

**Adeno associated virus (AAV) mediated Lysyl Oxidase gene
therapy for Keratoconus and the constructs thereof**

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Priority Claim:

[0001] This application claims priority from the provisional application numbered
202341034428 filed with Indian Patent Office, Chennai on 17th June 2023 entitled
“Adeno associated virus (AAV) mediated Lysyl Oxidase gene therapy for
10 Keratoconus and the constructs thereof”, the entirety of which is expressly
incorporated herein by reference.

Preamble to the Description

[0002] The following specification describes the invention and the manner in which
it is to be performed:

15 **DESCRIPTION OF THE INVENTION****Technical field of the invention**

[0003] The present invention relates to Adeno associated virus (AAV) mediated
Lysyl Oxidase (LOX) gene constructs for treatment of the corneal disease
Keratoconus. More particularly, the invention discloses AAV mediated LOX
20 delivery, which improves the collagen expression hence enhancing the collagen
crosslinking. The present invention aids in effective management of Keratoconus.
The Adeno associated virus (AAV) mediated Lysyl Oxidase gene constructs is safe
and biocompatible.

Background of the invention

25 [0004] Keratoconus commonly referred to as conical cornea is characterized by the
progressive thinning of the cornea causing eventual bulging of the cornea in a
conical shape. Keratoconus causes glaring, light sensitivity, altered refractive error,
and blurred vision. Most severe cases of Keratoconus can lead to blindness. The

progression of Keratoconus is classified as form fruste or suspect Keratoconus, mild to moderate Keratoconus and severe Keratoconus.

[0005] The common causes of Keratoconus include genetic and environmental factors, stress, atopy, inflammation and rubbing of the eyes. The rubbing of the eyes excessively and vigorously causes trauma to the cornea, that may lead to ectasia. Pre-existing conditions including Retinitis Pigmentosa, Down syndrome, Ehlers-Danlos syndrome, Marfan syndrome, allergy and asthma increase the risk of Keratoconus. The symptoms of Keratoconus consist of blurred and distorted vision, irregular bilaterally asymmetric astigmatism, glare, sensitivity to light, and clouding. Severe cases of Keratoconus lead to loss of vision, swelling of the cornea and hydrops, and corneal scarring.

[0006] The genetic factors responsible for Keratoconus are described under several phenomena including specific loci from familial studies, gene-gene interactions, genetic heterogeneity, associated variants from genome wide studies. Variations in several genes have been associated with developing Keratoconus. The genes associated with the occurrence of Keratoconus are the genes involved in eye development, the formation and structure of the cornea, the Extracellular Matrix (ECM), inflammation, and the regulation of cell growth.

[0007] Most of the variations in gene expression are associated with the synthesis of Extracellular Matrix proteins including collagen. Collagen belongs to a family of proteins, which functions to support and strengthen the tissues. The assembly of multiple collagen fibrils involving various enzymes forms the collagen protein. Keratoconus is associated with alterations in the Extracellular Matrix (ECM) leading to degradation due to enhanced ECM degrading enzymes. Deregulated collagen levels and deficient production of collagen fibril-maturing enzyme Lysyl Oxidase (LOX) influence the pathogenesis of Keratoconus.

[0008] The treatment options for Keratoconus focus on the management of distorted vision caused by the thinning and bulging of the cornea. The existing treatment options for Keratoconus are the use of soft contact lenses to correct near-sightedness and astigmatism, rigid permeable contact lenses to correct the vision

for progressive Keratoconus, intact implants to flatten the shape of bulged cornea, corneal crosslinking to prevent progression and corneal transplant surgery for late-stage Keratoconus.

[0009] The Patent Application **KR102466887B1** entitled “*Optogenetic vision restoration using Chrimson*” discloses a composition comprising a vector expressing chrimson protein fused to Td-Tomato (TdT). The vector system enables effective protein expression for the treatment and prevention of ocular diseases. The composition comprising chrimson proteins Chrimson 88 and Chrimson R is expressed in an adeno-associated virus vector. The composition effectively aids in preventing ocular diseases including glaucoma, cataract, corneal dystrophy, Keratoconus, blinding disorders caused by photoreceptor degeneration, dysfunction, loss or death, retinal dystrophy, retinitis pigmentosa (RP), retinal degeneration due to loss of photoreceptor function, macular degeneration (MD), congenital non-progressive night blindness, and age-related macular degeneration. The level of light stimulation induces retinal ganglion cell response below the radiation safety limit. The composition comprises AAV2 and AAV2.7m8 vectors and a promoter.

