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Synergy of nanocarriers with CRISPR-Cas9 in an emerging technology platform for biomedical appliances: Current insights and perspectives

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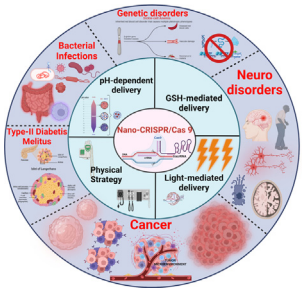
HIGHLIGHTS

- Synergistic action of nanocarriers and CRISPR can be used for ablation of many genomic disorders.
- The enhanced delivery system is mediated by the endosomal escape mechanism.
- The synergistic strategizes is used for many neurodegenerative disorders as well as infection system.

GRAPHICAL ABSTRACT



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ABSTRACT

Genetic editing technologies have emerged as a potential therapeutic tool in various biomedical fields owing to their applications against cancer, neurological diseases, diabetes, autoimmune disorder, muscular dystrophy, bacterial infections (AMR), and cardiovascular diseases. CRISPR is one such valuable genetic editing tool with extensive therapeutic appliances but with a major challenge in terms of delivery. Herein, we have strived to exploit a synergy of nanocarriers and CRISPR against the aforementioned diseases for their medical applications and explicated their clinical significance including the enhanced delivery via endosomal escape and environmental factors such as light, pH, and stimuli. In addition to highlighting the delivery strategies of nano-carriers for CRISPR and their characterization, we have expounded on the reliant factor of the CRISPR-Cas Complex.

1. Introduction

The rapid progression of genome editing in recent years has transformed human genome research to an advanced level, providing an enhanced comprehension to the researchers about the contribution of single-gene product in regulation of different diseases [1]. The invention of genetic engineering in the 1970s ushered a

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new era in biomedicine, applied biotechnology and pharmaceutical fields through the introduction of both synthetic and bacterial nucleases in *in vitro* or *in vivo* models; prompting addition, deletion, or neutralization of the genes for targeted genomic modifications without any off-target mutation [2,3]. The induction of nuclease-induced double-stranded breaks (DSBs) due to these modifications often leads to the stimulation of effective cellular DNA recombination processes in mammalian cells [4]. The different types of DNA repair mechanism like homology-directed repair (HDR) and non-homologous end-joining (NHEJ) can repair nuclease-induced DNA DSBs in cells for targeted integration or gene disruptions [1].

CRISPR has evolved as the most successful and favourable technology for gene editing, with varied usages in scientific research and clinical trials [5]. Although the application of CRISPR technology for DNA editing has progressed significantly in recent years, there is yet room for improvement. The CRISPR-Cas complex requires components to be transported into the nucleus for their effect on the nuclear genome to overcome tissue as well as cell membrane barriers, and its conveyance to specific tissues or cells is a difficult and time-consuming endeavour [6]. Among the existing strategies, non-viral vectors, viral vectors, and physical delivery are often deployed for the delivery of CRISPR-Cas9 [7,8]. The most popular physical tactic for transferring the CRISPR-Cas complex into cells is electroporation, and single-cell microinjection, which are widely employed in embryonic gene editing and the production of transgenic animals [9]. The Cas DNA or protein components have been transferred with good efficiency and little or no cytotoxicity. Microinjection, is a long-drawn and arduous method, limiting its use to a few numbers of species to deliver CRISPR-Cas complex [10]. Viral vectors are widely used as CRISPR-Cas 9 delivery vectors because they are efficient and effective, particularly for *in vivo* studies. Current consensus suggests that viral vectors are the undisputed masters of *in vivo* CRISPR delivery because of their superior cellular absorption and editing effectiveness. Full-sized adenoviruses and lentiviruses, as well as genetically modified adeno-associated viruses (AAVs), are examples of viral delivery vectors. Adeno-associated viral vectors (AAVVs) have emerged as the preferred *in vivo* delivery route for CRISPR components because of their minimal immunogenicity, cytotoxicity, and restricted incorporation into the host cell [11]. Another viral vector used to transport CRISPR components is the lentiviral vector (LV). Compared to the AAV vector, it can clone more efficiently. It is a platform for delivering the most prevalent CRISPR/Cas protein (Cas 9) and sgRNA cassette in a single viral transfection event since it can package two copies of an RNA genome (approximately 10 kilobases) [12]. However, LVs present a safe approach in therapeutic applications due to insertional mutagenesis and the persistent production of site-specific nucleases, both of which can lead to off-target mutations [13]. Non-integrating lentivirus vectors (NILVs) have recently been developed for the delivery of CRISPR components by scientists in an effort to mitigate the risk of integration through either a mutation in the viral integrase gene or a modification to the attachment sequence of lengthy terminal repeats (LTRs) [14]. Each viral vector has its own set of benefits and downsides, making it impossible to recommend a single viral vector as the ideal way to transport CRISPR components. There are significant issues concerning the clinical application of viral vectors, including immunogenicity, integration, and off-target effects, despite the fact that they have a high *in vivo* transfection efficiency, new techniques are constantly being developed.

Nanocarriers have recently demonstrated distinct advantages in gene delivery of the CRISPR-Cas complex [15]. Fig. 1 exhibits the timeline of the CRISPR-Cas system for its application in therapeutics. Certain nano-delivery technologies such as cationic liposomes, lipid nanoparticles (LNPs), cationic polymers, vesicles, and gold nanoparticles have been produced and utilized for transporting the CRISPR-Cas complex [16]. In preclinical studies, non-viral vectors have proven to be a promising technology for the delivery of CRISPR-Cas 9 systems as one-of-a-kind vehicles for expanding the application of this technology, thus instigating strong gene-editing in the life sciences and therapeutic settings [9]. To cite an example, Mout *et al.* used cationic arginine gold nanoparticles to deliver the Cas 9 protein for targeting the human AAVS1 gene to cause tumor regression in CC cells [17]. Zhou *et al.* used a biodegradable 2-D delivery platform utilizing black phosphorus nanosheets (BPs) with Cas 9-RNPs targeting EGFP via cytosolic delivery in a murine model to cause tumor regression [18].

This review highlights the synergy of nanocarriers and CRISPR deliberated for numerous biomedical appliances including cancer, diabetes, autoimmune disorder, neurological disorders, cardiovascular disorder, and muscular dystrophy with special emphasis on the clinical significance of both, the chemical and environmental strategies that can enhance the efficiency of CRISPR delivery. Additionally, the varied factor is underscored that affects the CRISPR-Cas complex efficiency and the characterization of the nanocarriers that influence CRISPR delivery.

2. Factors influencing CRISPR-Cas 9 activity

The efficiency of CRISPR Cas is dependent upon several factors like targeted DNA site selection and sgRNA design, off-target cutting, Cas activity, and occurrence or efficacy of HDR for gene editing [19](Fig. 2).

