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Review

Delivery strategies for CRISPR/Cas genome editing tool for retinal dystrophies: challenges and opportunities

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ABSTRACT

CRISPR/Cas, an adaptive immune system in bacteria, has been adopted as an efficient and precise tool for site-specific gene editing with potential therapeutic opportunities. It has been explored for a variety of applications, including gene modulation, epigenome editing, diagnosis, mRNA editing, etc. It has found applications in retinal dystrophic conditions including progressive cone and cone-rod dystrophies, congenital stationary night blindness, X-linked juvenile retinoschisis, retinitis pigmentosa, age-related macular degeneration, leber's congenital amaurosis, etc. Most of the therapies for retinal dystrophic conditions work by regressing symptoms instead of reversing the gene mutations. CRISPR/Cas9 through indel could impart beneficial effects in the reversal of gene mutations in dystrophic conditions. Recent research has also consolidated on the approaches of using CRISPR systems for retinal dystrophies but their delivery to the posterior part of the eye is a major concern due to high molecular weight, negative charge, and *in vivo* stability of CRISPR components. Recently, non-viral vectors have gained interest due to their potential in tissue-specific nucleic acid (miRNA/siRNA/CRISPR) delivery. This review highlights the opportunities of retinal dystrophies management using CRISPR/Cas nanomedicine.

1. Introduction

Prokaryotes, especially bacteria, dwell in a variety of environments, including many unfavorable conditions. This means they have many methods by which they adapt to survive in the harsh habitat. The defense systems acting in an undefined, natural way include the restriction-modification, abortive infection, and surface exclusion systems [1]. Recent studies have also shown an acquired immune system, such as the clustered regularly interspaced short palindromic

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repeats (CRISPR), in prokaryotic organisms, both in bacteria and archaea. These are repeat sequence elements with, 21–37 bp in length, separated by spacers of similar size but varying composition. It forms a part of the adaptive immune system developed for protection against the attacking phage. The bacterium cleaves the genome of the invading virus and assimilates short viral genetic segments amongst its CRISPR sequences, which constitutes the pathogen-specific spacer elements. Thus, when the same virus attacks the bacterium subsequently, the CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) guide the organism's CRISPR-associated (Cas) endonuclease to the foreign DNA complementary to its sequence, thereby degrading the invading viral genome [2]. A protospacer adjacent motif (PAM) that is present only in the viral genome and not in the bacterium helps it to differentiate self from non-self, thus cleaving and inactivating the virus [3].

CRISPR/Cas was discovered in 1987 and firstly demonstrated as therapeutic gene editing tool in mammalian cells in 2013 by Zhang and Church [4]. Since then, it has been identified as a potential therapeutic tool for genome editing and has been extensively studied for its application in many genetic and non-genetic diseases, including retinal dystrophies, cancer, hematological disorders, muscular dystrophies, neurodegenerative diseases, etc. The updated classification of CRISPR-Cas systems is based on the sequences of the Cas genes, the order of the repeats within the CRISPR arrays, and the organization of the Cas operons [5]. According to this system, there are three classes of CRISPR-Cas *i.e.* types I–III. Each type is further divided into subtypes, ranging from I-A to I-F, II-A to II-C, III-A and III-B. The Cas1 and Cas2 genes are present in all CRISPR-Cas types and the presence or absence of specific Cas proteins is the main basis of classification. For example, the Cas3, Cas9 and Cas10 proteins are hallmarks of CRISPR/Cas types I, II and III, respectively. There are systems that do not have the distinct Cas proteins of types I-III, are termed as unclassified (type U) [6].

The mechanism of action involves the formation of a ribonucleoprotein complex (RNP) that consists of the Cas9 protein and a guide RNA (gRNA) that can bind to the location directed by the gRNA on the genomic DNA. Upon binding, Cas9 cleaves the viral DNA, creating a double-stranded break that allows additional DNA modifications on the site [7]. The Cas9 nucleases are designed to lead to a DNA double-strand break (DSB) at the target site. Repair of the strands takes place through error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR). When a template is absent, NHEJ is activated usually, resulting in insertions and/or deletions (indels) that damage the target genome loci. When a donor template is present with homology to the targeted locus, the HDR pathway follows, enabling precise edits [8].

Recently, the CRISPR-Cas system has progressed as a remarkable engineering tool for carrying out precise and regulated genetic modifications in many microorganisms such as *Escherichia coli, Staphylococcus aureus, Lactobacillus reuteri, Clostridium beijerinckii, Streptococcus pneumonia* and *Saccharomyces cerevisiae* [9]. There are many steps involved in the application of CRISPR/Cas for bacterial genome editing. The first one being the selection of the target space in the genome which will also decide the guide RNA to be developed. Until now, various parameters such as sequence setting, gRNA binding stability, chromatin accessibility and PAM sequence have been discussed as important factors. Many software tools have been developed to forecast the on-site and off-site cleavage efficiency of sgRNAs, including CRISPOR, JATAYU and CHOPCHOP, amongst many others. The tool generates a series of sgRNA at different PAM sites within the targeted gene, which are then aligned based on their efficiency in terms of the expected on-target and off-target binding potential, as well as the other variables discussed above [10]. There are also many other parameters, such as specificity and mismatch concerns that must be investigated while creating it as a therapeutic tool. Moving forward, CRISPR/Cas9 system was adopted in three major forms i.e. plasmid, mRNA, and purified active RNP. All these forms have their inherent advantages and limitations (Fig. 1) and are therefore utilized accordingly for the therapeutic purposes.

Eye related diseases, especially retinal dystrophies are degenerative conditions marked with clinical and genetic heterogeneity and affect 1 out of every 2000 people all over the globe. More than 238 mutant genes that decide the phenotype are explored till now. The complexity of the neuronal pathways, physiological barrier due to anatomy of the eyes, the structure of each cell, and the diversity of functions of each retinal layer create many challenges in development of therapeutic strategies for these diseases. Most common site where the therapeutic agents need to work is the posterior part of the eyes which is quite inaccessible through conventional routes. Intravitreal route is beneficial in such cases with some risk of eye damage and requires expertise. There are available treatments for dystrophic conditions, such as wet age-related macular degeneration (wAMD), wherein anti-VEGF antibodies are injected through intravitreal route and were found to be beneficial. But the treatment needs multiple dosing over time and can cause eye damage due to multiple intravitreal injections. Therefore, a more relevant system needs to be developed to overcome such hurdles. Gene editing in recent times has grown to treat diseases characterized with gene mutation. CRISPR/Cas9 system could be directed towards a specific gene sequence to correct a mutation. This technique has been explored for retinal diseases, since the unique anatomical position, immune-privileged nature, blood-retinal barrier and identified underlying mutation makes eye, and specifically retina, amenable for therapeutic gene editing [11,12]. Further, it provides immense potential because of its one-time treatment possibilities *via* gene editing. CRISPR/Cas9 tool, however, is facing several delivery difficulties due to its large molecular weight. Although some viral vectors are available with limitations (**Table 1**), development of an efficient delivery vehicle for CRISPR/Cas is the need of the hour. Nanotechnology based non-viral carriers such as polymeric nanoparticles, liposomes, micelles, dendrimers etc. are currently being explored for delivery of CRISPR/Cas9. This review highlights the recent scenario of retinal dystrophic conditions and potential CRISPR/Cas based nanomedicines used in the treatment.

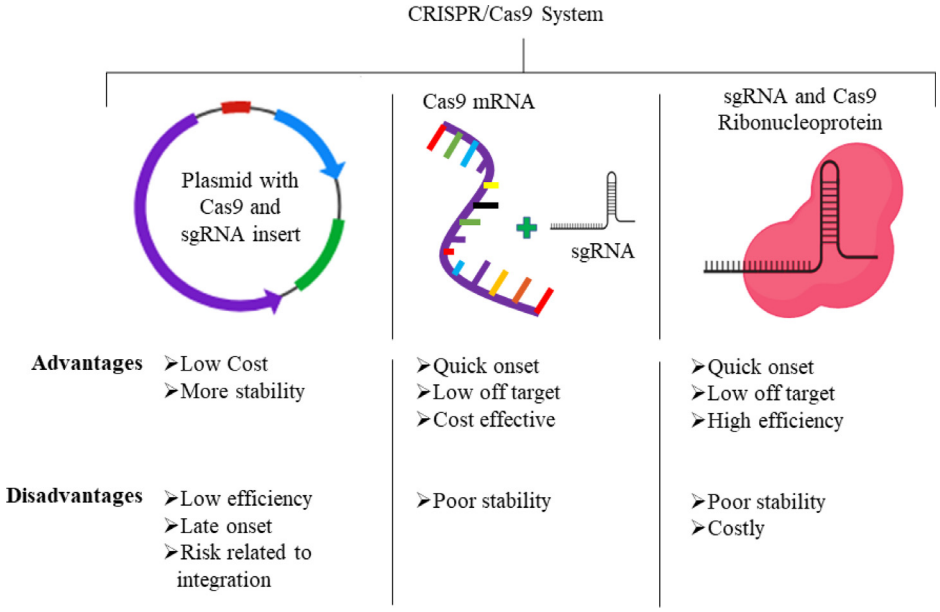

Fig. 1 – Various forms of CRISPR/Cas9 including plasmid, mRNA and RNPs that could be delivered to achieve significant gene editing to treat retinal dystrophies.

Table 1 – Pros and cons of the viral and non-viral delivery carrier used for CRISPR/Cas9 delivery to the eye.

Delivery vehicle	Pros	Cons
Viral vectors (Lentivirus, Adenovirus, baculovirus)	• High transfection *ex vivo* • High efficiency	• Risk of insertion mutagenesis [13] • Immune response [13] • Low loading efficiency • Low *in vivo* efficiency • Difficult to handle • High cost • Cannot deliver RNPs
Non-Viral vectors (Polymeric nanoparticles, dendrimers, exosomes, Liposomes, Lipid nanoparticle, polymeric micelles)	• Low cost • Ease of handling and • Ease of preparation • High loading • Can be prepared for target delivery • Low immunogenicity • Can deliver RNPs • *In vivo* stability • Less risk of mutagenesis • Flexibility	• Comparative low efficiency and transfection • Toxicity • Scalability

2. Applications of CRISPR/Cas system: Beyond double-strand break

After the success of the spCas9 as a gene-editing tool, it has been explored more for several other applications as well. Despite the specificity, the large size of spCas9 makes it difficult to deliver using viral vectors. Therefore, ample variants or orthologs have been discovered till now. *Campylobacter jejuni* (CjCas9) (984 amino acid) is a smaller Cas9 nuclease discovered in 2017 [15]. Later in 2017, the Zhang group discovered Cas13 as a new orthologue having RNA targeting potential [16]. In 2013, Qi et al. used a dead version of Cas9 (i.e. dCas9) RNPs to suppress the gene expression by interfering with the RNA polymerase binding mechanism [17]. Additionally, the same group reported the application of dCas9 protein fused with transcriptional repressor KRAB (dCas9-KRAB) in gene silencing (CRISPRi) [18]. dCas9 protein fused with transcriptional activators VP64 was explored as gene activator i.e. CRISPRa, and therefore make CRISPR a suitable tool for transcriptional programming [19]. In 2017, the Liu group reported CRISPR as a base editor without introducing DSB [20]. dCas9 fused with epigenome