[0010] The Patent Application **WO2010091279A1** entitled “*METHODS AND COMPOSITIONS FOR TREATMENT OF NEOVASCULARIZATION*” discloses a composition comprising Lysyl Oxidase type enzymes for the treatment of ocular neovascularization. The Lysyl Oxidase type enzyme binds to an antibody including Lysyl Oxidase (LOX) and LOX 2. The composition and method aid in the prevention of neovascularization in age-related macular degeneration (AMD), diabetic retinopathy (DR) and retinopathy of prematurity. The composition comprising a polynucleotide encoding the antibody is introduced into the eye consisting of retinal cells. The polynucleotide encoding antibody is encapsulated in an adeno associated viral (AAV) vector system comprising AAV type 2 and AAV type 4.

[0011] Although several courses of action are available for the treatment of Keratoconus, the course of action focuses on the management of symptoms caused

by Keratoconus. The course of action for Keratoconus aims at rectification of vision caused by thinning and bulging of cornea. Hence, there is a requirement for long-term and effective treatment for Keratoconus aiding in the efficient regulation of collagen synthesis.

5 Summary of the invention

[0012] The present invention overcomes the drawbacks of the existing systems to provide an adeno associated virus (AAV) mediated gene constructs for Keratoconus. The invention discloses a method for the generation of a recombinant adeno associated virus (rAAV) comprising Lysyl Oxidase (LOX) gene, wherein the
10 recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector is transduced into the fibroblast cells for expression of the Lysyl Oxidase (LOX) gene. The adeno associated virus (AAV) mediated Lysyl Oxidase (LOX) delivery enhances the expression of Lysyl Oxidase (LOX) by improving the collagen crosslinking.

15 [0013] According to the invention, the process of constructing the recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector comprises the steps of culturing the human corneal lenticule tissue to obtain human corneal fibroblast cells, wherein the human corneal lenticule tissue is washed with an antibiotic-antimycotic solution and the washed tissue is cut into minute pieces. The cut pieces
20 are incubated at 37°C using a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM) and Nutrient Mixture F12 supplemented with 10% fetal bovine serum (FBS), 0.3 mg/mL L-glutamine 0.1 mg/mL streptomycin, and 1,000 IU/ mL penicillin.

[0014] Further, the cultured human corneal fibroblast cells are subjected to ribose
25 nucleic acid (RNA) extraction, wherein the extracted RNA is converted to complementary deoxyribose nucleic acid (cDNA) for the analysis of gene expression of Lysyl Oxidase (LOX) enzyme. The gene expression of Lysyl Oxidase (LOX) gene is analyzed by the quantitative polymerase chain reaction (qPCR) by converting the extracted ribose nucleic acid (RNA) to complementary deoxyribose
30 nucleic acid (cDNA) using a kit, for example: cDNA synthesis kit. Further, the

analysis of expression of the Lysyl Oxidase (LOX) protein is performed by electrophoresing the protein sample on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with an anti-LOX antibody. From the cDNA, the LOX open reading frame is cloned into the AAV inverse terminal repeats (ITR) containing construct to generate the AAV.LOX constructs.

[0015] In further steps, the recombinant adeno associated virus (rAAV) LOX vector is generated by adenoviral-free triple plasmid transfection by the calcium phosphate method, wherein the generated recombinant adeno associated virus (rAAV) LOX vector is purified and the recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector cells are harvested and subjected to centrifugation to obtain pellets. The cells are resuspended in 10mM Tris-HCl buffer followed by sonication, where the cells are further treated with DNase I followed by the addition of trypsin and Sodium Deoxycholate and incubated for 30 minutes at 37°C. The cell suspension is further mixed with cesium chloride and is subjected to centrifugation, wherein the clear suspension is further ultracentrifuged at 46000 rpm for 40 hours and the fractions from the ultracentrifuge are collected and analyzed by quantitative PCR.

