly enhanced “bovine corneal transport” were reported for both drugs, compared to the eye drops. The higher uptake in the dendrimeric hydrogel formulations explained the temporary decomposition of the corneal epithelial tight junctions [97]. The same group also developed a novel “hybrid PAMAM dendrimer hydrogel/poly(lactic-co-glycolic acid) (PLGA) nanoparticle platform (HDNP)”, again for codelivery of brimonidine and timolol maleate. The formulations were also evaluated in terms of their *in vitro* potential toxicity and it was found that the formulation was noncytotoxic to human corneal epithelial cells. Following topical administration of the HDNP in adult “normotensive Dutch-belted male rabbits,” formulation was found to be effective and maintained significantly higher concentrations of both drugs up to 7 days in aqueous humor and cornea compared to saline. Furthermore, it was reported that dendrimeric hydrogel and PLGA nanoparticles were not inducing any ocular inflammation or discomfort. This study demonstrated that this new formulation is able to enhance drug bioavailability, and following topical administration, it is capable of sustaining drug activity [98]. Wathier and coworkers also developed an *in situ* gel formulation using Lys_xCys_y dendritic polymers to be used in cataract incisions instead of nylon sutures. It was reported that the hydrogel sealant procedure was simple and required less surgical time than conventional suturing and no additional tissue trauma was inflicted [99].

7. Conclusion

Effective treatment of ocular diseases is still a challenge in pharmaceutical research, because of the unique physiology of eye and presence of the ocular barriers especially in posterior segments of the eye. Research in ophthalmic drug delivery during the last two decades has undergone major advancements from the use of conventional formulations such as solutions, suspensions, and ointments to viscosity-enhancing *in situ* gel systems, different inserts, colloidal systems, and so forth.

Given their structural features, most of the ocular diseases would benefit from long-lasting drug delivery of dendrimers and dendrimer-based drug delivery systems. It was already reported that dendrimers present practical solutions to drug delivery issues such as solubility, biodistribution, and targeting. Since it is easy to control the features of dendrimers such as their size, shape, generation, branching length, molecular size, and surface functionality, these compounds are ideal carriers in pharmaceutical applications. Recent researches have shown that dendrimers are able to

- (i) enhance the corneal residence time of drugs administered topically,
- (ii) target retinal neuroinflammation and provide targeted, sustained neuroprotection in retinal degeneration,
- (iii) deliver drugs to the retina upon systemic administration,

(iv) be effective as corneal glues to potentially replace sutures following corneal surgeries.

Even though dendrimers are not approved yet for clinical use in the eye, their promising preclinical results can provide significant opportunities.

Conflict of Interests

The authors would like to declare that they have no conflict of interests and have received no payment in the preparation of this paper.

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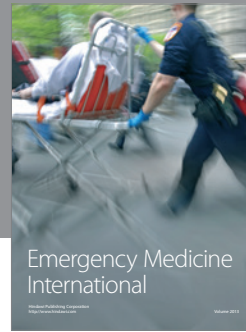
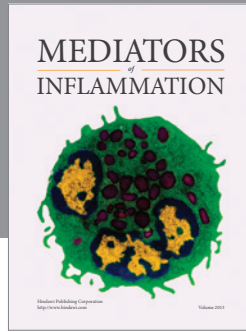
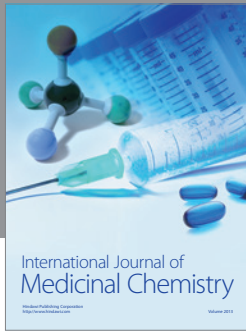
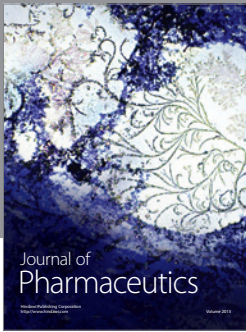
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