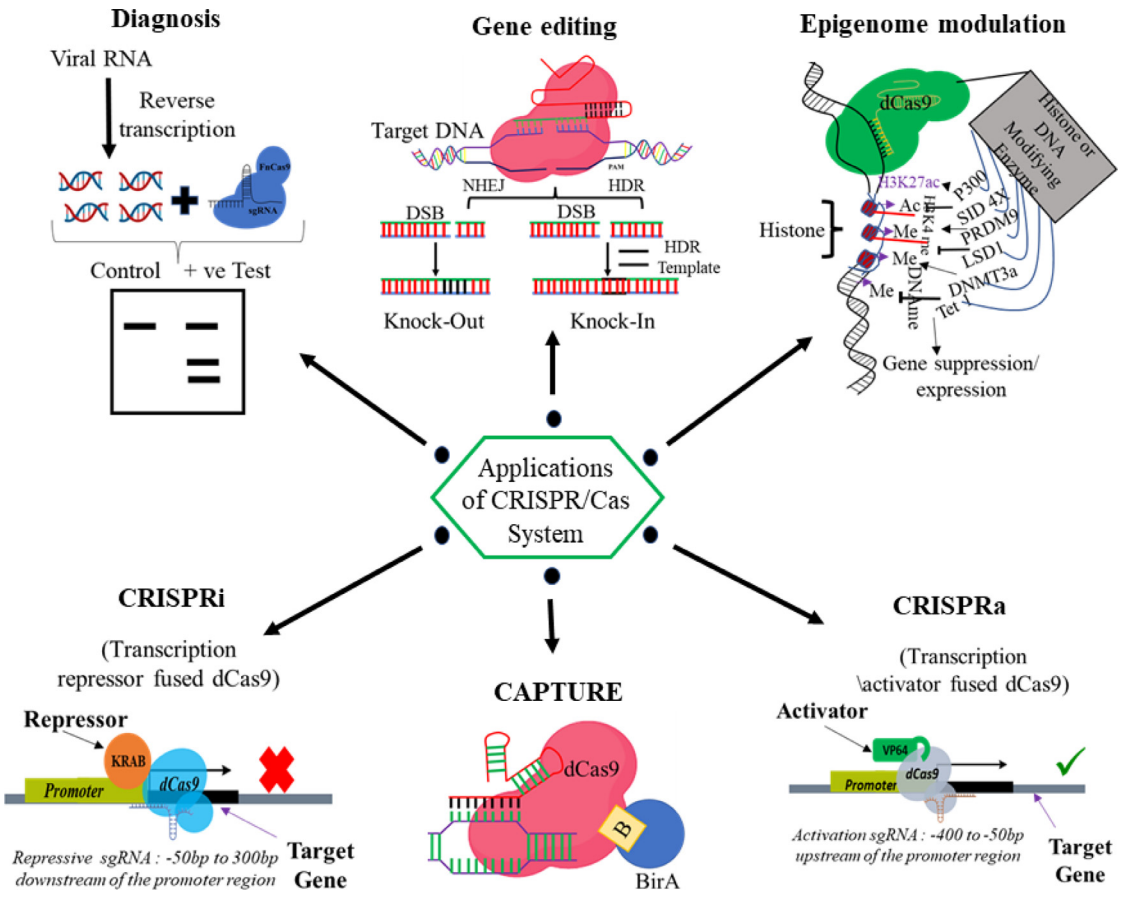

Fig. 2 – Applications of CRISPR/Cas technology.

modifier or the fluorescent tag was also used for the epigenome editing [21] and imaging [22] respectively. Recently, CRISPR/Cas system was also utilized as a diagnostic tool for the detection of Covid-19 infection [23]. Collectively, CRISPR/Cas has ample application beyond the DSB mediated gene editing. CRISPR has been potentially recognized as a tool for diagnostics, epigenome editing, gene regulation (CRISPRi/CRISPRa), imaging, etc. (Fig. 2).

3. Retinal dystrophies

The mammalian retina is being widely studied for genetic disorders for several reasons. Multiple phenotypes of the retina can be directly observed, and photographs can be recorded. The effects of psychophysical parameters (acuity, field, color contrast) can be documented and retinal electrophysiology can be used to assess retinal functions [24]. Lastly, the ease of visualization of the retina has led to the development of many animal models that have led to a better understanding of pathways leading to photoreceptor death [25].

Worldwide, 1 in every 2000 people suffers from inherited retinal dystrophies (IRD). Individuals with IRD typically present with progressive vision loss that ultimately results in blindness. Since these diseases are genetic and clinically heterogeneous, hardly any effective treatments are available. Multiple cells, genes and drug-based therapies are in different phases of clinical trials for IRD [26]. IRD are distinguished by continuous degeneration of retinal pigment epithelium (RPE) and the neural retina. These dystrophies are of various types such as cone-dominant dystrophy (cone-rod/cone dystrophy), rod-dominant dystrophy (retinitis pigmentosa/rod-cone dystrophy), pattern dystrophy, macular dystrophy (Best macular dystrophy, Stargardt disease, Sors by fundus dystrophy), photoreceptors and bipolar cells abnormality (congenital stationary night blindness, X-linked retinoschisis), hereditary choroidal diseases, and vitreoretinopathies (Stickler syndrome, Wagner syndrome) [27,28].

3.1. *CRISPR/Cas for correcting retinal dystrophies*

Genome therapy using CRISPR-Cas in ophthalmic diseases may be promising, considering the scale of impact on society and various monogenic disorders of the eye [29]. Hopes are high to attenuate inherited retinal disorders due to the multiple clinical trials which have been initiated for specific retinal conditions with advancing gene therapy technology [30]. Table 2 showing various pre-clinical studies related to the use of CRISPR/Cas system for the treatment of retinal dystrophies. Over the last two decades, eye tissue has become

Table 2 – Retinal dystrophies treated via genome engineering approach using different delivery strategies.

RD	Mutant Gene	Therapeutic approach	Host	Mode of delivery	Result	Year	Ref
Leber's Congenital Amaurosis (LCA10)	RPE65	Gene therapy	Homo sapiens	AAV Vector	Slight visual function improvement	2008	[77]
	RPE65	CRISPR/Cas9	Rd12 mice	AAV Vector	RPE65 mutation correction	2019	[78]
	CEP290	CRISPR/Cas9	iPSCs	Plasmid vector	Successful repairment of mutations	2017	[79]
Age-related macular degeneration (wAMD)	VEGFA gene	CRISPR/Cas9	Mouse	Subretinal injection of Cas9 RNPs	Reduction in CNV area after Cas9 RNPs injection in mice bearing laser induced AMD	2017	[48]
	VEGFA gene	CRISPR/Cas9	Mice	Lentiviral vector	*In vivo* disruption of VEGFA gene *in vivo* with 84% indel efficiency	2017	[80]
	VEGFA/HIF1a gene	CRISPR-LbCpf1	Mouse	AAV vector	A long term effect was seen when LbCpf1 targeted to Vegfa or Hif1a were introduced as a therapeutic in CNV to avoid the hurdles during multiple injection strategies. CNV, potentially avoiding repetitive injections.	2018	[81]
	RP1L1	CRISPR/Cas9	Zebrafish	Direction injection of gRNA and Cas9 into the embryo	Generated model of RP1L1-associated photoreceptor disease and the first zebrafish model of photoreceptor degeneration with reported subretinal drusenoid deposits, a feature of age-related macular degeneration.	2020	[82]
Retinitis Pigmentosa (RP)	RPGR gene (exon 8)	CRISPR/Cas9	Mice	AAV vector	Successful development of Rpgr knock out mouse model	2020	[83]
	RHO gene (P23H mutation)	CRISPR/Cas9	Mice	Plasmid vector	Successful editing in mutant P23H allele with a rate of ~45%.	2018	[84]
	PDE6B gene	CRISPR/Cas9	Mice	Plasmid Vector	Repaired mutation efficiently	2016	[85]
	RHO gene	CRISPR/Cas9	Xenopus laevis	Co-injection of Cas9, eGFP mRNAs, and sgRNAs into fertilized eggs.	introduced and characterized in-frame and out-of-frame Indel in three genes encoding rhodopsin.	2017	[86]
	RHO gene	CRISPR/Cas9	Rat	Plasmid vector	Improvement in visual function by preventing retinal degeneration	2016	[61]
	NRL gene	CRISPR/Cas9	Mouse	AAV vector	Successfully preserve cone cell function and improved survival of rod cells	2017	[87]
	RPGR	CRISPR/Cas9	Patient-derived iPSCs	Plasmid vector	Approx 13% of RPGR gene copies showed mutation correction and conversion to the wild-type allele.	2016	[62]
Usher Syndrome	USH2A gene	CRISPR/Cas9	HEK293 cells	pX330 vector	Repaired mutations efficiently	2017	[88]
	USH2A gene	CRISPR/Cas9	iPSCs,HEK293T Cells	Plasmid vector	Repaired mutations efficiently with minimal off target effects.	2020	[89]
Best disease	BEST1 gene mutations	CRISPR/Cas9	Ipsc	Lentiviral vector	Successfully reversal of the mutation with minimal off target effects	2020	[73]
X-linked Juvenile retinoschisis (XLRS)	*RS1* gene mutations (C625T)	CRISPR/Cas9	hiPSCs	Plasmid vector	Showed a high efficiency of mutation repair	2019	[74]
	RS1gene mutation (p.Y65X)	TALEN	Mice	TALEN mRNA was co-injected into fertilized eggs	RS1-KI mice were viable, fertile and did not show notable physical abnormalities.	2018	[90]
Achromatopsia	CNG B3	Gene therapy	Mouse and Dog	AAV-hCNGB3 vectors via injection	Successfully rescued the function of cone photoreceptors.	2016	[91]
Progressive cone and cone-rod dystrophies	*RPGIP1*	Gene therapy	Dog	rAAV mediated *Rpgrip1* gene transfer via injection	Successfully rescued both cone and rod functions.	2014	[92]

a frontline organ for gene therapy. It is achieved either by using viral vectors to transfer correct cDNA copies or through the use of RNA intrusion to knockdown proteins with dominant-negative traits or toxic inclusion of functionalities via gene silencing [31].

Before the arrival of gene therapy, retinal dystrophies were incurable [32]. Indeed, IRDs have been demonstrated as ideal candidates for gene therapy because: (i) they are inherited diseases linked to multiple genes and a subset of them show monogenic inheritance [33], (ii) the cells which are affected (PRs and RPE) can be accessed by various clinical and surgical procedures [34], (iii) the non-invasive diagnosis methods used in the clinics for IR patients could be translated to animal models and (iv) availability of animals models to study the eye conditions. The Phase III data for Spark Therapeutics' gene therapy product (i.e. SPK-RPE65) for the treatment of patients with visual impairment caused by RPE65 gene defects provides hope for clinical translation opportunities. SPK-RPE65 is an AAV2 gene therapy that delivers the RPE65 gene via subretinal injection to patients with a defective RPE65 gene. Clinical trial outcomes were found beneficial and the therapy, SPK-RPE65, was approved by FDA in 2019 with trade name of LUXTURNA for the treatment of vision loss in the patient [35]. The therapy is based on recombinant adeno-associated virus (AAV) vectors expressing the human *RPE65* cDNA using a viral promoter as a control [35]. Although, gene therapy provides immense potential for treatment of various genetic diseases, it poses some disadvantages like off-target effects and risk of mutation in the DNA. These disadvantages limit their application in several cases. Therefore, gene editing tools such as zinc finger nuclease (ZFN) and transcription activator-like effector nucleases (TALEN) have been developed for the treatment of genetic diseases. Moreover, in recent times CRISPR/Cas9 based gene editing tool is being explored for the treatment of genetic disease through its unique site-specific gene editing efficiency. Here, multiple guide RNAs are being used to simultaneously target various sites in the genome, which is a striking feature of the CRISPR/Cas system [36]. A major advantage of deploying CRISPR-Cas is that it is a RNA-based system; thus, custom guide RNAs can be easily designed to target within the genome. Whereas ZFN and TALEN systems, which are protein-DNA interfaces, are protein-dependent making it difficult to engineer for a given target [37]. The potential for multiplexed genome surgery is another interesting feature of the CRISPR-Cas system using several gRNAs for the concomitant editing of multiple sites within the genome [36,38].

Some of the major mutation based retinal dystrophic conditions are discussed below.