[0016] Additionally, the extracted viral DNA from the recombinant adeno associated virus (rAAV) LOX vector is subjected to slot blot analysis, and the purified recombinant adeno associated virus (rAAV) LOX vector is subjected to viral DNA extraction followed by slot blot hybridization, wherein the slot blot is hybridized for a positive signal by the LOX probe. Further, the recombinant adeno associated virus (rAAV) LOX vector is transduced to evaluate the infection, where the transduction and the rate of infection are analyzed using human stromal fibroblast cells. Further, the recombinant adeno associated virus (rAAV) LOX vector is processed and analyzed for polymerization of collagen and the analysis of LOX activity was performed by measuring the relative fluorescence.

[0017] The present invention is advantageous as the recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) gene constructs are safe, biocompatible, and efficient. The recombinant adeno associated virus Lysyl Oxidase (rAAV LOX)

gene constructs help to prevent the ECM degradation caused by the progression of Keratoconus. Further, the transduction of endogenous collagen crosslinking enzyme LOX by rAAV facilitates in crosslinking of collagen, thereby preventing the degradation of ECM. Additionally, the present invention facilitates reduction of the expression of ECM degrading enzyme MMP9 and enhancing the expression of ECM proteins including LOX, fibronectin and CTGF. Further, the invention enhances the percentage of gel contraction of collagen along with improving the elasticity, hence improving collagen crosslinking, tensile strength and corneal stiffness.

10 **Brief description of the drawings**

[0018] The foregoing and other features of embodiments will become more apparent from the following detailed description of embodiments when read in conjunction with the accompanying drawings. In the drawings, like reference numerals refer to like elements.

15 [0019] **Figure 1** illustrates the flowchart for constructing the recombinant AAV LOX vector.

[0020] **Figure 2** illustrates the expression of LOX by rAAV LOX.

[0021] **Figure 3** illustrates the percentage contraction of rAAV LOX in human fibroblast cells.

20 [0022] **Figure 4** illustrates the LOX enzyme activity in rAAV LOX transduced human fibroblast cells.

[0023] **Figure 5** illustrates the IHC staining of rAAV LOX.

[0024] **Figure 6** illustrates the collagen expression and lamellar structure upon transduction of rAAV LOX.

25 [0025] **Figure 7** illustrates the gene expression of LOX, collagen-I, α - Smooth Muscle Actin (α - SMA), fibronectin and MMP9 upon rAAV LOX transduction.

[0026] **Figure 8** illustrates the relative gene expression of LOX, collagen-I, collagen-IV and MMP9 in the cornea.

[0027] **Figure 9** illustrates the immunofluorescence staining of LOX in corneal tissues.

5 [0028] **Figure 10** illustrates the elastic modulus of transduced with rAAV LOX.

[0029] **Figure 11** illustrates the ocular tissue biocompatibility of rAAV LOX.

[0030] **Figure 12A** illustrates the slit-lamp biomicroscopy examined rabbit eyes for pre- and post-12 weeks of AAV.LOX and AAV.eGFP (Control) intra-stromal injections.

10 [0031] **Figure 12B** illustrates the central corneal thickness (CCT) and pachymetry pre- and post-injections measured using Anterior Segment Optical Coherence Tomography (AS-OCT) for assessing the changes in corneal thickness.

15 [0032] **Figure 12C** illustrates the posterior segments including the retina and optic nerve analysed using Fundus and Polarization-Sensitive Optical Coherence Tomography (PS-OCT), before and after injections.

[0033] **Figure 12D** illustrates the corneal and intraocular structures analysed using in-vivo confocal examination with the Heidelberg Retinal Tomograph (HRT), pre- and post-injections.

20 [0034] **Figure 12E** illustrates the examination of the corneal endothelial cells using specular microscopy, pre- and post-injections, assessing density, morphology, and cell size.

[0035] **Figure 12F** illustrates the corneal thickness, curvature, and parameters assessed using intra-stromal measurements, post-week 12, providing insights into corneal structural integrity and treatment efficacy.

25 **Detailed description of the invention**

[0036] In order to more clearly and concisely describe and point out the subject matter of the claimed invention, the following definitions are provided for specific terms, which are used in the following written description.

[0037] The term “*Adeno-Associated Virus or AAV*” refers to non-enveloped virus,
5 which can be engineered for DNA delivery to target cells.

[0038] The term “*Gene Constructs*” refers to modification of genes in the cells to treat a disease.

[0039] The term “*Extracellular Matrix (ECM)*” refers to a network of proteins that provide structure and support to the cells and tissues.