2.1. Target DNA site selection as a sgRNA design

The CRISPR-Cas 9 system possesses the potential to target any 23 base pair (bp) sequence containing a PAM motif on either strand of the target DNA that occurs at every 8 bp for the SpCas 9 PAM [20]. The PAM sequence for Cas 9 proteins differs slightly from species to species, as in the case of *Neisseria meningitidis* [21]. The enhanced flexibility in selecting the target sequence increases with the identification of Cas 9 proteins with varying PAM motifs. Novel Cas 9 variants having different specificities of PAM sequences could be engineered owing to directed evolution and structure-guided ration design of VGR, EQR, and VRER varieties of SpCas9 with altered PAM sequences [22]. Single, as well as multi-base mismatches, are permitted while multiple-base mismatches encompassing increased distances from the PAM sequence are additionally preferred for CRISPR-Cas [23]. It bears lower specificity than ZFNs or TALENs owing to its short targeting sequences with no significant off-target gene alteration in a gRNA-dependent manner [24]. The emergence of different computational tools and software packages facilitates the sgRNA design.

2.2. Off-target cutting

To enhance the specificity and reduce off-target cleavage mutant, Cas 9 systems have been designed for improving the specificity of sgRNA. However, mutating the Cas 9 protein can be modified to introduce ss-nicks. Upon modification, CRISPR-Cas binds with forward and reverse DNA flanking sequences of the target site for DSB through cooperative nicks. Simple DNA ligases can be used to repair single-stranded nicks produced due to off-target cleavage. The use of the aforementioned system reduces off-target cutting in mammalian cells significantly without compromising the target gene cleavage [25]. Another mutant Cas 9 system encompasses a fusion protein of inert dCas9 and Fok1 nuclease dimer using ZFN and TALEN as a template wherein sgRNAs have been engineered to bind to forward and reverse flanking the target sequence. The

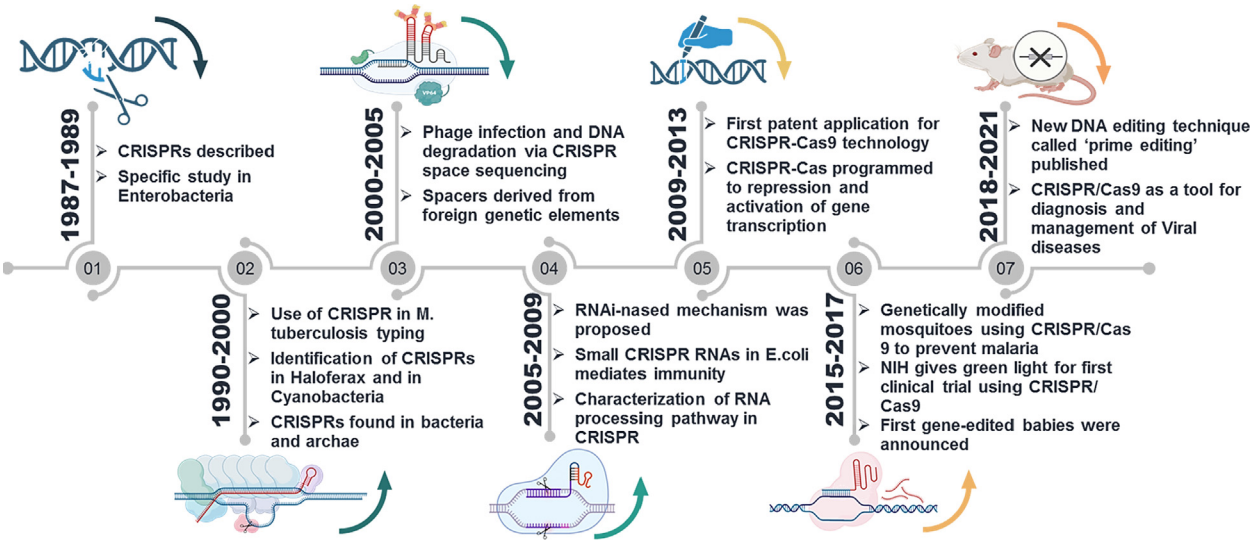

Fig. 1. Schematic diagrams showing a timeline of the CRISPR-Cas system and when the first nano delivery of the CRISPR-Cas system was used.

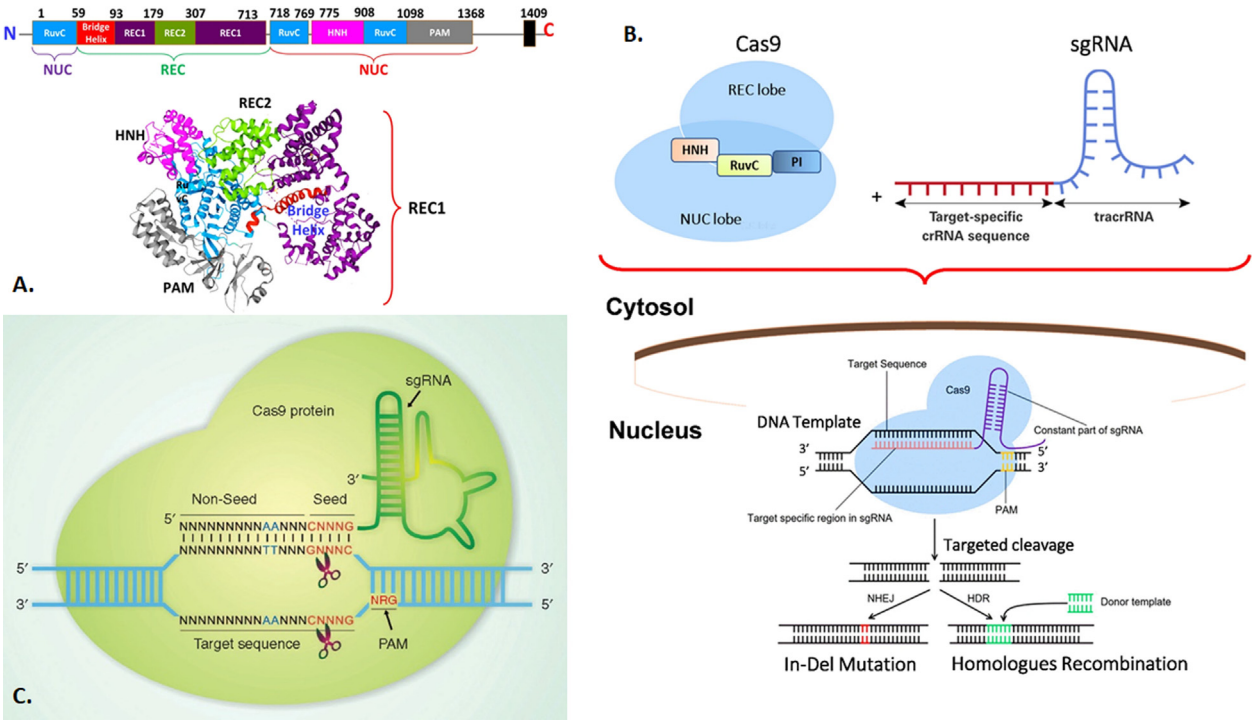

Fig. 2. Factors modulating CRISPR-Cas 9 activity. (i) Schematic model depicting the organization of CRISPR-Cas 9 domain (PDB ID: 4CMP) [31]. (ii) Graphical model depicting the phenomena of off-target cutting by CRISPR-Cas 9 system [32]. (iii) Mechanism of CRISPR-Cas 9 cleavage of target DNA through either NHEJ or HDR [31].