3.2. *Leber's congenital amaurosis*

Leber's Congenital Amaurosis (LCA) has been known to be the most severe retinal dystrophy as it potentially leads to congenital blindness in less than one year of age. Fourteen mutated genes have been identified by homozygosity mapping, linkage analysis and genome analysis in LCA patients and children with retinal degeneration constituting approximately 70% of the cases [39]. LCA is mostly associated with severe defects which includes roving movements of the eye called nystagmus. Also, slow reactions of the pupil and lack of electroretinographic reactions are some of the symptoms in children [40,41]. Genes involved in LCA encode proteins which are responsible for retinal functions includes photoreceptor morphogenesis (CRB1, CRX), vitamin A cycling (LRAT, RPE65, RDH12), phototransduction (AIPL1, GUCY2D), guanine synthesis (IMPDH1), and outer segment phagocytosis (MERTK) and also intra-photoreceptor ciliary transport processes (CEP290, RPGRIP1, LCA5, TULP1) [39]. The most prominently studied gene for LCA is mutations in the RPE (RPE65) gene, which is responsible for encoding retinoid isomerase [29] whereas, the most frequently occurring mutations are associated with the CEP290 (15%), GUCY2D (12%), and CRB1 (10%). Around 20% of patients in north-western Europe have an intronic CEP290 mutation (p.Cys998X). An AVV-CRISPR system has been developed for *in vivo* treatment of autosomal dominant retinitis pigmentosa (adRP) and LCA10 in mice. In this study, AAV-SpCas9 vector was delivered via subretinal-injections that targets the rhodopsin (RHO) or CEP290 and Nrl (neural retina leucine zipper transcription factor) gene in mouse models for adRP. The outcomes of the study showed the expression of spCas9 protein in retinal cells of the mice for 9.5 months. While the authors have deployed different AAV serotypes as well as different vector doses, the results proved effective restoration of RP or LCA10 phenotype without off-target effects and adverse toxic reactions [42,43]. Later, the strategy was adopted to resolve the RHO gene mutation in human cells successfully. Collectively, CRISPR/Cas9 technology has shown to be effective at targeting gene/alleles in an efficient and specific manner in this study, demonstrating that it could be used in the treatment of RP and other genetic disorders, including dominant human conditions.

3.3. *Age-related macular degeneration*

Age-related macular degeneration (AMD) is a multi genetic disorder [29]. Wet AMD, which is the neovascular form of AMD, is marked by abnormal growth of the choroidal vessels in the macula region of the retina, resulting in a loss of central vision. Macula is enriched with cone photoreceptors and is responsible for the bright light activities and color vision [44]. Neovascularization in wAMD occurs due to overproduction of vascular endothelial growth factor (VEGF); hence anti-VEGF agents becomes the therapy of choice [45]. Currently, wet AMD patients are treated with intravitreal injection of anti-VEGF agents such as ranibizumab, aflibercept and bevacizumab [46]. For the treatment of AMD, AAV-CRISPR systems have also been developed based on CjCas9 (*Campylobacter jejuni*) [47] and LbCpcf1 nucleases (nucleases which are a member of the type-V CRISPR-Cas systems). In the study, authors packaged the CjCas9 gene, its corresponding sgRNA sequence, along with a marker gene into an AAV vector. Being highly specific, CjCas9 can cleave only a restricted number of sites in the human or mouse genome. Hence, when delivered using AAV, CjCas9 lead to targeted mutations in the RPE cells. CjCas9 can be specifically targeted to the Vegfa or Hif1a gene in RPE cells thereby, decreasing the size of laser-induced choroidal

neovascularization, making *in vivo* genome editing with CjCas9 a new advancement in the therapy of AMD. Further, the results indicated an Indel efficiency of 22±3 and 31±2%, for Vegfa and Hifla genes, respectively at 6-weeks post injection of AAV-CjCas9 intravitreally. Moreover, the effect of Indel was seen at protein level as well where significant decrease in VEGF-A protein was observed in RPE cells with respect to control group. In another study, sgRNA/Cas9 expressing plasmid and Cas9 RNPs were delivered using lipofectamine 2000, wherein Cas9 RNPs showed 82%±5% and 57%±3% of indel in NIH3T3 and ARPE-19 cells, respectively. Comparative study indicated that the Cas9 RNPs were more effective w.r.t plasmid at 2nd day of transfection. Further, it was observed that level of VEGF A mRNA and protein reduced to 40%±8% and 52%±9%, respectively in adult retinal pigment epithelial cells (ARPE) after Cas9 RNPs treatment. For *in vivo* efficacy evaluation, Cy3 labelled RNPs were delivered via intravitreal injection. The results indicated the accumulation of Cy3 dye into RPE cells after 3 days post injection and 25%±3% of indel was also detected in RPE cells at delivery site. Moreover, CNV model was also developed in mice using laser (to mimic wAMD) followed by subretinal injection of Cas9 RNPs. After 3 d, 22%±5% indel was observed in RPE cells for Vegfa gene. Additionally, Cas9 RNPs treatment significantly reduced CNV area by 8%±4%, and VEGF A protein level as well (Fig. 3) [48].

3.4. *Retinitis pigmentosa*

Retinitis pigmentosa (RP), affecting 1 in 4000 people, has become the leading cause of progressive blindness [49]. Classical RP, also known as the rod-cone dystrophy, is identified by a “tunnel vision”, which is a progressive loss of peripheral vision. The first signs include nyctalopia, which is the development of night blindness and difficulties in adapting to the dark that occurred through the loss of rod function in the early years of life [50]. The RPE starts losing its pigment due to loss of photoreceptor that ultimately leads to the accumulation of intraretinal melanin deposits, which look like a “bone spicule” conformation. However, the central vision remains intact until the last stages. It can be inherited through different transmission modes such as autosomal dominant, autosomal recessive or X-linked and is heterogeneously related with mutations in at least 79 genes [49]. RP is mainly of two types, MERTK associated and RPGR X-linked. The RPE apical membrane contains photoreceptors that are light sensitive and their turns over is enabled by MERTK (Mer tyrosine kinase), the receptor involved in phagocytosis of the rods and cones [51]. These photoreceptors must be continuously recycled for efficient working, which is disrupted by mutations in MERTK, leading to degradation and loss of photoreceptors [52]. Meta analyses have revealed that only 3% of MERTK type RP are due to autosomal recessive transmission [53,54] and causes macular atrophy and early-age photoreceptor abnormality [55,56]. Mutations in RP GTPase regulator (RPGR), an X-linked RP (XLRP) is seen in 1 in 3500 people. RPGR, along with the delta subunit of rod cGMP phosphodiesterase, regulates the proteins, and its dysfunction leads to progressive loss of central vision and night blindness [57–60]. Some *in vitro* and *in vivo* studies have been reported where RP has been treated using CRISPR/Cas9 technology. For example, CRISPR/Cas9 tool was used in rat model of adRP by Bakondi et al., in 2016 to ablate mutation in rhodopsin gene (RhoS334). In this study, sgRNA/Cas9 plasmid (targeting exon 1 immediately upstream of a PAM unique to the RhoS334 locus) was administered intravitrealy in S334ter-3 rats. Genome analysis of transfected retinal cells confirmed a cleavage efficiency of 33% and 36% in two different rats. Also, improved visual acuity and extensive preservation of retina was observed *via* immunohistology following sgRNA/Cas9 plasmid injection [61]. In addition, a CRISPR/Cas based strategy was developed for editing RHO gene mutations. In this study, a plasmid was designed which contained an insert for two sgRNAs targeting RHO gene to cause DSB followed by NHEJ. The outcome of the study dictated successful editing of RHO gene, which further downregulated the expression of RHO protein. Further, Bassuk et al. treated XLRP by correcting RPGR point mutation using CRISPR/Cas9 in iPSCs. In this report CRISPR was used to treat the pathogenic mutation in iPSCs obtained from a patient with photoreceptor degeneration. The authors screened 21 different sgRNAs for editing where g58 was found most effective. Therefore, g58/Cas9 expressing plasmid was designed and transfected into iPSCs along with a RPGR single-stranded oligo deoxy ribonucleotide (ssODN), which acts as a donor during HDR pathway. Further, deep sequencing was performed, and the data revealed the successful correction of mutation in 13% of transfected cells [62]. Moreover, the results showed that TAG (premature stop codon) gets replaced by GAG (wild type codon), which encode glutamate at residue 1024. On the other hand, no changes in the mutation were seen in the untransfected iPSCs. Further it was concluded that the correction rate of 13% was significantly fruitful and can be improved by minimizing error-prone NHEJ by inhibiting DNA ligase IV at the DNA cleavage site.

In addition, a CRISPR/Cas-based strategy was developed for editing RHO gene mutations in a mouse model of ADRP. In this study, a plasmid was designed that contained an insert for two sgRNAs, targeting RHO gene (exon 1) having P23H dominant mutation. Firstly, the gene editing was performed *in vitro* in HeLa cells, where 70%, 76% and 82% of indel frequency was observed with sgRNA1, sgRNA3 and 2sgRNA, respectively, Additional, the RHO expression was also observed using Real time Taqman PCR wherein 35%, 25% and 20% of reduction in expression was seen with cells treated with sgRNA1 sgRNA3, and 2sgRNA, respectively. Later, the electroporation of CRISPR/Cas plasmid containing 2sgRNA along with green fluorescence protein (GFP) expression was performed subretinally in P23 RHO transgenic mice. The GFP expressing section of the retina was isolated and evaluated for the Cas9 expression wherein the Cas9 expression was limited to the cells expressing GFP along with 84 edited sequences [63].

3.5. *Choroideremia*

Choroideremia (CHM) is named from the complete loss of the choroid, retina and RPE, exposing the underlying white sclera, which is unique to the disease [64]. CHM is an X-

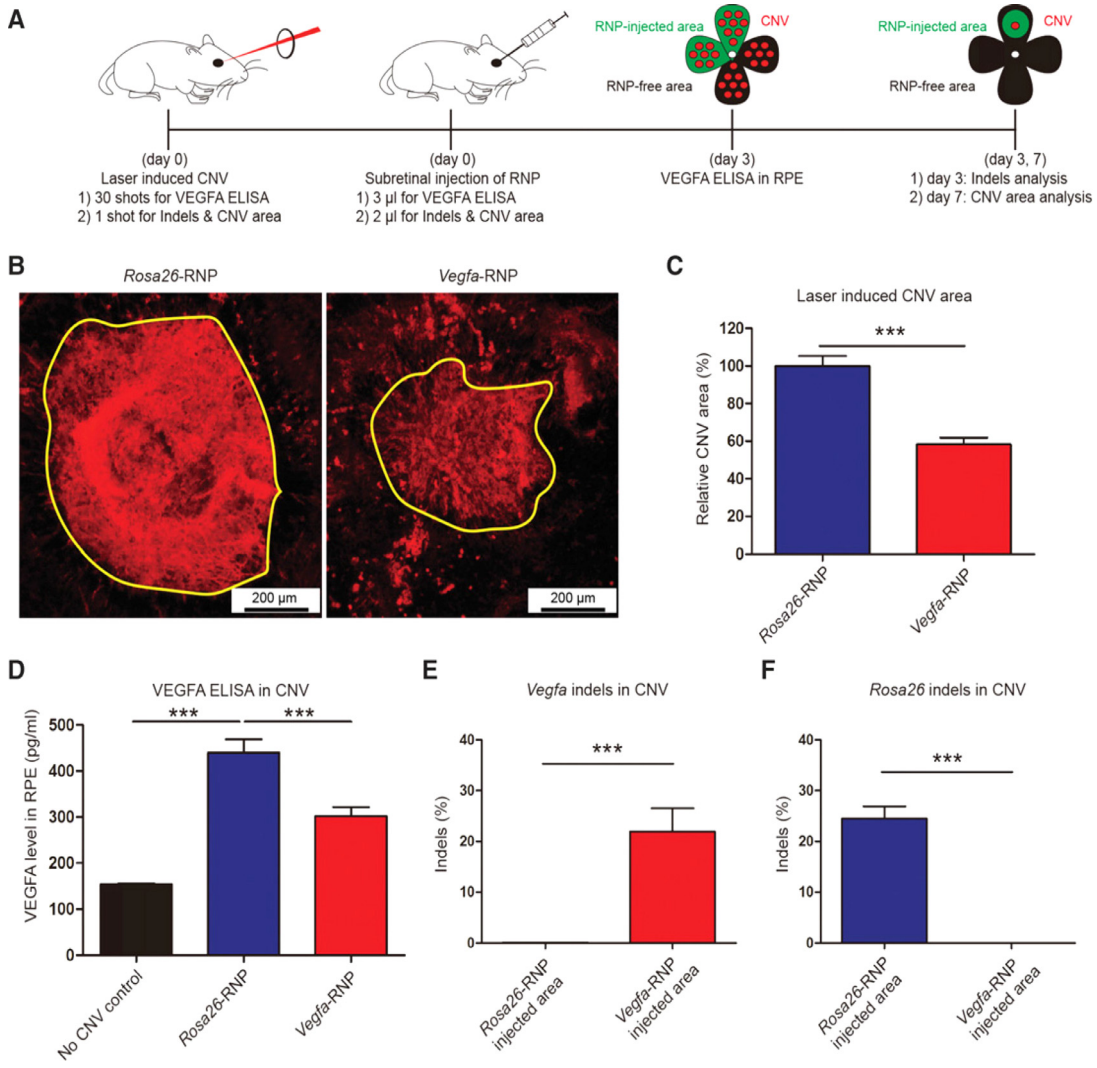

Fig. 3 – VEGF A gene editing efficiency of Cas9 RNPs in retinal dystrophy in mice. (A) The overall study outline, herein, CNV model was developed in mice using laser followed by subretinal injection of VEGF A targeting Cas9 RNP. After 7 d injection of RPE complexes were analyzed for CNV area and deep sequencing was performed to evaluate gene editing in the targeted cells/tissues. Meanwhile, after 3 d injection of VEGFA ELISA was also performed. (B) Representative laser-induced CNV stained with isolectin B4 (IB4) in C57BL/6J mice injected with the Rosa26-specific Cas9 RNP (as a control) or the VEGFA-RNP. The area of CNV is demonstrated as yellow line. (C) CNV area. (D) level of VEGF A in the CNV area. (E) Gene editing in terms of indel in RPE cells at VEGFA targeted site. Reprinted from [48], licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/legalcode). Copyright © 2017 the authors. Published by Cold Spring Harbor Laboratory Press.