10 [0040] The present invention discloses an adeno associated virus (AAV) mediated gene constructs for Keratoconus. The present invention discloses a method for the generation of recombinant AAV comprising Lysyl Oxidase (LOX) gene. The rAAV LOX vector is transduced into fibroblast cells for the expression of LOX. The AAV mediated LOX delivery enhances the expression of LOX and decreases the
15 expression of matrix metalloproteases hence improving the collagen crosslinking.

[0041] FIG 1 illustrates the flowchart for constructing the recombinant AAV LOX. The process (100) begins with a step (101) where the human corneal lenticule tissue is cultured to obtain human corneal fibroblast cells. The human corneal lenticule tissue is cultured by washing the tissue with an antibiotic-antimycotic solution. The
20 washed tissue is cut into minute pieces followed by incubating in at 37°C using a 1:1 mixture of Dulbecco’s modified Eagle medium (DMEM) and Nutrient Mixture F12 supplemented with 10% fetal bovine serum (FBS), 0.3 mg/mL L-glutamine 0.1 mg/mL streptomycin, and 1,000 IU/ mL penicillin.

[0042] At step (102), the cultured human corneal fibroblast cells are subjected to
25 ribose nucleic acid (RNA) extraction. The extracted RNA is converted to complementary deoxyribose nucleic acid (cDNA) for the analysis of gene expression of Lysyl Oxidase (LOX) enzyme. The gene expression of LOX is analyzed by quantitative polymerase chain reaction (qPCR) by converting the extracted RNA to cDNA using cDNA synthesis kit. The analysis of LOX protein

expression is performed by electrophoresing the protein sample on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with an anti-LOX antibody. From the cDNA, the LOX open reading frame is cloned into the AAV inverse terminal repeats (ITR) containing construct to generate the
5 AAV.LOX constructs.

[0043] At step (103), the recombinant adeno associated virus (rAAV) LOX vector is generated by adenoviral-free triple plasmid transfection by the calcium phosphate method. At step (104), the generated rAAV LOX vector is subjected to purification. The rAAV LOX cells are harvested followed by centrifugation at a low speed to
10 obtain pellets. The cells are resuspended in 10mM Tris-HCl buffer followed by sonication. The cells are further treated with DNase I followed by the addition of 0.25% Trypsin and 10% Sodium Deoxycholate and incubating for 30 minutes at 37⁰ C. The cell suspension is further mixed with cesium chloride at a density of 0.6814 g/ml and centrifuged. The clear suspension from the centrifuge is
15 ultracentrifuged at 46000 rpm for 40 hours. Further, the fractions from the ultracentrifuge are collected and analyzed by qPCR.

[0044] At step (105), the extracted viral DNA from rAAV LOX vector is subjected to slot blot analysis. The purified rAAV LOX vector is subjected to viral DNA extraction followed by slot blot hybridization. The slot blot is hybridized for a
20 positive signal by LOX probe. At step (106), the rAAV LOX vector is transduced to evaluate the infection. The transduction and the rate of infection is analyzed using human stromal fibroblast cells.

[0045] At step (107), rAAV LOX vector is subjected to collagen gel contraction assay and human corneal lenticule extraction and transduction. The transduced
25 LOX to human fibroblast cells is detached by the addition of 0.25% trypsin and 0.02% ethylene diamine tetra acetate (EDTA). The human fibroblast cells are analyzed for polymerization of collagen by incubation for 48 hours.

[0046] At step (108), rAAV LOX vector is subjected to collagen gel contraction assay and human corneal lenticule extraction and transduction. The collagen gel
30 contraction assay and human corneal lenticule extraction is analyzed by the

intraoperative collection of lenticules and culturing in DMEM media supplemented with 10% FBS, glutamine, streptomycin and penicillin. The analysis of LOX activity was performed by standard kit and protocol comprising β -aminopropionitrile for the specific inhibition of LOX activity. The relative fluorescence determines the activity of LOX.

[0047] The following examples are offered to illustrate various aspects of the invention. However, the examples are not intended to limit or define the scope of the invention in any manner.

[0048] The transduction of rAAV LOX vector into cells is followed by performing several analyses to determine the gene expression of LOX. The analyses including immunohistochemical (IHC) studies, histopathological studies, gene expression studies, relative gene expression studies, differential expression studies and LOX overexpression studies were conducted.