Fok1 nuclease dimers became functional for the formation of DSB through this configuration instigating enhanced target specificity by reducing off-target cleavage and reducing possible target sites owing to the constraints imposed by PAM and sgRNA designs [26]. The *in-vivo* transport of these gene-altering tools became a challenge owing to their large size. Non-specific DNA contacts could be reduced by another type of Cas 9 mutants where binding of the target and non-target DNA strand with the sgRNA could be weakened while maintaining robustness on target cutting [27].

2.3. Incidence or efficiency of homology directed repair (HDR)

The occurrence of HDR-induced DSB repair via Cas 9 is significantly low in a mammalian murine model (Fig. 2). NHEJ is used more often as a repair method for HDR even in the presence of a donor template [28]. To enhance HDR efficiency, NHEJ was suppressed by using small inhibitors of NHEJ gene silencing for cell cycle synchronization. Scr7 could be used as an inhibitor of the NHEJ component DNA ligase IV to enhance the efficacy of HDR-mediated gene editing 19-fold [29]. Due to the toxic effects of NHEJ inhibitors on host cells, cell synchronization into the late-S and G_2 phase involved HDR action which renders direct nucleofection of

Cas 9 ribonuclease complex as an effective alternative to chemical inhibition of NHEJ [30].

2.4. Cas 9 activity

Various Cas 9 proteins have been used for gene editing that has been isolated from different species like SaCas9 from *Staphylococcus-aureus,* NmCas9 from *Neisseria meningitides,* and St1Cas 9 from *S. thermophiles.* Each of these possesses different PAM sequences and varying activity, thus, making specific Cas 9 orthologs more useful via specific target sequences [25]. The inherent action of a Cas 9 protein influences its activity in the translocation of eukaryotic cells into the nucleus through nuclear localization signals (NLS). The addition of a 32 amino acid spacer between the Cas 9 protein and the NLS enhances the DNA cleaving efficacy [33]. The on-target DNA cutting efficiency could be enhanced via the relative concentration of sgRNA to Cas 9 protein that ensures the formation of active RNPs by all Cas 9 proteins based on increased sgRNA concentration beyond the threshold level [33]. The binding efficiency of Cas 9 activity with the target DNA sequence to displace Cas 9 from the DNA strand is significantly less in comparison with other enzymes during the DSB formation [33].

3. Nano-CRISPR synergy

The deliverance of CRISPER to the target is must criteria prior to the use of CRISPR-based genome editing as therapeutics against various diseases, especially when the therapeutic alteration has to be made in *in-vivo* [1]. In the case of CRISPR Cas complex-based genome modification, the cargo is usually delivered via systemic injection, to overcome the series of challenges on their way to the cellular compartment. This can be performed either by viral vector or non-viral vectors. Viral vectors possess limitations like high immunogenicity, huge production cost, and low packaging capacity, that need to be addressed before consideration for their deployment as a vector for the CRISPR-Cas complex [34]. Consequently, nano-based non-viral vectors are utilized to deliver the cargo Cas 9 in the form of protein, mRNA, or plasmid DNA by packing either electrostatically or covalently.

The nanoparticle, nanocarriers or nano-formulation shell is not only used to protect the CRISPR-Cas complex from aggregation, immunological clearance, renal elimination, and pre-mature release but also leads the cargo to the target cells by coating with the appropriate targeting ligands [35]. It encloses genome editing materials to overcome the next obstacle for targeting the plasma membrane of the cell. The genome-editing material of the CRISPR-Cas complex can either burrow directly or enter via endocytosis completely via their protective shell for alteration of homologous dependent recombination or non-homologous recombination that required gene against the respective disease [36].

Non-viral vectors have already been utilized in multiple platforms for delivering CRISPR-Cas systems in a relatively safe manner owing to their ability to accommodate a wide range of cargos while lowering delivery obstacles without triggering any immunogenicity in the host system. For example, Ray *et al.* illustrated cancer immunotherapy utilizing arginine nanoparticles (ArgNPs) to deliver CRISPR-Cas 9 protein. The cargo was used as a gene modification apparatus for SIRP-a knockout in macrophage, which prevents phagocytosis of cells by enhanced attack and destruction of cancer cells [37]. In a similar investigation, a polymeric nanoformulation with encapsulated CRISPR-Cas9/ sgRNA plasmid was used for targeting and cleavage of neurofibromatosis 1 (Nf1) *in vivo* murine model. It was developed using a localized intrathecal administration route that led to downregulation of cytosolic regulatory protein collapsin response mediator protein 2 (CRMP2) as a therapeutic target against NF1 [38]. Lipid NPs have been functionalized with DSPE-PEG to overcome the issues of cytotoxicity for utilizing them as a vector for CRISPR-Cas delivery. The use of lipid NPs is preferred due to their enhanced transfection efficiency and ability to facilitate endosomal escape. In another study, Zhang *et al.* utilized biodegradable amino-ester lipid-like nanoparticles for delivering the Cas 9-mRNA [39].

Several strategies have been applied for the delivery of CRISPR-Cas 9 using nanocarriers to enhance the efficiency of the delivery system and to improve the gene-editing efficiency; endosomal escape, stimulus-induced release, and physical transportation of genome-modifying are used to overcome the limitations of CRISPR-Cas [40]. The majority of nanoparticles enter cells via endocytosis by fusion to early endosomes for maturation in late endosomes and destruction at lysosomal compartments to cause endocytosis-mediated internalization [40]. Therefore, early escape from endosomes is critical for the effective delivery of Cas 9 into the nucleus via PEI-mediated osmotic pressure, liposome-mediated membrane fusion, pH-responsive polymer-mediated swelling, and cationic peptide-mediated membrane destabilization. Thus, inducing the rapid release of nucleic acids or proteins into the cytoplasm [41]. Herein, the chemical or molecular delivery and physical strategies for delivering CRISPR are expounded.

3.1. Endosomal escape

Chemical or peptide-enhanced delivery uses chemicals and peptides to enhance endocytosis or endosomal escape. The peptide pardaxin is a membrane-penetrating peptide that has been used for the non-lysosomal intracellular transport of CRIPSR-Cas [42]. The endosomal escape, mediated by glucuronylglucosyl, has been investigated for efficient genome editing in brain cells [43]. Wang *et al.* deployed PEGylated nanoparticles for the delivery of CRISPR-Cas9 protein with the sgRNA-aided α-helical polypeptide. This formulation conferred superior membrane penetration capacity due to the retention of its helical structure aiding to cellular uptake, and endosomal escape followed by translocation into the nucleus. Thus, Cas 9 plasmid or sgRNA (as a complex or separately) was efficiently delivered in HeLa cells for targeting Plk1 using CRISPR-Cas 9 based gene editing increasing survivability and negligible toxicity [44]. Similarly, a fluorinated polymer (PF33) core bound to the CRISPR-Cas 9 system (PF33/Cas 9-hMTH1 nanoparticles) and having a versatile multifunctional shell (RGD-R8-PEG-HA, RRPH) was utilized for *in vivo* delivery targeting the nucleus of ovarian cancer with minimal side effects [45].