linked recessive degenerative retinal dystrophy, affecting 1 in 50,000 individuals and is only associated with males. Due to mutations in CHM gene, which encodes for Rab escort protein 1 (REP1) and its dysfunction leads to progressive loss of vision and choroid atrophy. It starts with night blindness in the early years of life with a gradual decline in peripheral vision and legal blindness by 50–70 years of age [65]. The CHM disease is characterized by retinal thickening, resulting from Müller cell activation and photoreceptor layer hypertrophy. This further causes RPE depigmentation, degeneration of photoreceptors and retinal remodeling. Hence, retinal remodeling is being considered as a possible strategy for *in vivo* studies [66].

3.6. *Stargardt disease*

Stargardt disease is an autosomal recessive genetic disorder majorly caused by mutations in the ABCA4 (ATP-binding cassette, subfamily A, member 4) gene and is the most common form of juvenile-onset macular degeneration. It is characterized by the loss in central vision due to the gradual accumulation of cytotoxic lipofuscin within the RPE [67]. This disease affects at least 1 in 10,000 people, with approximately 31,000 cases in the United States. The disorder consists of a quite fast degeneration of the macula resulting from the deposition of lipid enriched deposits called lipofuscin (comprised mainly of A2E, a vitamin A derivative) in the RPE

Table 3 – Factors affecting the use of CRISPR/Cas for gene editing in RDs.

Factor	Description
Ethical Issue [98]	While using CRISPR/Cas9, the ethical issues will be there, since CRISPR/Cas based gene editing could result in serious off-target gene manipulations.
Selection of gene [99]	For example, in LCA there are 14 genes that have mutations. Therefore, one should be clear about the gene that needed to be edited for improvement of the complications associated with the RD.
Knock out [100]	Knock out is not always beneficial, until and unless the role of the gene has been vastly understood. For instance, knockout of Vegfa gene is shown in wAMD to stop angiogenesis. But it cannot be applicable for every gene because one gene can be involved in various cellular functions and knockout could cause the loss of some important cellular functions.
Knock in [101]	Some of the RDs require HDR. Since NHEJ is more prominent w.r.t HDR, therefore, one should consider this factor while utilizing the CRISPR/Cas technique.
Vitreal barrier [102]	The presence of vitreous fluid may retard the diffusion of the CRISPR/Cas components toward the posterior segment of the eye. Additionally, the nucleases/proteases present in the vitreous fluid could degrade the CRISPR components.
Targeted Delivery	RDs requires editing of the gene in retinal cells only, therefore, delivering the CRISPR/Cas component specifically to retinal cells could be challenging.
Off-target effect	While designing sgRNA for a specific gene, one should ensure the specificity of the sgRNA toward the gene of interest. Else, it could lead to undesired gene editing.
PAM sequence [100]	As it is known that the CRISPR/Cas perform DSB near the PAM sequences (NGG, GGG), and however, it is not always possible to have a PAM sequence at the desired gene editing site.
Limited delivery route	Blood retinal barrier limits the distribution of therapeutic agents to the eye tissue, therefore localized injection is the only potential approach left. Localized injection such as intravitreal (IVT) injection has the risk of eye damage and requires trained healthcare personnel.

cell layer. Due to this, the interaction between photoreceptors and RPE is affected, causing the death of photoreceptors by hampering with their ability to uptake nutrients and perform the visual cycle [68].

3.7. *Usher syndrome*

With a prevalence of 1 in 20 000, usher syndrome is one of the common forms of syndromic IRD. Its unique features include RP and hearing loss [69]. The heterogenous syndrome is classified into three subtypes depending on the progression and severity of the hearing loss and the age of onset of the RP. Usher syndrome type 1 (USH1) is the most critical; usher syndrome type 2 (USH2) presents moderate to severe symptoms and is most frequently observed. Lastly, usher syndrome type 3 (USH3) is characterized by a moderate phenotype, and the onset of the disease and its progression could vary on a case by case basis [70]. USH1 is the most common cause of deaf-blindness in humans, characterized by vestibular dysfunction, profound congenital deafness and RP and is inherited in an autosomal recessive manner. USH1 is caused due to mutations in myosin VIIA, which encodes for an organelle transport protein within the RPE [45]. In 2017, Fuster-Garca et al., proposed the use of CRISPR/Cas9 gene editing to restore c.2299delG mutation in the USH2A gene. Human dermal fibroblasts (HDFs) cells were isolated from an USH2 patient with c.2299delG mutation and used for gene editing. Briefly, using nucleofection, a Cas9 RNPs (comprising Cas9 (15 µg) and sgRNA (20 µg)) was transfected into HDFs of the normal patient, yielding 18% indel frequency. Subsequently, RNPs were co-delivered with ssODN-2299, which yielded HDR efficiency of 5%. Similarly, HDFs of the patient with c.2299delG mutation were transfected with ssODN with a WT sequence and the PAM sequence ablated. As per the results, 6% indel frequency and a 2.5% HDR were detected [71].

3.8. *Best disease*

Bestrophin, encoded by the BEST1 (VMD2) gene, is a transmembrane protein expressed on the basolateral aspect of the RPE cells and is responsible for the conduction of chlorine across the RPE. Mutations in the BEST1 (VMD2) gene, and hence bestrophin, hampered the fluid transport across the RPE thereby causing debris to build up between the RPE and photoreceptors. Consequently, atrophic macula scar and central visual loss occur in a short span of time, leading to best disease or Vitelliform macular dystrophy. It affects between 1 and 9/100 000 people and is inherited in an autosomal dominant manner. Many other retinal dystrophies can also occur due to BEST1 mutations, including RP and ADVIRC (Autosomal Dominant Vitreo Retino Choroidopathy). Biallelic mutations lead to multifocal small egg yolk deposits leading to Recessive Best Disease. Neovascularization in the choroid, along with hemorrhage and leak into the retina, further aggravates the disease condition and intravitreal anti-VEGF agents can be used for successful therapy [72]. In the year 2020, Sinha et al. demonstrated the effectiveness of gene augmentation in the treatment of the Best disease. Induced pluripotent stem cell-derived RPE (iPSC-RPE) was used as an *in vitro* Best disease model for this objective. Gene augmentation restored BEST1 gene activity and improve rhodopsin degradation. Meanwhile, some of the mutations did not respond to the gene augmentation, therefore CRISPR/Cas9 was used to investigate the efficiency of site-specific gene editing in iPSCs RPE models. The findings revealed that CRISPR/Cas9 edit the mutant BEST1 gene while leaving the wild-type BEST1 gene intact. Off-target indels were

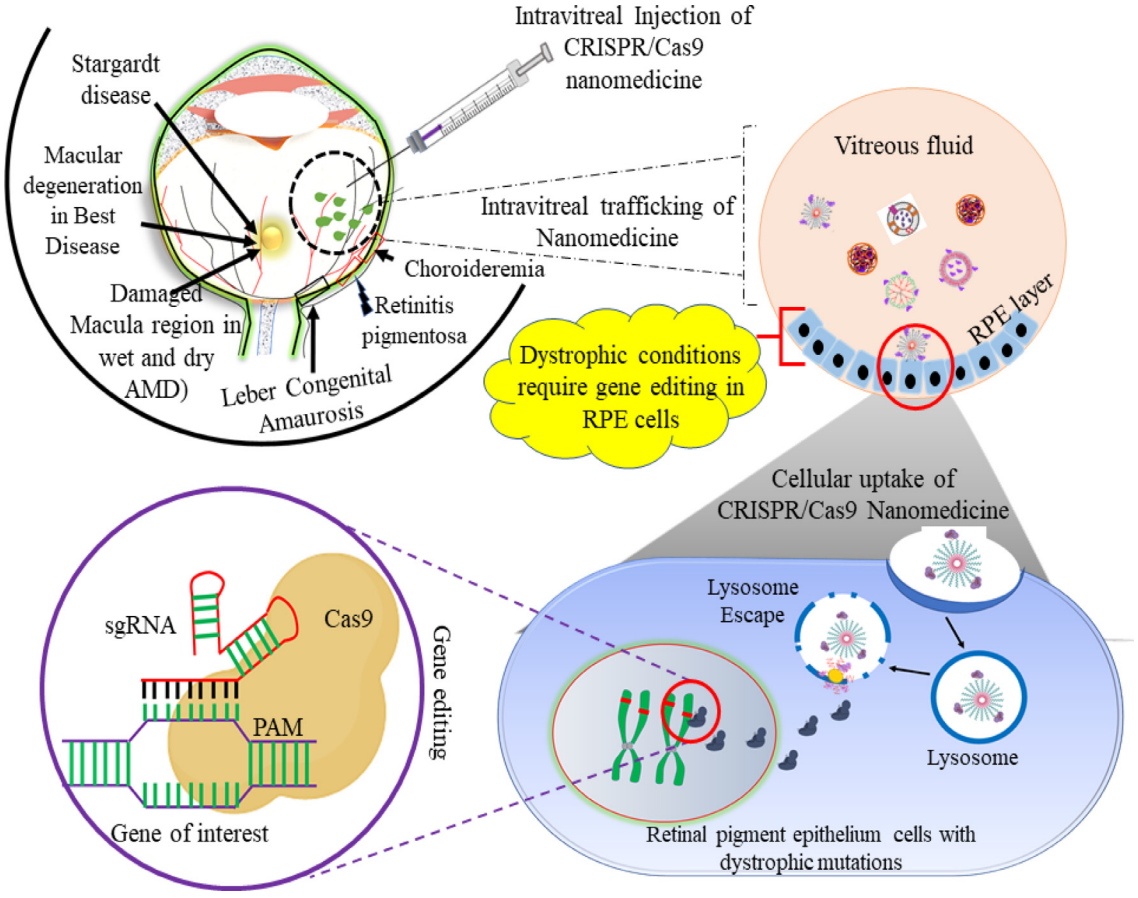

Fig. 4 – Schematic representation of nanomedicine trafficking in the treatment of retinal dystrophic conditions starting from (1) intravitreal injection, (2) diffusion through vitreous fluid, (3) cellular uptake, (4) endosomal escape and (5) interest specific gene editing.

also tested, however, no evidence of off-target gene editing was reported. The study overall revealed the application of CRISPR/Cas based precise and specific gene editing for the management of retinal dystrophies [73].