Example 1: Analysis of gene expression of LOX in fibroblast cells

[0049] The rAAV LOX vector transduced tissues were subjected to immunohistochemical (IHC) studies to detect the presence of specific proteins in 4- μ m thick tissue sections. Antigen retrieval steps were performed and tissues were incubated with primary antibodies for LOX overnight. The incubation was followed by washing the primary antibodies and addition of secondary antibodies and counterstaining with hematoxylin. The intensity of staining indicates the level of LOX protein expression. **FIG 5** illustrates the IHC staining of rAAV LOX. The LOX protein expression is analyzed by IHC staining comprising negative staining, and hematoxylin and eosin staining. The IHC staining indicates upregulated expression of LOX protein in corneal stroma upon intrastromal injection of AAV.LOX compared to control AAV vector.

[0050] The rAAV LOX vectors were subjected to histopathological analysis to evaluate signs of tissue abnormalities followed by visualization under light microscopy. The transduced corneas with rAAV LOX were embedded with 4- μ m thick paraffin followed by dewaxing and rehydration. **FIG 11** illustrates the ocular

tissue biocompatibility of rAAV LOX. The ocular tissue biocompatibility was analyzed by hematoxylin and eosin staining of rAAV LOX transduced cells followed by visualization. The visualization did not indicate any structural abnormalities.

5 [0051] FIG 6 illustrates the collagen expression and lamellar structure upon transduction of rAAV LOX. The AAV.LOX vectors aid in enhanced corneal compactness or density. The enhanced gene expression was indicated by the increase in stromal density represented graphically. The enhanced gene expression of LOX further aids in the management of Keratoconus effectively. In addition to
10 enhancing the gene expression of LOX, the transduction of rAAV LOX aids in the reduction of gene expression of matrix metalloproteinase 9 (MMP9). FIG 7 illustrates the gene expression of LOX, collagen-I, α - Smooth Muscle Actin (α - SMA), fibronectin and MMP9 upon rAAV LOX transduction. The overexpression of LOX resulted in 4-fold reduction in MMP9 expression. The ECM proteins collagen-I
15 exhibited 3-fold increase and fibronectin exhibited a 2.5-fold increase in expression compared to respective controls. FIG 8 illustrates the relative gene expression of LOX, collagen-I, collagen-IV and MMP9 in the cornea. The graph indicates enhanced relative gene expression of LOX, collagen-I, and collagen-IV. Additionally, the graph indicates reduced relative gene expression of MMP9. The
20 enhanced gene expression of ECM proteins LOX, collagen-I, and collagen-IV and reduced relative gene expression of MMP9 effectively prevents the degradation of ECM proteins in the context of Keratoconus.

[0052] The rAAV LOX vector was administered topically to the corneas of mice. FIG 9 illustrates the immunofluorescence staining of LOX in corneal tissues. The
25 immunofluorescence staining exhibits the intensity of expression of LOX in cornea. The differential expression of ECM proteins indicates a significant reduction in ECM degrading enzyme MMP9 and a significant increase in the expression of collagen I and collagen IV. FIG 10 illustrates the elastic modulus of corneas transduced with rAAV LOX. The transduction of rAAV LOX gene aids in
30 enhancing the corneal strength by increasing the elastic modulus. The addition of

rAAV LOX aids in 5-to-10-fold increase in the elastic modulus indicating the restoration of the tensile strength of the ECM by collagen crosslinking activities.

[0053] The rAAV LOX vectors were injected intrastromally to the eye inside a laser created pocket within the cornea. The rAAV LOX is injected at a concentration of 5×10^{10} multiplicity of infection (MOI) of virus. Further, the cornea with the rims were excised and cultured in DMEM F12 media enriched with penicillin and streptomycin. **FIG 2** illustrates the expression of LOX by rAAV LOX. The control AAV vector injected stromal cells indicates minimal expression of LOX. The transduction of LOX caused other Extracellular Matrix (ECM) proteins including fibronectin, Connective Tissue Growth Factor (CTGF) to exhibit enhanced expression levels at concentrations of 50k MOI, 25k MOI and 10k MOI. The western blot analysis indicates notable enhancement in the expression of ECM proteins including LOX, fibronectin and CTGF.