In a similar investigation, Sun *et al.* designed a novel CRISPR–Cas 9 delivery mechanism utilizing DNA nano-clew (NC) wrapped with polyetherimide based on the principle of rolling circle amplification with palindromic sequences encoded to guide nanoparticle self-assembly in a murine model by targeting EGFP expression via endosomal escape for tumor regression [46]. Thus, these studies depict the utility of chemical or peptide-enhanced delivery for enhancing efficiency and improving gene-editing machinery.

3.2. Light-enhanced delivery

Phototherapy, also known as light-enhanced delivery, is another strategy that is efficiently used to promote the endosomal escape or initiate the release of encapsulated cargo in a photon-dependent manner. Several biomaterials have been designed for the delivery of small drugs or genes using phototherapy [47]. One of the major advantages of this strategy is the possession of no adverse side effects on exposed tissues compared to conventional used stimuli like radio radiation. The release of gene-editing cargoes by light has been proved to have more advantages

as it facilitates remote control of gene editing to the target tissue and enables the endosomal escape for the carrier allowing the cargo to be released. A non-intrusive, temporal and spatial solution is provided by the controlled transport and activation of gene editing tools by light (Fig. 3) [48]. Different materials can be altered physically or chemically by the exposure of light. For examples, light-induced photothermal effects caused by photothermal agents, ROS production caused by photosensitizing agents, and photon-up conversion by luminescent materials [48].

Recent research has utilized the characteristics of light mediated delivery to transport and activate CRISPR-Cas 9. Notably, near-infrared (NIR) light (>800 nm) has a greater penetration depth (up to 3.2 cm) than ultraviolet or visible lights (1 mm), making it a desirable light source for photothermal therapy [49]. To cite an example, a NIR-triggered CuSRNP/DOX@PEI nano-platform for combined gene therapy, photothermal therapy, and chemotherapy was described by Chen *et al.* The core of CuS NPs was functionalized with thiol group which was further conjugated with DNA fragments with attached Cas9/sgRNA using the base complementary pairing for loading of doxorubicin. To release doxorubicin and Cas9/sgRNA RNP, CuS NPs, acting as a photothermal agent, converted NIR light (808 nm) into heat, which led to hastened the breakdown of hybridization between DNA fragments and sgRNA. Expression of Hsp90α, a subunit of heat shock protein 90 (Hsp90) was successfully downregulated by RNPs targeting the Hsp90 gene, thus reducing the capacity of tumor cells for thermal tolerance and metastasis. Furthermore, tumour cells could also be killed by CuS NP-triggered thermal effect. Thus, the combination of light-triggered genome editing, photothermal therapy, and DOX chemotherapy substantially suppressed tumorigenesis in the A375 melanoma mouse model [50].

Moreover, an increase in temperature can result from the conversion of light into heat energy via photothermal agents, which include inorganic (such as gold NPs, CuS NPs, and graphene) and organic (such as indocyanine green and polydopamine) materials [51]. For example, an AuNP-based photothermally regulated CRISPR-Cas9 delivery system was described by Wang *et al.* where electrostatic attraction was used to condense Cas9-sgPlk-1 plasmids onto the positively charged cell-penetration peptide-functionalized with AuNPs to generate AuNPs/CP. When exposed to light at a wavelength of 514 nm, AuNPs generated hot electrons that could cleave Au-S bonds, releasing peptides and Cas9-sgPlk-1 plasmids [52]. This initiated penetration of the tumor cells for the release of CP into the cytosol using laser-induced thermo-effects of AuNPs resulting the entry of CP in the nuclei using TAT guidance. The phenomenon was achieved after successful knock-out of the