3.9. *X-linked juvenile retinoschisis*

RS1 is a retinoschisin gene that encodes a protein responsible for the cell adhesion. Mainly observed in males, RS1 mutations cause the development of cystic cavities in the center of the retina that enlarge gradually with age, along with decreased visual acuity. As the dystrophy progresses in the retinal periphery, the condition of the split retina worsens with large atrophic holes; hence, the residual retinal blood vessels left hanging in the vitreous cavity above the retina which may result in vitreous hemorrhage. It has been observed that people with this disease usually have a refractive error of long-sightedness. Worldwide, about 1 in every 5000 to 25,000 suffer from the condition. Children who suffer slowly lose out on central vision; however, most children can complete a fully sighted education, with the help of magnified texts and teachers for visual support. The main therapy for the retinal cysts is the carbonic anhydrase inhibitors, although a significant improvement in symptoms is rare. Huang et al. developed a base editing strategy to cure X-linked juvenile retinoschisis (XLRS) in 2019. Using human induced pluripotent stem cells (hiPSCs) from patients, a 3D retinal organoid model with XLRS characteristics was created *in vitro*. To evaluate the model, CRISPR/Cas9 targeting the C625T mutation in the RS1 gene was introduced as a plasmid using a viral vector. According to the findings, CRISPR effectively repairs gene mutations while also correcting the phenotypes by up to 50%. The findings also revealed the existence of off-target indel, which is a CRISPR constraint [74].

3.10. *Congenital stationary night blindness*

Congenital stationary night blindness (CSNB), also called nyctalopia, is a non-progressive type of night blindness. Patients suffering from this condition have difficulty observing in low light. The symptoms start early in children along with low amplitude nystagmus, strabismus and reduced visual acuity [75]. Associated with 17 genes, CSNB is a polygenic disease, and diagnosis involves electroretinography to measure photoreceptor function. The ERG results may show lack of rod functioning or incomplete functioning of both cone and rod as well as abnormal fundus upon examination. The state of complete CSNB is caused when

bipolar cell signaling is disrupted, leaving a single intact alternate pathway [76]. CSNB is of four subtypes - Schubert Bornstein (branched into complete and incomplete), Riggs, Fundus Albipunctatus and Oguchi Disease, of which the last two types show abnormal fundus. Myopia and photophobia are two of the prominent features observed in patients. Children with 'incomplete' CSNB may not be aware of the condition as the symptoms are mild and central vision is reduced from normal [72].

3.11. *Achromatopsia*

Affecting only 1 in 30 000 to 40 000 people, achromatopsia is a rare autosomal recessive disease. Mutations in six different genes have been identified that are responsible for this disease. 75% cases are due to CNGB3 and CNGA3, while the rest are accounted by GNAT2, PDE6C, PDE6H and ATF6. There is complete color blindness and central vision is diminished. In the early months, patients have to deal with profound photophobia and nystagmus. However, the nystagmus in achromatopsia patients is pendular or horizontal unlike the roving nystagmus of LCA. The diagnosis involves electrophysiology where it is observed that the function of cone photoreceptors is absent, and rods functions normally. Usually, the complete form is seen, and the incomplete form with a milder phenotype tends to be much rare. The symptoms of the disease are mostly constant, and the glare and photophobia can be managed by incorporating red/brown shade glasses.

3.12. *Progressive cone and cone-rod dystrophies*

As the name suggests, cone cell degeneration (COD) or cone followed by rod degeneration (CORD) are progressive retinal dystrophies and are seen from a young age. The major difference between them is that rod involvement increases the severity of the disease and by age 40, these people reach the stage of legal blindness [93]. Examination of the fundus and macula reveals an atrophic appearance or deposits of retinal pigments seen variably in different patients. Mutations in over 30 genes have been found as well as molecular causes identified in around 20% and 74% of autosomal dominant and X-linked COD/CORD respectively, while 23%–25% of autosomal recessive types have been worked out [94].

A number of ongoing clinical trials are living proof that gene therapy has made retinal dystrophies curable [29]. Furthermore, constraints such as multiple intravitreal injections resulting in physical retinal damage and resistance render the current therapy ineffective. Fortunately, the eye, and specifically the retina, is accessible to therapeutic gene editing due to its unique anatomical position, immune-privileged nature, presence of the blood-retinal barrier, and known underlying mutations [12]. As a result, the eye has been extensively studied for gene editing. However, just a few RDs in terms of preclinical evidence related to effective gene editing utilizing CRISPR/Cas have been published, and more research in this field is needed. Interestingly, some preclinical studies have been published wherein wAMD was treated by employing a CRISPR/Cas-based tool to knock out the VEGF A gene in RPE cells. Off-target effects and the deletion of some uncleared functions of the concerned gene are two key pitfalls that could be encountered with CRISPR. On the other hand, several groups are working to integrate data and screen for off-target effects [95–97]. It will be intriguing to observe if a CRISPR/Cas-based gene editing method can prevent angiogenesis in wAMD in clinical trials as it directly eliminates the fundamental cause of RDs. Further, the CRISPR/Cas-based therapy could also treat RDs with a single dose injection. We have discussed various factors that should be considered while adopting CRISPR/Cas9 for the treatment of RDs (Table 3).

4. Use of CRISPR for genome editing in RDs

The three main approaches for CRISPR based genome editing include the use of (a) plasmid DNA (pDNA) that expresses the Cas9 protein and sgRNA, (b) mRNA that encodes the Cas protein, and (c) RNP which is a complex of Cas protein and sgRNA. Among reported approaches, the plasmid-based approach is the simplest while the RNP based approach showed minimal off-target effects. Nevertheless, these CRISPR components ought to be delivered to the target cells followed by their translocation to the nucleus. Owing to the nature of the cargo, different challenges including packaging, immunogenicity, mutagenesis, extra- and intra-cellular barrier, etc., needs to be overcome to achieve efficient genome editing (Fig. 4).

4.1. *Vector packaging*

The major challenge faced in the delivery of CRISPR components for therapy is their packaging into a single vector system. Through the AAV approach, the maximum possible size for the cargo gene is ~4.7 kb, whereas that of the SpCas9 gene alone is ~4.3 kb. Hence, for AAV method, it becomes a hurdle to insert additional CRISPR components like sgRNAs, or extra genes. To solve this issue, various techniques such as using a smaller sized Cas9 (SaCas9) or splitting Cas9 into two vectors, have been propagated, but their feasibility for therapeutic applications have to be evaluated [103]. For the packaging of RNPs, viral vectors cannot be used. The use of non-viral vectors possesses a multitude of problems, be it the high molecular weight of the protein, highly negative charge and/or the stability of the RNPs.

4.2. *Immunogenicity*

CRISPR/Cas9 is a bacterial immune system, and therefore, being bacteria-derived they could lead to the immune response in the host. Specifically, if the gene-based approach is used, it could lead to the integration of Cas9 protein into the cells of the host. The expression of ectopic Cas9 protein in the individual could cause an MHC class I mediated immune response thereby, eliminating Cas9 expressing cells [104]. A study by Chew et al. showed that the *in vivo* delivery of AAV vector prompted immunogenicity not against the viral antigens but against the Cas9 protein. According to this result, immunogenicity of AAV-CRISPR-Cas9 has been considered as a key property that destabilizes the host system and

will negatively impact its application *in vivo* [105]. The best results have been found with a protein-based delivery of the CRISPR-Cas system, which has shown the least potential immunogenicity, as the ectopic Cas9 protein is present only transiently in the host cells [103].

4.3. *Insertional mutagenesis*

A lot of times, the vectors may get inserted in random sites within the genome thereby causing mutagenesis of essential genes. When trials were conducted using a retroviral vector based gene-therapy approach to treat Severe combined immunodeficiency (SCID), it caused leukemic transformation, as the virus got integrated into the host DNA [106] and triggered abnormal expression of the targeted gene. Tumorigenesis can result from vector insertions, when the integration occurs near a protooncogene, thus posing a greater risk for integrating viral vector based CRISPR delivery systems. This issue has been circumvented using non-integrating viral vectors such as AAV-based systems and by protein/RNA based CRISPR delivery systems [103].

4.4. *Systemic delivery*

CRISPR/Cas system is required to be delivered in cells or tissue of interest without any off-target effects. Therefore, localized delivery route advantageous than systemic delivery of CRISPR/Cas, especially to reduce immunogenicity, avoid off target effects and to improve the target cell edit efficiencies. In the case of retinal dystrophies, localized injections are given via intravitreal or subretinal administrations.

4.5. *Targeted delivery*

Targeting may be defined as a preferential accumulation of the active agent at a predetermined site which could be a tissue or organ (first order targeting), a specific cell type (second order targeting) or an intracellular site of targeted cells (third order targeting). Targeting is important because the therapeutic product can cause many adverse effects and damage the non-target cells. It also decreases the concentration of drug required to produce desired effect at the site of action. One of the advantages that viral vectors provide is tissue tropism, which will be beneficial for targeted CRISPR/Cas9 delivery [107] . But, if non-viral vectors are employed, specific moieties such as peptides and antibodies will be required for targeting [108]. However, such targeting is quite difficult to achieve due to complications in packaging that may arise due to the insertion of extra biomolecules to a delivery vector along with the CRISPR components.

4.6. *Transfection and editing efficiency*

CRISPR/Cas9 components need to be successfully delivered in sufficient quantity into the target cells by transfection, a prerequisite for efficient genome editing. Transfection methods are of three types, viral, chemical, and physical. Among these, the most used non-viral method is electroporation. But, due to the high electric field strength and accompanying electrochemical reactions, electroporation often causes high post-transfection mortality [109]. The editing efficiency for CRISPR/Cas9 obtained *in vivo* is much lower than that of those achieved *in vitro* in cell lines. In another study, when Cas9-RNP was delivered locally into the mouse inner ear, it caused 20% GFP fluorescence loss. This small percentage may work in some diseases such as liver tyrosinemia and muscular dystrophy. The editing efficiency is also linked to delivery efficiency. Recently Cas9-RNP delivery efficiency up to ~95% in cultured cells has been attained although the *in vivo* delivery efficiency requires further investigation [110].

4.7. *Off-target effects*

One of the biggest setbacks for genome editing technology is the off-target effects. Off-target effects are occurring when the specially engineered sgRNA, apart from targeting the gene of interest, targets the non-specific genes [111,112]. TALENs usually have lesser toxicity and greater specificity than ZFNs. Also, with CRISPRs, different cells may carry different edits even if they get edited by a single gRNA. It has been shown that even a single mismatch between base pairs can decrease binding to a great extent. A mismatch occurring at the 5′ end of the target is much more destructive than the 3′ end. In case of CRISPR therapy as well, off-target effects have been a significant problem. Secondary targets of the sgRNA, which have multiple mismatches with respect to the sgRNA undergo mutations at rate similar to the desired target [113]. The CRISPR/Cas off-target effects are further amplified when viral gene delivery method is employed, possibly due to the long-term constitutive expression of Cas9/sgRNA that leads to continued exposure of Cas9/sgRNA to non-specific genes. Various techniques are being developed to eliminate off-target effects such as designing sgRNA of high specificity [114,115]. *In vivo* effects for these systems have not been fully developed for the off-target effects. The best technique in this regard remains protein-based delivery of CRISPR since there is only a transient exposure of the host genome to the Cas9/sgRNA, thus decreasing off-target events [116].