[0054] **FIG 3** illustrates the percentage contraction of rAAV LOX vector in human fibroblast cells. The transduction of corneal cells with LOX is associated with improved contractile ability. The graph indicates the percentage contraction in primary healthy donor corneal fibroblast cells and Keratoconus corneal fibroblast cells. The healthy corneal fibroblast cells and Keratoconus corneal fibroblast cells exhibit an average of 75% gel contraction at concentrations of 50k MOI, 25k MOI and 10k MOI. The gel contraction assay indicates an increased percentage of contraction of the collagen gel with human corneal fibroblast cells on transduction with AAV.LOX vectors.

[0055] The rAAV LOX improves LOX activity in a dose-dependent manner. The secretion of LOX enzymes, hence the enzyme activity in corneal epithelial cultures is enhanced by the transduction of rAAV LOX in human healthy donor primary corneal fibroblast cells and Keratoconus patient corneal fibroblast cells. **FIG 4** illustrates the LOX enzyme activity in rAAV LOX transduced human fibroblast cells.

[0056] The present invention discloses sequences of Lysyl Oxidase and codon optimized Lysyl Oxidase for the expression of rAAV vector. The LOX open

reading frame for expression from AAV vector comprising AAV2 5' inverted terminal repeats (ITR) comprising 111 base pairs and AAV2 3' ITR comprising 116 base pairs depicted by **SEQ ID NO: 1**, a cytomegalovirus (CMV) promoter comprising 527 base pairs depicted by **SEQ ID NO: 2**, an LOX open reading frame (ORF) comprising 1254 base pairs depicted by **SEQ ID NO: 3**, and a poly adenine comprising 192 base pairs depicted by **SEQ ID NO: 4**. Additionally, codon optimized LOX for expression from AAV vectors comprising AAV2 5' ITR comprising 111 base pairs and AAV2 3' ITR comprising 116 base pairs depicted by **SEQ ID NO: 5**, a CMV promoter comprising 527 base pairs depicted by **SEQ ID NO: 6**, a codon optimized LOX comprising 1254 base pairs depicted by **SEQ ID NO: 7**, and a poly adenosine comprising 192 base pairs depicted by **SEQ ID NO: 8**.

Example 1: Studying the viscoelastic properties after intrastromal injection of AAV.LOX Virus in rabbit eye corneas.

[0057] According to some embodiments of the invention, the intrastromal injection of AAV.LOX virus was injected in the rabbit eye corneas, to determine the viscoelastic properties of the cornea. The process involved in delivering the AAV.LOX virus to the rabbit eye corneas involved giving intrastromal injection to the cornea by injecting 30 microliters of virus including AAV.LOX virus (sample) and AAV.eGFP (control), and monitoring the rabbit for 12-week period with weekly imaging till week-6 and bi-weekly imaging till week-12. The imaging techniques used for weekly and bi-weekly imaging involved slit-lamp biomicroscopy, Anterior Segment Optical Coherence Tomography (AS-OCT) examination for CCT and pachymetry, corneal and intraocular structure analysis by Heidelberg Retinal Tomograph (HRT), specular microscopy examination, and fundus and PS-OCT examination.

[0058] **Figure 12A** illustrates the examination of the rabbit eyes using slit-lamp biomicroscopy for pre- and post-12 weeks of AAV.LOX and AAV.eGFP (Control) intra-stromal injections. The slit-lamp biomicroscopy examination revealed normal corneal anatomy and transparency in both AAV.LOX and AAV.eGFP injected

corneas. The intra-stromal delivery of AAV.LOX and AAV.eGFP did not pose any negatively impact on corneal morphology or result in any clinically significant adverse effects on corneal physiology. It was observed that, throughout the 12-week period, subjective clinical evaluations showed no signs of intraocular inflammation, redness, ocular discharge, corneal or conjunctival edema, or infection in the rabbit eyes.

[0059] **Figure 12B** illustrates the central corneal thickness (CCT) and pachymetry before and after AAV.LOX and AAV.eGFP (Control) intra-stromal injections measured using Anterior Segment Optical Coherence Tomography (AS-OCT) for assessing the changes in corneal thickness. The AS-OCT examination for CCT and pachymetry involved measurement of the corneal thickness at the central region using optical coherence tomography, as disclosed in **Figure 12B**, and it revealed that in both the AAV.LOX and AAV.eGFP groups, the central and overall corneal thickness significantly increased from week 1 to week 3 following the delivery of the viruses. The corneal thickness further decreased around week 4 and remained stable for the duration of the 12-week study period.