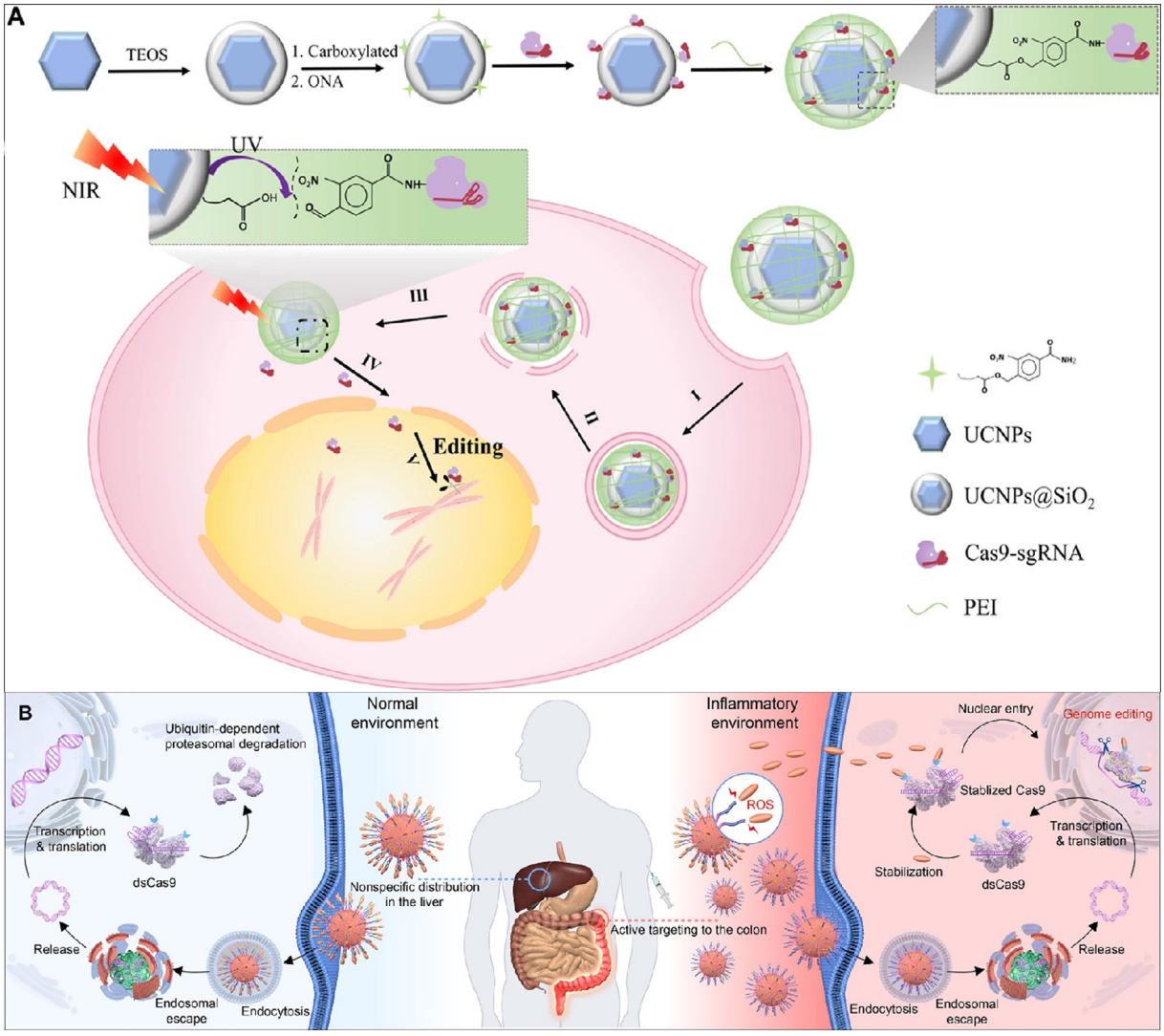

Fig. 3. (A) A-Schematic model depicting the synthesis of PEI conjugated Cas9 system with UCNPs that are triggered by NIR responsive treatment and are utilized for genome editing [57]. (B) Schematic model depicting the ROS mediated targeted delivery of CRISPR-Cas system on the colonic lesion [58].

target gene (Plk-1) and melanoma regression in both *in vitro* and *in vivo*. A deletion of the Plk-1 gene could be successfully obtained from the intratumoral injection of LACP and exposure to 514 nm light into a mouse model of A357 melanoma [52]. Thus, overexpression of Plk-1 can be seen in disruption of tumor cells by apoptosis [52].

It has been confirmed that photothermal effects can also stimulate endo/lysosomal escape and the breaking of thermosensitive chemical bonds to achieve the desired payload release [53]. Li *et al.* used the principle to administer CRISPR-Cas 9 plasmids and dexamethasone (Dex) using a semiconducting polymer (SP) functionalized with fluorinated polyethyleneimine (PF). Cas 9 plasmids were attached to PF polymers, and Dex through electrostatic and supramolecular interactions. Dex (a compound that interacts with the nuclear glucocorticoid receptor to enlarge the nuclear pores) was encased within the hydrophobic core of the NPs. Precise site-specific genome editing could be achieved by activating the SP. Which can cause heat-mediated endo/lysosomal escape of NPs leading to NPs disruption using NIR-II radiation of 950–1700 nm. Furthermore, the inclusion of Dex in this system facilitated the nuclear translocation of the Cas 9 plasmid for improved genome-editing effectiveness [54]. In a similar investigation, physically adsorbing and π-stacking of Cas 9/sgRNA onto the surface of PEI-decorated silicene nanosheets was used in synergy which has been a favourable material for their biocompatibility and biodegradability, high drug-loading capacity, and rapid photothermal conversion. The periodic atomic grooves on the surface of 2D silicene can serve as a multitude of anchoring sites to load proteins and RNA. The rapid endo/lysosomal escape of NPs and release of RNP were both facilitated by the photonic hyperthermia effect induced by NIR-II light of 1064 nm. Through protein processing in the endoplasmic reticulum signalling pathway, the TXNDC5 gene suppresses the efficiency of photothermal therapy (PTT) causing knocked down of the TXNDC5 gene through CRISPR-Cas 9 and thereby enhancing the photothermal hyperthermia action against tumor [55]. Thus, based on the aforementioned studies it could be concluded that phototherapy provides an ideal condition through various strategies for precision delivery of CRISPR-Cas systems via nano systems in biomedical disease.

3.3. Glutathione (GSH)-responsive delivery

Glutathione (GSH)-responsive delivery has been extensively investigated as a method to improve the release of genetic cargo for enhanced delivery and genome editing efficiency. The GSH concentration in the cytosol is 1000 times greater than that of the extracellular compartment [56]. This difference is used to prepare stable nanomaterials containing encapsulated or covalently linked gene-editing machinery in which the release of the CRISPR intracellularly is triggered by the high levels of GSH. The disulfide bridge is one of the most well-studied GSH-responsive linkers, and it can target an intracellular GSH-induced thiol-disulfide exchange [56]. It is equally important to give importance to design details of GSH-responsive features for the effectiveness of GSH-responsive systems. In the case of tumors, the GSH-bonds can be partially broken during blood circulation before the nanoparticles reach the target tissue in high intracellular GSH levels as the GSH is abundant in the blood as well as in the microenvironment of tumors [56].

3.4. pH-dependent delivery

The pH difference mainly triggers the activation of delivery system between tissues and intracellular organelles [59]. The acidic pH of tumor tissue and endosomes triggers the delivery of CRISPR-Cas 9 (DNA plasmids, mRNA) via membrane penetration and endosome escape [59]. Consequently, unless the delivery system escapes to the cytoplasm, it undergoes degradation by lysosomal enzymes after entering into the endo- lysosome. Thus, a variety of pH-responsive polymers facilitate the release of genome-editing constituents from endosomal lysosomes [59].

3.5. Physical strategy

Physical delivery strategies have been extensively explored to introduce therapeutic genome-editing components into the cytoplasm [60]. Most of the cargoes in receptor-mediated delivery methods undergo degradation and loss of functions in the lysosomes post-endocytosis. However, physical delivery systems have the advantage of being able to transfer gene-editing tools into the cytoplasm rapidly and efficiently without being getting degraded [60]. Various stimuli, which include mechanical or electrical forces, have been used to temporarily disrupt the cellular membrane for intracellular delivery [61]. It carries the advantage of being able to induce efficient the tedious genome editing in HSC cells. However, the determination of the potential risk involving modification of cellular activity and viability requires further research. One of the major concerns is the cell stress and damages that may ensue from the physical puncture of the cell membrane by a nano-needle or ultrasound [36]. The pH difference mainly triggers the delivery system activation between tissues and intracellular organelles. The acidic pH of tumor tissue and endosomes triggers the delivery of CRISPR-Cas9 (DNA plasmids, mRNA) via membrane penetration and endosome escape. Consequently, unless the delivery system escapes to the cytoplasm, it undergoes degradation by lysosomal enzymes once it enters the endo- lysosome. Thus, a variety of pH-responsive polymers facilitate the release of genome-editing constituents from endosomal lysosomes.

4. Application of nano-CRISPR

The combinational therapy of nano-conjugates and nano-formulation are utilized to deliver CRISPR-Cas complex and has garnered interest owing to its vivid theragnostic application in the field of biomedical science like neurological disorder, antimicrobial infection, cancer, genetic disorder, auto-immune and paracrine disorder. Herein, the clinical significance of nano-CRISPR against all the aforementioned disorders is elucidated.