5. Ocular delivery: Challenges and opportunities for nanomedicine

Anatomically, anterior and posterior segments of the eye are affected by vision threatening disorders. Most of the currently available ophthalmic preparations are eye drops possessing poor bioavailability through conjunctival route [117]. There are ample of physiological and anatomical barrier which impede the delivery of active pharmaceutical ingredient (API) to affected areas of the eye. Tear film, eye blinking, efflux pump, nasolacrimal drainage, are some of the barrier to drug absorption [118]. Most of the dystrophic conditions require delivery of the therapeutic agent to the posterior portion of the eye and is limited by static barriers including blood-retinal barrier, Bruch's membrane, sclera choroid, and the dynamic barriers *i.e.* lymph and choroidal blood flow [119]. Intravitreal route is the most common

mode of administration of drugs to the posterior chamber of the eye [120]. On the same note there are some limitations such as patient compliance, need expertise, risk of retinal detachment, risk of cataract and hemorrhage [121]. Attempts have been made to overcome existing problems related to the delivery of molecules towards the posterior portion of the eye using nanotechnology-based delivery carriers. Nano size and surface charge of nanoparticle helps target specific retention and conjugation *in vivo*. Also, nanoparticles with higher zeta potential are supposed to have higher stability. Additionally, cationic nanoparticles are considered more applicable for topical ophthalmic delivery, as conjunctiva and cornea have negative charge on the surface [122]. Therefore, electrostatic interaction helps in the internalization of nanoparticle into eye. For intravitreal injection, anionic nanoparticle diffuses more effectively through vitreous as it is composed of anionic hyaluronic acid which helps anionic particle to reach posterior chamber of the eye without any interaction [123]. On the other hand, cationic nanoparticles interact with anionic hyaluronic species and remain undiffused and are entrapped in the vitreous. Therefore, anionic charge of nanoparticles ease the intravitreal delivery of cargo to posterior part of eye [124]. Till now, several nanocarrier systems have been explored for ocular delivery *viz*., polymeric micelles, liposome, polymeric nanoparticles, nano-emulsion/suspension, solid lipid nanoparticles etc. These nanocarrier systems provide advantages such as targeted delivery, enhanced bioavailability, sustained release, improving residence time in ocular space etc. [125]. Additionally, for ocular retinal delivery, intraocular route has significant benefits over systemic route. Systemic route poses hurdles such as blood retinal barrier, systemic toxicity of the drug administered, poor target specificity, rapid clearance, off target effects, and only1%−2% drug reaches to the eye *via* systemic route. Hence intravitreal route can provide distinct advantages and nanomedicines could serve as potential therapeutics for the treatment of retinal dystrophic conditions. On the same note, there are some clinical complications with intravitreal injection such as patient compliance, infectious endophthalmitis, intraocular inflammation, rhegmatogenous retinal detachment, intraocular pressure elevation, ocular hemorrhage, glaucoma, cataract, non-infectious uveitis etc. These complications can be minimized by reviewing patient medical history, appropriate ocular examination, ancillary diagnostic testing, individualized medical decision-making, and proper follow-up by a clinician.

6. Delivery strategies used for CRISPR/Cas9 components

Many genome engineering applications have been developed for CRISPR/Cas systems for *in vitro* studies in cell lines; however, achieving an efficient *in vivo* delivery of CRISPR/Cas system is a major challenge as multiple components need to be delivered to the target cell to produce the desired effect. Nucleofection, electroporation and lipid-based deliveries have been tried for plasmid DNA (that encodes for the Cas9-gRNA) through the cell membrane [126]. Electroporation is a technique in which high voltage is applied to create pores in the cell membrane so that, direct transfection can occur into the cells both *in vitro* and *in vivo* [127]. Electroporation can be extremely toxic as it disturbs the cell membrane and may even lead to cell death [128]. Microinjection is preferred in rapidly dividing single cells, specifically in larger cells such as fertilized embryos, wherein the CRISPR components are directly injected into the single cell to create varieties of knockout and transgenic animals. However, this is a technically demanding procedure [129]. These mechanical methods are preferred for *in vitro* editing as they are reproducible, simple and have high levels of gene expression. Further, delivery through these direct methods has been used *ex vivo* in cells harvested from patients and then reintroduced into their body. Collectively, existing literature revealed the accuracy and efficiency of direct methods, but these methods are limited to *in vitro* or *ex vivo* applications. However, efficient *in vivo* delivery systems need further research and technical advancements.

Delivering payload specifically to any organ in the body should have some common factors that need to be considered, such as immune response, hematological toxicity, targeted delivery, distribution, etc. However, there are additional factors to be considered for retinal delivery of the therapeutic agent. The blood-retinal barrier limits the amount of payload that reaches the eye after intravenous injection thus localized injections (such as subretinal, intravitreal, corneal permeation, etc.) are mostly preferred for a retinal delivery route. Fortunately, the anatomical location also makes the eye more feasible to the localized injection. Vitreous fluid is one of the major barriers to retinal delivery. The viscosity and anionic charge of the vitreous fluid must be considered while developing a nano carrier-based delivery system [130]. As reported earlier, the high positive charge nanoparticle gets accumulated within the vitreous due to electrostatic interaction with anionic hyaluronic acid present in the vitreous [131]. Similarly, particle size is also having a considerable impact on vitreous diffusibility [132]; reports say that the particle size below 50 nm showed rapid clearance from the vitreous. Since the volume of the vitreous is very less, the injection volume is limited to a certain amount (25–100 ul). Therefore, the nanocarrier should have sufficient payload capacity so that the desired concentration of the payload could be delivered in that limited injection volume. Being a sense organ, the eye is more sensitive to toxicity, and therefore while selecting a nano-carrier, the toxicity issue should be considered. Although the immune-privileged nature of the eye provides opportunities to use distinct biomaterial used in delivery, despite that, toxicity may lead to the loss of eye integrity and could cause vision loss. Chitosan is the best example, which is known to cause retinal toxicity by inducing an immune response in the eye [133]. An increase in intraocular pressure is also a major challenge in intravitreal delivery and certain measures must be taken to resolve this issue. The intravitreal injection dose must be given by a trained professional because any mistake could lead to a serious eye injury. Additionally, multiple frequent injections need to be avoided in case of retinal delivery.

6.1. Viral vectors

CRISPR therapy had been developed for the primary purpose of treating inherited genetic diseases. Thus, the carrier package needs to be structured with a high degree of specificity, with no toxicity and rapid elimination after payload delivery [134]. The most widely used method for the efficient delivery of nucleic acid that encodes for the required protein, has been the viral vectors. But, as we have seen in the earlier section, even the viral delivery of CRISPR/Cas components causes undesirable effects such as immunogenicity and insertional mutations, hence restricting their clinical application [135].

Broadly, three types of viral vectors *i.e.*, adenoviral, lentiviral, and AAV have been used to deliver genes that encode Cas9 into cells of interest. While the adenoviral vectors can elicit severe immunogenic reactions against the complex capsid proteins, the lenti and retroviral vectors have the risk of host gene integration and insertional mutations. AAVs are known for their low immunogenicity and target specificity based on their serotypes, and are preferred gene delivery vectors. They also show good transduction efficiencies and long-term transgene expression, without genome integration [136]. Various changes such as removing the endogenous Rep protein and encoding double self-complementary replicase of the viral genome (scAAVs) have led to reduced integration of the vector and improved their transduction efficiency by about 140 times [136,137]. However, the AAV-based viral vectors have limited packaging capacity and this limits the packaging of large gene cargos such as Cas9 and make it difficult to accommodate other regulatory elements such as promoters, polyadenylation signals and selection markers. Splitting the spCas9 into two parts will make the genes fit into the vector, but it reduces the delivery and edit efficiencies [138].

AAVs have been successfully used for *in vivo* gene delivery and shown long-term therapeutic effects for up to six years in LCA patients administered with AAV2 vector encoding RPE65 [77,139]. AAV and adenovirus delivered RPE65 in the rd12 mouse model of LCA2 have been shown to restore vision significantly [140,141]. The efficacy of AAV gene therapy was convincingly demonstrated in 2008 for the treatment of Leber congenital amaurosis. Many phase I and phase IIa trials for the subretinal delivery of AAV2-*RPE65* cDNA have shown no serious adverse effects, along with improved pupillary reflexes, visual acuity and mobility in few of the treated patients [77,142,143]. The first AAV-based gene therapy drug, Glybera, was approved by the European Medicines Agency (EMA) in 2012 for the delivery of LPLD gene, with Luxturna becoming the first AAV gene therapy product to receive US FDA approval five years later, for the delivery of RPE65 gene.

Since AAVs pose a significant problem of packaging, many smaller Cas9 orthologs have been isolated from *Streptococcus thermophilus* (StCas9) [144], *Staphylococcus aureus* (SaCas9) [145], *Campylobacter jejuni* (CjCas9) and *Neisseria meningitidis* (NmCas9) [146]. Kim et al. used a combination of SaCas9 and CjCas9 together along with gRNAs incorporated into a single AAV. Their results showed that the cleaving action was as efficient as SpCas9 *in vitro* applications. In another study, *Lachnospiraceae bacterium* (LbCpf1) was used to prepare Cpf1 nuclease which was put together with the crRNA into a single AAV vector [81] showcasing excellent prospects for its use as an *in vivo* genome editing tool in the therapy of angiogenesis-related disorders.

Adenoviral vectors (AVs) are not the most used delivery vectors due to immunological concerns; however, their bigger genomes, episomal nature of intracellular maintenance and efficient transduction are advantages for *in vivo* delivery systems. The have a high packaging capacity (~30–40 kb pairs), which can fit all the required elements. Thus, a single virus vector can express the Cas protein and one or many sgRNAs. To facilitate homology-directed repair, large donor DNA sequences can also be co-delivered. Hence, the Cas proteins and sgRNA are expressed proportionately within cells and the episomes may get lost in dividing cells thereby allowing only transient Cas9 expressions and reduced off target risks. AVs have been successfully used for *in vivo* genome editing in mice, although immune-related toxicities were observed [147]. However, immunogenicity is not a concern for *in vitro* editing of cell lines and stem cells. One of the first studies on AV was conducted in 1996 by Bennett et al. to study retinal disease in an animal model. They delivered the cDNA encoding phosphodiesterase β subunit into photoreceptors of the rd1 mouse model and shown successful delay of photoreceptor degeneration by six weeks. The disadvantage in AV therapy is their relatively high immunogenicity and the existence of neutralizing antibodies in humans against certain serotypes such as Ad5, renders the vector ineffective in most patients [148]. Studies have shown that subretinal delivery causes a lower T cell-mediated immune response than that of intravitreal injections [149,150]. AVs are being used to inhibit retinoblastoma growth in a mouse model and reduce retinal and choroidal neovascularization in rat and rabbit models [151–153].

Lentiviral vectors (LVs) are currently one of the most used vectors in the clinical application where long term effects are desired. Lentiviruses belong to the family of viruses known as Retroviridae; they are RNA viruses, which integrate into the host DNA using reverse transcriptase and integrase enzymes. Studies have shown that the LVs are safe and effective for gene delivery into photoreceptor cells of humans [154–157]. LV packaging capacity is higher than AAVs in the range of approximately 8 to 9 kb. LVs can transduce both non-dividing and dividing cells with very high efficiency and can integrate into the host cell genomes to enable long-term transgene expression. However, long-lasting expression of Cas proteins may increase the risk of unwanted off-target edits [158]. To address this concern, self-inactivating constructs were engineered with two sgRNAs: one against the Cas9 gene and one against the target sequence of interest, thus allowing only transient expression of Cas9 to achieve the desired target site edits.

Among the viral delivery methods, AAV vectors are most preferred because of their mild immune response and absence of pathogenicity. AAVs can target non-dividing cells but have a limited packaging size. The development of the shorter dCas9 of 1 kb size has overcome this limitation to some extent [14]. Newer approaches are now being explored to decrease cytotoxicity, and to escape neutralizing antibodies for an overall improvement *in vivo* transduction efficiencies. As a results, some of the AAV based gene therapy products

are already in clinical trials for the treatment of RDs (Table 4).

6.2. Non-viral vectors

The problems associated with viral vectors such as immunogenicity and packaging issues have paved way for the development of non-viral systems that are usually better characterized and can be modified chemically to meet the delivery requirements [159]. However, non-viral vectors have concerns related to toxicity, biocompatibility, adverse immunological reactions, risk of release of therapeutic material into non-targeted sites. They also suffer from low *in vivo* delivery efficiency, although this problem is being solved with the advancements in material sciences. Nanotechnology-based formulations are expected to provide ample benefits over existing approaches [160]. These include (i) sustained release of payload, (ii) improved uptake in retinal cells, (iii) better vitreous penetrability, (iv) could be tailored to achieve cell-specific delivery and (v) reduce vitreal clearance leading to improved exposure time. Improved success has been achieved with newer polymer- and lipid-based complexes that have the required properties for effective transport of their genetic cargo across multiple physiological barriers. As a results, ample of nanobased products are approved by FDA or under investigation (Table 5).

For the delivery of CRISPR/Cas components *via* non-viral vectors, mechanisms of direct conjugation of the active molecule to the excipient has been adopted, such as gRNA or Cas protein conjugation with cell-penetrating peptides (CPPs). Studies by Ramakrishna et al. in HEK293T cells have demonstrated that the conjugation has led to 72% and 62% editing efficiencies with plasmids and RNPs, respectively. But, these CPP systems have not proven efficient to cross all delivery barriers [116]. The following are the current non-viral delivery systems that are being used for CRISPR/Cas9 delivery (Fig. 5).