[0060] **Figure 12C** illustrates the posterior segments including the retina and optic nerve analysed using Fundus and Polarization-Sensitive Optical Coherence Tomography (PS-OCT), before and after AAV.LOX and AAV.eGFP (Control) intra-stromal injections. With reference to **Figure 12C**, the fundus and Polarization-Sensitive Optical Coherence Tomography (PS-OCT) were performed to assess the posterior segment, including the retina and optic nerve, wherein the evaluations facilitated determination of any potential impact on the retinal structure following the intra-stromal delivery of the vectors. It was observed that, throughout the time period including multiple follow-up assessments, and there were no changes detected in the thickness of the retinal sub-layers. The results indicate that the administration of AAV.LOX and AAV.eGFP into the rabbit corneas did not adversely affect the retinal morphology or compromise the integrity of the optic nerve.

[0061] Figure 12D illustrates the corneal and intraocular structures analysed using *in-vivo* confocal examination with the Heidelberg Retinal Tomograph (HRT), before and after AAV.LOX and AAV.eGFP (control) intra-stromal injections. The *in-vivo* confocal examination was performed using Heidelberg Retinal Tomograph (HRT) to assess the corneal and intraocular structures both before and after the administration of AAV.LOX and AAV.eGFP injections, and the detailed analysis revealed that the corneas remained normal throughout the 12-week period. Further, specifically the superficial epithelial cells were observed to be flat and healthy, indicating no abnormalities on the surface of the cornea. The distribution of corneal stromal keratocytes was consistent with normal physiology, suggesting that the structural integrity and cellular arrangement within the stroma were maintained. Additionally, the endothelial cells exhibited normal morphology, which is crucial for maintaining corneal transparency and overall health. Further, no evidence of stromal fibroblast transformations was observed throughout the 12 week follow up period.

[0062] Figure 12E illustrates the examination of the corneal endothelial cells using specular microscopy, pre- and post-injections, assessing density, morphology, and cell size, before and after AAV.LOX and AAV.eGFP (control) intra-stromal injections. The specular microscopy examination was used to examine the corneal endothelial cells, to determine the cell density, morphology, and size. The results indicated that the endothelial cells maintained normal morphology throughout the 12-week period, where the cell density remained stable, suggesting that there was no significant loss or proliferation of endothelial cells following the injections, as disclosed in Figure 12E.

[0063] Figure 12F illustrates the corneal thickness, curvature, and parameters assessed using intra-stromal measurements, after week 12 of AAV.LOX and AAV.eGFP (control) intra-stromal injections, providing insights into corneal structural integrity and treatment efficacy. The analysis was conducted to examine the corneal structural integrity and treatment efficacy, wherein the uniaxial tension test experiments revealed that clear improvement was observed in the mechanical characteristics of rabbit eye corneas following intrastromal injection of the

AAV.LOX virus when compared to the control group. It was observed that, with AAV.LOX injection, it was observed that there was a substantial increase in the tensile strength, wherein the remarkable elevation in the tensile strength serves as a clear indicator of improved collagen crosslinking within the corneal stroma, indicating enhanced tissue integrity and strength, and suitable to treat the pathologic functional problem in Keratoconus eyes.

[0064] The present invention discloses adeno associated virus (AAV) mediated Lysyl Oxidase gene therapy for Keratoconus and the constructs thereof. The present invention aids in prevention of ECM degradation by the progression of Keratoconus. The insufficiency of crosslinking in collagen fibrils leads to degradation of ECM and eventual corneal thinning. The transduction of endogenous collagen crosslinking enzyme LOX by rAAV aids in crosslinking of collagen hence preventing the degradation of ECM. The present invention further aids in reducing the expression of ECM degrading enzyme MMP9. The present invention enhances the expression of ECM proteins including LOX, fibronectin and CTGF. The present invention enhances the percentage of gel contraction of collagen along with improving the elasticity hence improving collagen crosslinking, tensile strength and corneal stiffness. The present invention is safe, biocompatible, and efficient.