4.1. Nano-CRISPR in cancer therapy

Cancer is the second largest cause of death in the world at this moment. Many chemotherapeutics has been evolved with time for the treatment of cancer however, owing to drug resistance of compounds by cells, the development of chemotherapeutics has come to a standstill [62] [159–163]. Thus, there is a need to develop therapeutic approaches to deal with it. CRISPR-Cas has been used in past to delete the MDR genes of cancer but the basic issue with that was its delivery and specificity. Accordingly, the deployment of nano-conjugates and nano-formulation to deliver the CRISPR Cas complex to the site of the tumor for the regression of cancer is described in this segment and summarized in Table 1. Among different strategies, numerous nanoparticles system has been utilized namely lipid-based nano-systems, polymer-based nano-systems, and cationic systems to deliver CRISPR-Cas system in cancer cells (Fig. 4**).**

Drug delivery systems rely heavily on polymers such as Polyplexes, nanoconjugates, micelles, nano-capsules, dendrimers [63]. In the field of targeted drug delivery, the poly-ethylenimine (PEI) polymer family is frequently employed because of its high charge density, branched PEI is useful for plasmid encapsulation. These

Table 1
Application of Nano-CRIPSR-Cas9 against Cancer.

NP	Form of Cas9	Functionalizing ligand	Cancer Therapy	Target gene/ Protein	Efficiency	Preclinical study	Interaction	Reference
LNP	Cas9/sgPLK-1 plasmids	NA	melanoma	PLK-1	>67 %	*In- vivo & in- Vitro*	Electrostatic	[65]
LHNPs	RNP	NA	Cancer gene therapy	PLK-1	>60 %	*In -vitro*	NA	[69]
ABTT NPs	Plasmid	NA	brain tumors				Hydrophobic	[69]
DOTAP	Plasmid DNA	Cholesterol and methoxy-Chol	ovarian cancer	DNMT1	84 %	*In-vivo*	N/A	[74]
lipopolymer	Plasmid	PEG-PEI-Cholesterol (PPC	osteosarcoma	VEGFA	>80 %	*In vitro & In -vivo*	N/A	[75]
carboxymethyl chitosan	Plasmid	protamine sulfate, KALA endosmotic peptide, aptamer AS1411	Tumor cell	CDK11, MMP-9, and VEGF	>80 %		NA	[67]
AuNPs, DOTAP, DOPE, PEG2000-DSPE	Plasmid	Cholesterol	melanoma	Plk-1	*79 %*	*in vitro and in vivo*	electrostatic interaction	[52]
yarn-like DNA nano-clew	DNA	NA	Tumor cell	EGFP	NA	*In vitro & In -vivo*	NA	[46]
Lipid NPs, DOPE	NA	cholesterol	Tumor cell	EGFP	50 %	*In-vivo*	electrostatic interaction	[70]
Fluorinated PAMAM dendritic polymer	NA	HEMA-ran GMA backbone	Cancer	MASPIN	90 %		intermolecular interaction	[71]

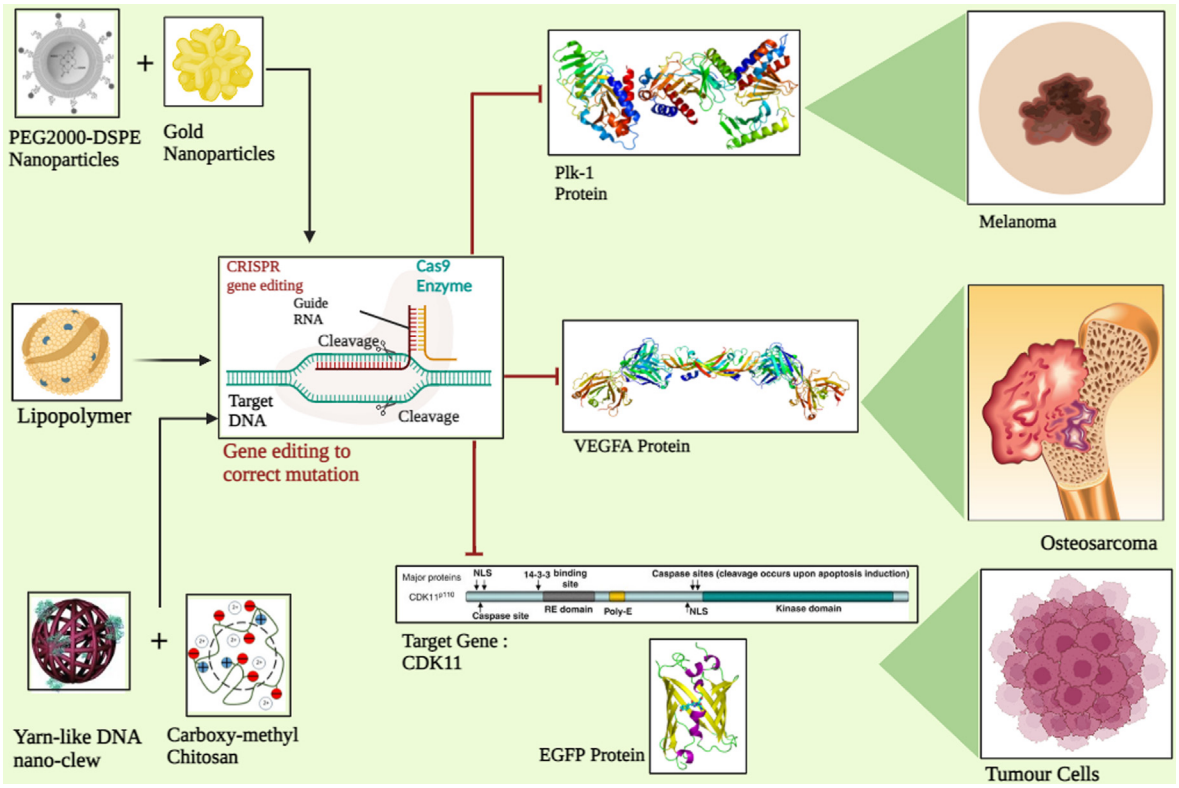

Fig. 4. Schematic diagram presenting synergy of nanoparticle - CRIPSR-Cas system for anti-cancer therapy.

properties aids to pack negatively charged nucleic acids into polyplexes, which protect them from degradation by nucleases. Branched PEI's high charge density also gives the nanocarrier pH-buffering qualities, which aids in its escape from the intracellular endosome [64]. As a result, this can lead to severe membrane damage and mitochondrial malfunction, which in turn can block ATP production and trigger cellular apoptosis and necrosis. Both the structure and the molecular weight of PEI are critical to its efficacy as a transfection agent, showing that the positive charge of these carriers correlates directly with the efficiency. Higher molecular weight PEIs have been shown to have higher transfection efficiency *in vivo*, however this comes at the expense of an increase in cytotoxicity due to their net positive charge [64] (see Table 2).

Zhang *et al.* developed a unique PLNP-based strategy to deliver a CRISPR-Cas9 complex against melanoma with enhanced efficacy than commercial transfection reagents like Lipofectamine 2000 in both, *in vitro* and *in vivo* studies. A polyethylene glycol phospholipid-modified cationic lipid nanoparticle (PLNP)-based delivery system condenses and encapsulates a Cas9/single-guide RNA (sgRNA) plasmid (DNA) thus forming a core–shell structure (PLNP/DNA) to target Polo-like kinase 1 (PLK-1) gene via intratumor injection of Cas9/sgPLK-1 plasmids for melanoma regression

Table 2
Application of Nano-CRISPR-Cas9 against Chronic Degenerative Diseases.