6.3. Lipoplexes

In 1987, the scientific expression "lipofection" was first used to describe a lipidic system used for gene transfection [161]. It is one of the oldest and widely used techniques for gene transfer. Lipids have been extensively studied for their characteristics as nanocarriers and to electrostatically complex with a negatively charged gene, a positively charged cationic lipid is incorporated in the carrier. Commercially available cationic lipids include N-[1-(2,3-*dioleyloxy*) *propyl*]-N,N,N-trimethyl-ammonium chloride (DOTMA), 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE). and 2,3-dioleyloxy-N-[2(*sperminecarboxamido*)*ethyl*]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) [162]. Wang et al. in their experiments showed that CRISPR/Cas RNP can be administered into the cell using biodegradable cationic lipid nanoparticles, leading to effective knockout of genes [163]. The delivery of supercharged Cre protein and Cas9:sgRNA complexed with bio-reducible lipids into cultured human

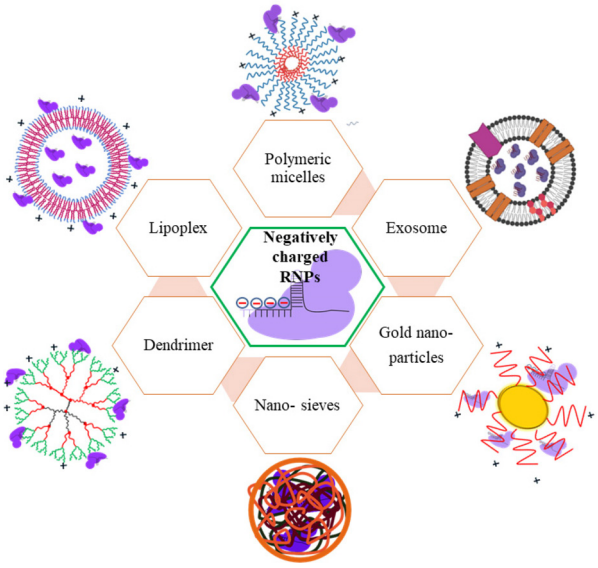

Fig. 5 – Non-viral vectors being explored for the delivery of CRISPR/Cas RNPs.

cells (HeLa-DsRed cells) enabled gene recombination and genome editing with efficiencies greater than 70%. Further, disulfide linkages in the lipid material can be used to trigger release by the degradation of endosomal particles leading to endosomal release. In addition, the authors demonstrated that these lipids are effective for functional protein delivery into mouse brain for gene recombination *in vivo* [164]. In 2020, Wei et al. used mixture of lipid (5A2-SC8, DOTAP, DMG-PEG, Chol, DOPE) to prepare a lipidic system i.e. 5A2-DOT with different concentrations of DOTAP (10 - 50 mol%). The nanoformulation showed sufficient payload for Cas9 RNPs and showed efficient, precise gene editing (for TdTomato gene) in mice brain, muscle, when administered locally. Further, formulation given intravenously also showed significant gene editing in liver and lungs tissues as well [165].

6.4. Polyplexes

There are various types of polymer complexes that are being used for CRISPR/Cas delivery. Cationic polymers pose lesser immunogenicity problems as well as are much easier to synthesize on a large scale. Those which have gained attraction include polyethylenimine (PEI), poly(L-lysine) (PLL), poly[2-(dimethylamino) ethyl methacrylate] (PDMAEMA), and polyamidoamine (PAMAM) dendrimers [166]. Among polymeric vectors, polyethylenimine (PEI) has been extensively researched upon. Scientists in 1995 carried out the first successful transfection using PEI, after which it is regarded as a gold standard in the study of polymeric non-viral carriers due to their high transfection efficiency. The characteristics of PEI which make it suitable for gene transfer are its "proton sponge" nature and high charge density [167]. Like cationic lipids, PEI can be complexed with nucleic acids, induce endosomal uptake and release in the cytoplasm of the cell. A study by Zhang et al. shows a formulation made up of PEI-β-cyclodextrin to deliver plasmids coding for

Table 4 – List of therapeutic molecules in clinical trials for the treatment RDs.

Retinal dystrophy	Targeted gene	Therapeutic approach	Phase	Year	NCT ID
LCA	CEP290 Intron 26(IVS26)	subretinal EDIT-101 (CRISPR/Cas9)	I/II	2019–24	NCT03872479
	CEP290 p.Cys998X	Intravitreal QR-110(Antisense oligonucleotides)	I/II	2017–19	NCT03140969
			I/II	2019–21	NCT03913130
			II/III	2019–21	NCT03913143
	RPE65	Subretinal rAAV2-CBSB-hRPE65	I	2007–26	NCT00481546
		Subretinal tgAAG76(rAAV2/2.hRPE65p.hRPE65)	I/II	2007–14	NCT00643747
		rAAV2-CBSB-hRPE65	I/II	2009–17	NCT00749957
		Subretinal AAV2-hRPE65v2	I	2007–18	NCT00516477
			III	2012–29	NCT01208389
			I/II	2010–26	NCT00999609
		Subretinal rAAV2-hRPE65	I	2009–17	NCT00821340
		Subretinal rAAV2-CBSB-hRPE65 Applied	I/II	2009–17	NCT00749957
		Subretinal rAAV-2/4.hRPE65	I/II	2011–14	NCT01496040
		Subretinal AAV2/5-OPTIRPE65	I/II	2016–18	NCT02781480
			I/II	2016–23	NCT02946879
CHM	REP1	Subretinal rAAV2.REP1	I/II	2015–25	NCT02077361
		Subretinal rAAV2.REP1	I/II	2011–17	NCT01461213
		Subretinal AAV2.REP1	II	2016–21	NCT02407678
		Subretinal rAAV2.REP1	II	2016–18	NCT02671539
		Subretinal rAAV2.REP1	II	2015–18	NCT02553135
		Subretinal BIIB111(AAV2-REP1)	II	2018–22	NCT03507686
		Subretinal AAV2-REP1	III	2017–20	NCT03496012
	CHM	Intravitreal 4D-100	I	2020–23	NCT04483440
		Subretinal AAV2-hCHM	I/II	2015–22	NCT02341807
RP	PDE6B	Subretinal AAV2/5-hPDE6B	I/II	2017–24	NCT03328130
	RLBP1	Subretinal CPK850	I/II	2018–26	NCT03374657
	PDE6A	Subretinal rAAV.hPDE6A	I/II	2019–25	NCT04611503
	USH2A	Intravitreal QR-421a	I/II	2019–22	NCT03780257
Advanced RP	ChR2	Intravitreal RST-001	I/II	2015–35	NCT02556736
adRP	RHO	unilateral IVT injection QR-1123	I/II	2019–21	NCT04123626
XLRS	RS1	Intravitreal rAAV2tYF-CB-hRS1	I/II	2015–23	NCT02416622
		Intravitreal AAV8-scRS/IRBPhRS	I/II	2015–23	NCT02317887
XLRP	RPGR	sub-retinal BIIB112	I/II	2017–20	NCT03116113
		Intravitrea 4D-125	I/II	2020–23	NCT04517149
		Subretinal AAV2/5-RPGR	I/II	2017–20	NCT03252847
		Subretinal AGTC-501 (rAAV2tYF-GRK1-RPGR)	I/II	2018–26	NCT03316560
		Subretinal AAV5-RPRG	III	2021–22	NCT04671433
Achromatopsia	CNGB3	Subretinal rAAV2tYF-PR1.7-hCNGB3	I/II	2016–25	NCT02599922
		Subretinal rAAV.hCNGA3	I/II	2015–27	NCT02610582
Leber Hereditary Optic Neuropathy	ND4	GS010; Sham intravitreal injection	III	2016–19	NCT02652767
			III	2016–18	NCT02652780
		Intravitreal scAAV2-P1ND4v2	I	2014–23	NCT02161380
		Intravitreal rAAV2-ND4	—	2011–15	NCT01267422
USH1B	—	Subretinal SAR422459	I/II	2012–19	NCT01505062
	—	Blood draw for the laboratory assessment	I/II	2013–32	NCT02065011
Stargardt's Macular Degeneration	—	Long term follows up in all patients who received SAR422459 in previous study TDU13583	I/II	2012–34	NCT01736592
	—	SAR422459	I/II	2011–19	NCT01367444
Neovascular AMD	—	Subretinal RetinoStat	I	2011–15	NCT01301443
	—		I	2012–29	NCT01678872
	—	Intravitreal AAV2-sFLT01	I	2010–18	NCT01024998
	—	Subretinal rAAV.sFlt-1; Control (ranibizumab alone)	I/II	2011–17	NCT01494805

Abbreviations: RPE65: Retinal pigmented epithelium-specific protein with molecular mass 65 kDa; **CEP290:** Centrosomal Protein 290; **ChR2**: Channelrhodopsin-2; **PDE6B:** Phosphodiesterase 6B; **RLBP1:** Retinaldehyde-binding protein 1**; USH2A:** Usherin; **PDE6A:** Phosphodiesterase 6A; **RPGR:** Retinitis pigmentosa GTPase regulator; **REP1:** Rab escort protein 1; **RS1:** Retinoschisin.

Table 5 – List of nanomedicines approved or under clinical investigation for the treatment of RDs.

RD	Status	Nanomedicine	Product name	Molecule	NCT ID
AMD	Approved by FDA	Liposome	Visudyne®	Verteporfin	NCT00121407
wAMD	Approved by FDA	Aptamer–polymer nanoparticle	Macugen®	Pegaptanib sodium	NCT00549055
Macular edema	Approved by FDA	Suspension	Kenalog	Triamcinolone acetonide	NCT00101764
	Phase II	Lipid-based nanoparticle	TLC399 (ProDex)	Dexamethasone sodium phosphate	NCT03093701

sgRNA and Cas9 in HeLa cells, achieving gene knockout [168]. PEI has also been used in a formulation by Sun et al., in which they have used DNA as a nanomaterial for coating of CRISPR/Cas. These particles were encapsulated by PEI to enhance endosomal release. These particles were directly injected in mice which had EGFP tumors, and the resulting phenotypes showed knockout of EGFP [169]. Apart for PEI, the high charge density PLL, a synthetic polypeptide, has been explored for gene delivery. However, due to the absence of buffering capacity (required for endosomal escape), PLL displays lower transfection efficiency than other polyplex systems [170]. Further, the high-molecular-weight PLL leads to a high level of cytotoxicity as it interacts with serum proteins, causing rapid elimination of the complexes from the system. Studies have shown a PLL-PEG copolymer can help solve these issues and facilitates its duration in the system [171]. The success of PLL-PEG copolymer in gene delivery has been proved in both *in vitro* and *in vivo* studies. In a study conducted in 2004 on treating cystic fibrosis (CF), PLL-PEG was explored as a gene delivery construct carrying the cystic fibrosis transmembrane regulator encoding gene [172]. PDMAEMA is another non-viral delivery method. A water-soluble cationic polymer, polysaccharide modified PDMAEMAs are gaining significance in CRISPR/Cas delivery [135]. In 2020, Carlos et al., synthesized magnetite/silver-PDMAEMA conjugate and evaluated it for the delivery of CRISPR plasmid. It showed 16.4% loading efficiency with minimum toxicity. Further, colocalization studies were performed in SH-Y5Y cells with lysotracker and data indicated, the Pearson's correlation coefficient approached 0.240 ± 0.024 after 0.5 h and decreased to 0.215 ± 0.029 in a statistically significant manner after 4 h. Collectively, the magnetite/silver-pDMAEMA showed endosomal escape after 4 h of internalization and showed effective delivery potential *in vitro* [173]. In 2019, Chen et al. developed Cas9 RNPs loaded nanocapsule (NCs) composed of cationic polymer and liposome components along with a glutathione cleavable linker in between. NCs carry 40% of RNPs content with a particle size of 25 nm. The research focused on *in vivo* gene editing performed in eyes and muscles of transgenic Ai14 mice having three sv40 polyA transcription terminators as stop cassette for TdTomato gene. NCs were decorated with all-trans-retinoic acid (NTRA, binds to inter-photoreceptor retinoid-binding protein) to form NTRA-NCs, and evaluated for gene editing in RPE cells in the eyes of Ai14 mice (Fig. 6). Further, mice were injected with PBS, naked RNPs, NCs and all trans retinoic acid NCs (ATRA-NCs) subretinally and eyes tissue were excised after 12 dpost injection. NCs showed considerable and ATRA-NCs showed significantly higher gene editing in RPE cells in terms of TdTomato fluorescence. Moreover, the NCs were injected intramuscular and NCs showed gene editing and a strong TdTomato fluorescence was observed in muscle tissues. Collectively, the study explored the potential role of non-viral biodegradable NCs in the delivery of high molecular weight Cas9 RNPs for *in vivo* gene editing application [174].