[0065] According to an embodiment of the invention, the recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector facilitates the treatment of corneal ectasia where the expression of Lysyl Oxidase (LOX) protein enhances the Extracellular Matrix (ECM) structures by biological crosslinking. The recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector can be used in the treatment of Keratoconus by stabilizing the corneal protrusion and thinning. Further, recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector transgene for packaging in different serotypes of AAV capsids for diverse use in functional studies or human and clinical use. Alternatively, the recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vectors are used in conditions where the Extracellular Matrix (ECM) needs to be strengthened in cornea, skin and other applications.

Claims:**We Claim:**

1. An Adeno associated virus (AAV) mediated Lysyl Oxidase (LOX) gene therapy vector, the vector comprising Lysyl Oxidase (LOX) gene, wherein the gene therapy vector is capable of expressing Lysyl Oxidase (LOX) enzyme that acts as a biological crosslinker in the cells and tissues.
2. A method for construction of recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector, the method comprising the steps of:
 - a. culturing the human corneal lenticule tissue by washing the tissue with an antibiotic-antimycotic solution to obtain human corneal fibroblast cells;
 - b. subjecting the cultured fibroblast cells to RNA extraction followed by conversion to cDNA, isolation and cloning of Lysyl Oxidase (LOX) open reading frame in adeno associated virus vector backbone;
 - c. generating the recombinant adeno associated virus (rAAV) Lysyl Oxidase (LOX) vector by adeno-viral free triple plasmid transfection by calcium phosphate method performing adenoviral-free triple plasmid transfection by the calcium phosphate method;
 - d. subjecting the generated recombinant AAV (rAAV) Lysyl Oxidase (LOX) vector to purification, and extracting the viral DNA from the purified rAAV LOX vector;
 - e. subjecting the extracted viral DNA from the recombinant AAV (rAAV) Lysyl Oxidase (LOX) vector to slot blot analysis, and evaluating the infection by transducing the recombinant adeno associated virus (AAV) (rAAV) Lysyl Oxidase (LOX) vector; and
 - f. subjecting the recombinant adeno associated virus (rAAV) Lysyl Oxidase (LOX) vector to collagen gel contraction assay and human corneal lenticule extraction and transduction to construct recombinant adeno associated virus (rAAV) with Lysyl Oxidase (LOX) gene.

3. The method as claimed in claim 2, wherein the Lysyl Oxidase (LOX) open reading frame from the cDNA is cloned into the AAV inverted terminal repeats (ITR) containing constructs, where the Lysyl Oxidase (LOX) sequence is further codon optimized and all the optimized versions are cloned in a vector backbone having AAV inverted terminal repeat (ITR) constructs.
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4. The method as claimed in claim 2, wherein the extracted viral DNA from rAAV Lysyl Oxidase (LOX) vector analysed using slot blot analysis, where the purified rAAV Lysyl Oxidase (LOX) vector is subjected to viral DNA extraction followed by slot blot hybridization demonstrating efficient packaging in AAV capsids.
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5. The method as claimed in claim 2, wherein the recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector subjected to collagen gel contraction assay, human corneal lenticule extraction and transduction, where the transduced LOX to human fibroblast cells induce significantly greater collagen contraction relevant to more tensile function.
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6. The method as claimed in claim 2, wherein the recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector facilitates prevention of Extra Cellular Matrix (ECM) degradation, through the transduction of endogenous collagen crosslinking enzyme Lysyl Oxidase (LOX) by rAAV, enhancing crosslinking of collagen.
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7. The method as claimed in claim 2, wherein the expression of Lysyl Oxidase (LOX) in the cells and tissues facilitates increase in the tensile strength and corneal stiffness.
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8. The method as claimed in claim 2, wherein the recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector enhances the expression of Lysyl Oxidase (LOX), fibronectin and connective tissue growth factor (CTGF).
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9. The method as claimed in claim 2, wherein the recombinant adeno associated virus Lysyl Oxidase (rAAV LOX) vector decreases the expression of

Extracellular Matrix (ECM) degrading protein Matrix Metalloprotease 9 (MMP9).

10. The method as claimed in claim 2, wherein the recombinant AAV mediated
5 LOX gene constructs are biocompatible.

11. The method as claimed in claim 2, wherein the recombinant AAV mediated
LOX gene constructs may be used in the treatment of atleast one corneal
complications including corneal ectasia, where the expression of LOX protein
10 enhances the extracellular matrix structures by biological crosslinking; and
Keratoconus by stabilizing the corneal protrusion and thinning.

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