NP	Vector characterization	Target Gene/protein	Disease	Efficiency	Preclinical study	Interaction	Reference
lecithin	NA	DPP-4 gene	T2 DM	N/A	*In vivo*	electrostatic interaction	[79]
cationic lipid NPs	NA	NLRP3	T2DM	70.2 %	*In vitro*	N/A	[80]
cationic lipid aided PEG-*b*-PLGA NPs	NA	neutrophil elastase	T2DM	N/A		N/A	[67]
cationic lipid-assisted PEG-*b*-PLGA	plasmid	Ntn1 gene	T2 DM	36.7 %	*in vitro and in vivo*	N/A	[81]
nanocomplexes containing the R7L10 peptide	RNP	BACE1 gene	AD	N/A	*in vivo*	N/A	[94]
arginine-functionalized AuNPs	NA	AAPS1	AD	N/A	N/A	N/A	[85]
(PLGA) nanoparticles & human serum albumin (HSA) NPs	plasmid	HTT	Huntington disease	N/A	*In-vivo*	N/A	[85]
Gold nanoparticle	Plasmid	metabotropic glutamate receptor 5 (mGluR5) gene	FXS	40–50 %	*In vivo*	non-covalent interaction	[91]
BAMEA-O16B	plasmid	GFP	NA	90 %	*in vitro and in vivo*	electrostatic interaction	[95]
5A2-DOT-5 LNPs	NA	PCSK9	hypercholesterolemia	71 %	*In-vivo*	electrostatic interaction	[96]

in the *in-vivo* model [65]. The delivery of Cas9 protein and sgRNA-encoded plasmid DNA was aided with a PEI-based gene-delivery system by targeting the *Slc26a4* locus in Neuro2a cells for tumor regression. A nano-vector of hydroxyl-rich polycation functionalized with fluorinated acid (ARP-F) was utilized for the delivery of the Cas9 plasmid in a murine model. Furthermore, the conjugation of ARP-F/pCas9-surv with TMZ escalated tumor suppression by increasing cancer cell sensitivity to anticancer drugs without any toxicity [66]. Similarly, Liang *et al.* utilized, osteosarcoma specific aptamer LC09 that was functionalized with PEG-PEI-Cholesterol (PPC) lipopolymer by encasing with CRISPR/Cas9 plasmids coding for VEGFA gRNA and Cas9. LC09 facilitated the selective CRISPR/Cas9 distribution for the downregulation of VEGFA expression causing reduced orthotropic OS malignancy, lung metastasis, angiogenesis, and bone lesion without any toxicity [67].In a similar investigation, Liu *et al.* developed self-assembled natural polymers to deliver CRISPR-Cas9 plasmid against tumor-bearing cells for genome editing. This led to a significant downregulation in CDK11, MMP-9 and VEGF expression, responsible for the metastasis and upregulation of the tumor suppressor protein p53 causing tumor regression [67].

Lipid nanoparticles have proven to be a reliable and effective strategy to transport nucleic acids into cells. Although liposomes are biodegradable and biocompatible, their translational applications are often constrained by factors such as their short half-life, instability, low encapsulation efficiency, quick clearance by the reticuloendothelial system (RES), and intermembrane transfer [68]. Lipid nanoparticles can be employed in two different ways to transport CRISPR/Cas9 components: either by transporting the genetic material for Cas9 and sgRNA (plasmid DNA or mRNA) or by transporting the Cas9: sgRNA RNP complexes. Using a lipid-based nanoparticle system has been widely studied for its potential in the treatment of cancer (Fig. 5). For example, Chen *et al.* developed liposome-templated hydrogel nanoparticles (LHNPs) for CRISPR Cas delivery in a murine model for tumor regression via autocatalytic brain tumor-targeting (ABTT) mechanism designed for drug delivery by suppressing the polo-like kinase 1 (PLK-1) gene involved in cellular proliferation and invasion [69].

Wang *et al.* utilized an anionic Cas9-sgRNA complex with a bioreducible lipid nanoparticle for genome editing to target EGFP in HEK cells for tumor regression [70]. To circumvent the delivery issues associated with CRISPR, a highly regulated synthetic approach to produce a flexible dendritic polymer was deployed to deliver a pool of sgRNAs for activation of transcription of MASPIN (mammary serine protease inhibitor) in MCF-7 cell line; MASPIN is a tumor suppressor gene whose downregulation causes enhanced invasive potential and metastasis. CRISPR upregulated MASPIN using their optimized polymer design upon delivery [71]. A nanocarrier with a gold nanoclusters (GNs) core was functionalized with a lipid shell delivered Cas9 protein with sgRNA plasmid for targeting the Polo-like kinase-1 (Plk1) to cause melanoma regression [72]. Guo *et al.* fashioned an antibody-complexed tumor-targeted nano-lipo gel capable of delivering CRISPR/Cas9 plasmids to triple-negative breast cancer cells by knockout of lipocalin 2 genes with an efficiency of 81 % causing 77 % tumor regression without any toxicity [73].

ABTTPNP: Autocatalytic brain tumor-targeting poly(amine-*co*-ester) terpolymer nanoparticles; BP: Black phosphorus; ARP-F: Acid-labile polycation decorated with fluorinated alky chains; MASPIN: mammary serine protease inhibitor).

Furthermore, major development of nano-CRISPR was done in the field of CC. Lao *et al.* designed a self-assembled micelle for disruption of the human papillomavirus (HPV) E7 oncogene using CRISPR-Cas9 in both *in vitro* and *in vivo*. Seijo *et al.* used an amphiphilic penetrating peptide encompassing a hydrazone bond between a cationic peptide scaffold and a hydrophobic aldehyde tail for delivering Cas9 directly to cervical cancer cells to knock out EGFP in an EGFP-expressing HeLa cell line for CC regression [76].

Thus, based on the aforementioned studies it could be concluded that nanoparticle assisted CRISPR-Cas delivery is facilited via either lipid NPs or polymer NPs to cause regression in various carcinomas. The mechanism includes knockdown of the expression of gene/protein promoting carcinomas upon delivery (Fig. 10). Further investigation needs to be done to get a proper understanding of modes of delivery. Moreover, exploration of inorganic materials for targeted delivery of CRISPR-Cas system for anti-cancer therapy is required.