6.5. *Dendriplexes*

Polyamidoamine dendrimers (PAMAM) dendrimers are commercially available for gene transfer and one of the most frequently used systems [175]. The structure is made up of a core surrounded by polymeric branches with the surface expressing cationic primary amines that helps them complex to nucleic acids. PAMAM dendrimers are known to be generation-dependent; low-generation (G0–G3) dendriplexes have poor gene transfection efficiencies and are less cytotoxic, but higher generation (G4–G8) PAMAMs have improved gene transfection efficiencies and are more cytotoxic [176]. Yu et al. in their research, prepared a formulation made up of a lipid and dendrimer hybrid. The structure consisted of a long hydrophobic alkyl chain and a low-generation hydrophilic PAMAM dendron. In this way, the system exhibited the pros of both lipid and polymers in delivering siRNA and achieved the efficient gene-silencing effect *in vitro* and *in vivo* studies [177]. In another study, L-arginine was used to transform the surface of PAMAM enhancing gene transfection efficiency due to ease of complexation [178]. In 2019, Liu et al., synthesized boric acid rich 5 (G5) amine-terminated PAMAM (P4) for CRISPR/Cas9 RNPs delivery. The P4 dendrimer nanoparticle loaded with RNPs showed particle size of 300 nm. The functionalized dendrimer showed efficient delivery along with 45% of EGFP gene knock out in EGFP-HEK293T cells, analyzed *via* flow cytometry. Further, another set of experiments were performed to evaluate gene editing efficiency, and CRISPRMax was taken as standard along with P4 dendrimer. CRISPR/Cas9 RNPs were designed for targeting adeno-associated virus integration site 1 (AAVS1) and hemoglobin subunit beta (HBB) and transfection assay was performed followed by T7 endonuclease (T7E) assay, where results indicate indel frequency of 23.1% and 17.5% for AAVS gene with P4 dendrimer/RNPs and CRISPRMax/RNPs, respectively. Likewise, indel frequency of 1.1% and 9.7% was observed for HBB gene with P4 dendrimer/RNPs and CRISPRMax/RNPs, respectively. Collectively, this study explored the potential

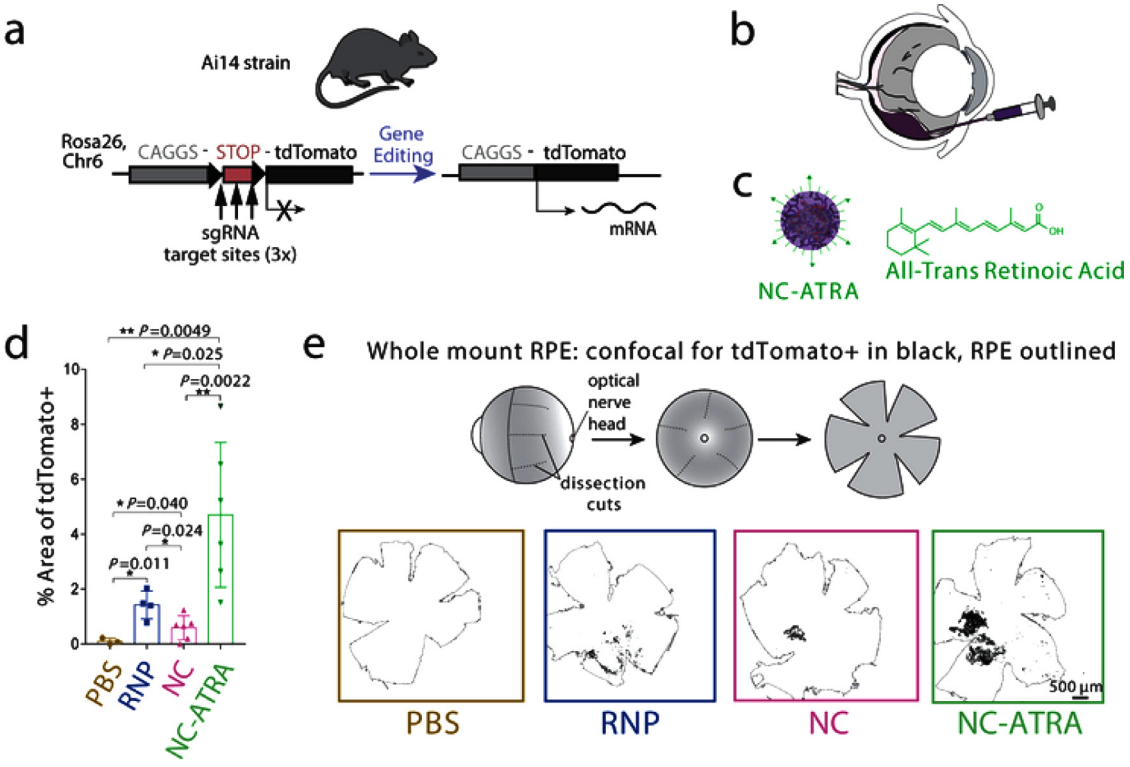

Fig. 6 – *In vivo* gene editing efficiency of RNP loaded nanocapsule in mice.Graphicalillustration of the (a) TdTomato gene, (b) site of intravitreal injection in mice eye, (c) ATRA targeted nanocapsule. (d) post injection *in vivo* gene editing in RPE layer quantified in terms of TdTomato positive area (%) in different treatment groups, (e) mounted RPE layer of the mouse treated with PBS, RNP, NCs and NC-ATRA, herein black area represents TdTomato signals after 12 d treatment. Reprinted by permission from [174]. Copyright ©2019 the authors under exclusive licence to Springer Nature Limited.

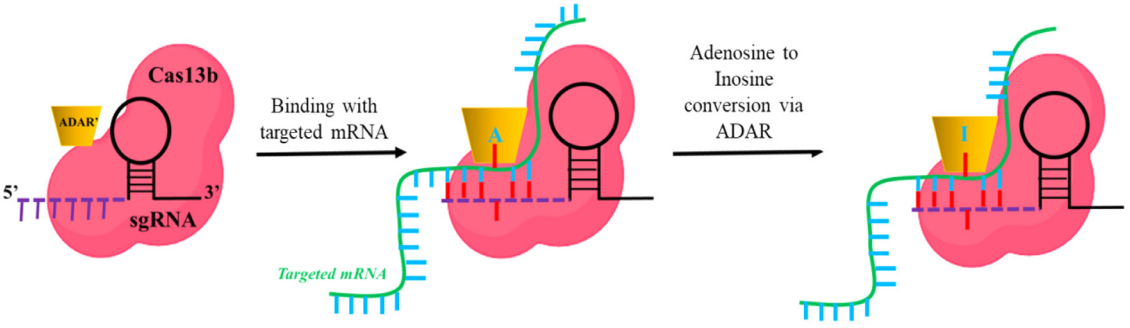

Fig. 7 – Schematic representation of mRNA editing using Cas13b in retinal dystrophic conditions.

role of dendrimers as a delivery vehicle for CRISPR/Cas9 [179].

7. CRISPR/Cas based RNA editing approach in retinal degeneration diseases

Theoretically, G>A or T>C single-base mutations in any gene related to inherited retinal degeneration could be corrected by using base editing [180]. Nevertheless, all the diseases are not equally responsive to RNA editing. AAV mediated gene replacement provides effective treatment for small gene deliveries. On the other hand, higher rate of mutant allele-specific editing is required for dominant diseases where the mutant alleles have to be efficiently knocked out. Interestingly, larger recessive genes (>4.2 kb) are difficult to correct by gene replacement using AAV and requires in-situ editing strategy either *in vitro* or *in vivo*. Stone et al. have listed the major recessive genes such as ABCA4, USH2A, CEP290, MYO7A, EYS, CDH23 along with their relative frequency in the patients with inherited retinal degeneration [181]. The most common single nucleotide mutation is G>A, amenable to currently available base editing techniques. The proportion of G>A or T>C mutation in CEP290 and ABCA4 gene was 9% and 32%, respectively [181]. Further, 6% of the mutations observed in USH2A gene results in the creation of premature stop

codon, while missense and donor/acceptor slice mutations were predominantly seen in ABCA4 gene. Collectively, the data suggested the dominancy of G>A mutation (~75 in number) in both the genes, that leads to premature stop codons. Humans have adenosine deaminase acting on RNA (ADAR) enzymes, which play a key role in adenosine to inosine conversion (A→I) [182]. Since inosine is read as guanosine by the splicing and translation apparatus, ADARs can also be used to alter RNA splicing to restore normal reading frames and modify amino acid sequences.

In 2017, Cox et al. explored a new system called 'REPAIR' (RNA Editing for Programmable A to I Replacement), which aimed to cleave RNA at a specific site by the means of a novel protein Cas13a, isolated form the *Leptotrichia wadei*. Cas13a binds to RNA-RNA hybrid unlike the DNA-RNA hybrid bound by Cas9 and is also a part of the bacterial CRISPR mediated RNA interference system [183]. The Cas13-based RNA editing technique may be advantageous over the conventional DNA editing, as DNA manipulations are permanent in cellular genomes and may have unanticipated long-term side effects. Currently, a synthetic RNA targeting system has been developed with a human protein, with Cas13 like ssRNA recognizing properties. This system termed as CIRTS (CRISPR/Cas inspired RNA targeting system) is an engineered modular RNA-guided RNA-targeting effector system, synthesized totally from human proteins and provide a new tool set to overcome the size and immunogenicity limitations of bacterial CRISPR-Cas systems. This system consists of an engineered gRNA, that carries sequences complementary to the target RNA and also carries a sequence to recruit an engineered hairpin binding protein and a non-specific ssRNA binding protein for complex stabilization and an ADAR2 like effector protein to enable base editing (Fig. 7). CIRTS8 system, when delivered in the form of a plasmid construct, could achieve 47% edit efficiency in HEK293T cells without significant off-target edits [180]. RNA editing thus provides immense potential in repairing pathogenic mutations in many diseases such as Duchenne's muscular dystrophy, cystic fibrosis, hurler's syndrome etc. RNA edits are also reversible and therefore offers improved safety in terms of therapeutic considerations. Inducible RNA editors with automatic switch off systems and external stimuli triggered switch on systems further improves the safety profiles of RNA editing [184].

8. Conclusions and future perspective

CRISPR/Cas system has emerged as a rapidly evolving therapeutic tool in genomic engineering. Genome therapy using CRISPR/Cas in ophthalmic diseases is a boon for the society considering the impact it has on the lives of thousands of people. It offers newer hopes of developing promising therapeutics for the treatment of inherited retinal disorders. In the past 20 years, the eye and ocular diseases have caught the limelight of gene therapeutic and cell therapeutic efforts, mainly for the unique anatomical location that enables easy interventions, detailed imaging and documentations; the immune privileged status of the eye; the existence of blood-retinal barrier safety that ensures long-term ocular retention and containment of therapeutics; and finally for the huge societal impact of even marginal improvements in vision to patient beneficiaries. Gene therapeutics either alone or when combined with gene editing strategies can enable delivery of normal gene copies; or *in situ* mutation editing for normal protein and RNA expression; and targeted disruption of dominant mutant alleles or their transcripts for the reversal of disease phenotypes. Several pre-clinical studies have been initiated using both the viral and non-viral vectors for delivering CRISPR components for therapeutic applications. While it is exciting to watch the fast pace of developments in this field, it is prudent to move forward responsibly, considering both the ethical and societal implications. It is important to exercise well-informed caution and consider both the intended and unintended outcomes, while designing gene editing based therapeutic strategies.

Conflict of interest

The authors declare no conflict of interest.

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