4.2. Nano-CRISPR in diabetes mellitus

Diabetes mellitus (DM) is a chronic endocrine and metabolic disorder marked by hyperglycemia causing 463 million cases worldwide with a high mortality rate [77]. Insulin, insulin analogs, and non-insulin oral hypoglycemic agents have been used to treat patients with DM. However, they were rendered ineffective, owing to inherent drug deficiencies and limitations in administration routes (subcutaneous administration oral delivery) causing enzy-

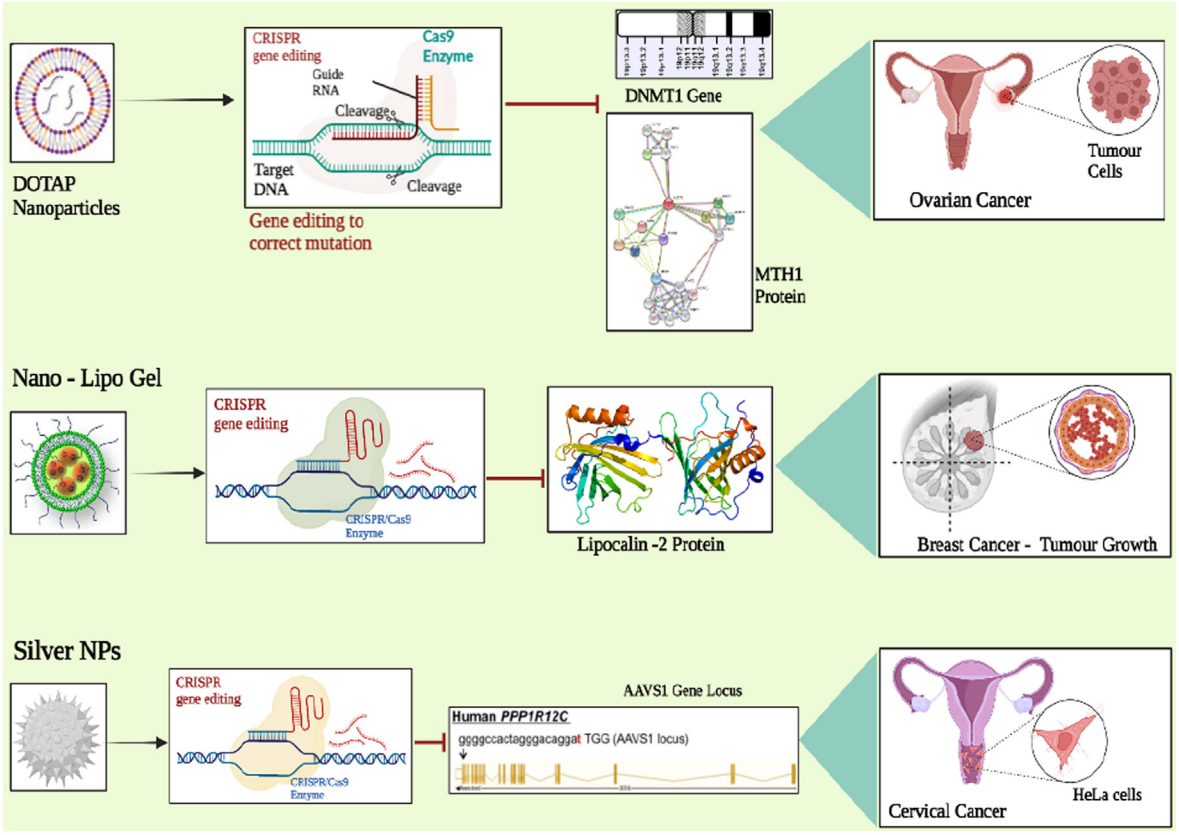

Fig. 5. Schematic model providing an understanding of delivery of CRISPR-Cas system via lipid assisted and cationic nano-system.

matic hydrolysis, chemical instability, and poor gastrointestinal absorption [77]. CRISPR gene therapy and nanocarriers offer a promising therapeutic strategy for DM treatment using liposomes, polymer-based nanoparticles, and inorganic nanoparticles for delivering CRISPR to the target sites.

Nano-carriers can be utilized for shielding the CRIPSR from an enzymatic breakdown in the gastric chambers, enhancing their stability *in vivo,* and increasing bioavailability (Fig. 6). The combination of nanocarrier and CRISPR-Cas complex replicates endogenous insulin administration via external stimuli while lowering the risk of hypoglycemia and improving patient compliance. This therapy can be more precise for targeted locations while also being released in a regulated and sustained manner over a lengthy period and thus minimizing the unwanted side effects and maximizing therapeutic impact for the treatment of diabetes [78].

Chou *et al.* utilized CRISPR along with a nanocarrier made from lecithin for the first time to treat DM. The nanocarrier encapsulates Cas9 gene-editing enzyme with the ribonucleoprotein complex (Cas9-RNP) to downregulate DPP-4 gene expression in a murine model causing restoration of blood glucose levels, and insulin responsiveness; a nano-liposomal delivery system with therapeutic Cas9-RNP improves the T2DM therapy considerably [79].

The NLRP3 inflammasome is a well-studied target for the treatment of a variety of auto-inflammatory diseases like diabetes, but current therapies have a lingering significant challenge. Xu *et al.* developed a promising strategy for treating NLRP3-dependent DM utilizing a cationic lipid- nanocarrier for delivering CRISPR-Cas9 into macrophages for the downregulation of NLRP3 via CLAN encapsulated mCas9 and gRNA-targeting NLRP3 in macrophages. This not only inhibits the activation of the NLRP3 inflammasome to reduce acute inflammation post- intravenous injection but also increases insulin vulnerability and lowers inflammation in the adipose tissue of high-fat diets (HFDs) induced type 2 diabetes [80].

Another study, conducted by Liu *et al.* delivered the CRISPR–Cas9 system to neutrophils in the epididymal white adipose tissue and liver using a library of cationic lipid-assisted PEG-*b*-PLGA nanoparticles (CLAN) with varying polymer compositions (PEG5K-*b*-PLGA11K, PLGA8K, and cationic lipid BHEM-Chol), surface density and surface charges causing downregulation of the neutrophil elastase secretion in a murine model for DM treatment [67]. Similarly, Luo *et al.* developed macrophage-specific CRISPR-Cas9 plasmids by replacing the original chicken β-actin promoter with CD68 promoter and sgNtn1 for targeting Ntn1 gene encased in cationic lipid-assisted PEG-*b*-PLGA nanoparticles (CLAN) via intravenous injection for instigating decreased Ntn1 gene expression and improvement of Type 2 diabetes (T2D) symptoms both *in vitro* and *in vivo* [81].

Based on the aforementioned studies it could be concluded that cationic and lipid nanoparticles assisted nano system aids in the precision delivery of CRISPR-Cas9 system to target Ntn1, elastase, NLRP3, DPP4- gene to cause regression of Type 2 DM. However, further studies in the animal model need to be conducted to receive a detailed etiology of their molecular mechanism.

4.3. Nano-CRISPR in neurological diseases

4.3.1. Alzheimer's disease (AD)

AD is a neurodegenerative disorder with 5.8 million cases in the USA marked by extracellular plaques containing β-amyloid (Aβ) and intracellular neurofibrillary tangles containing tau causing cognitive impairment, and short-term memory loss [82]. A vast majority of AD cases are either sporadic Alzheimer's disease (SAD) or familial Alzheimer's disease (FAD) which occurs due to

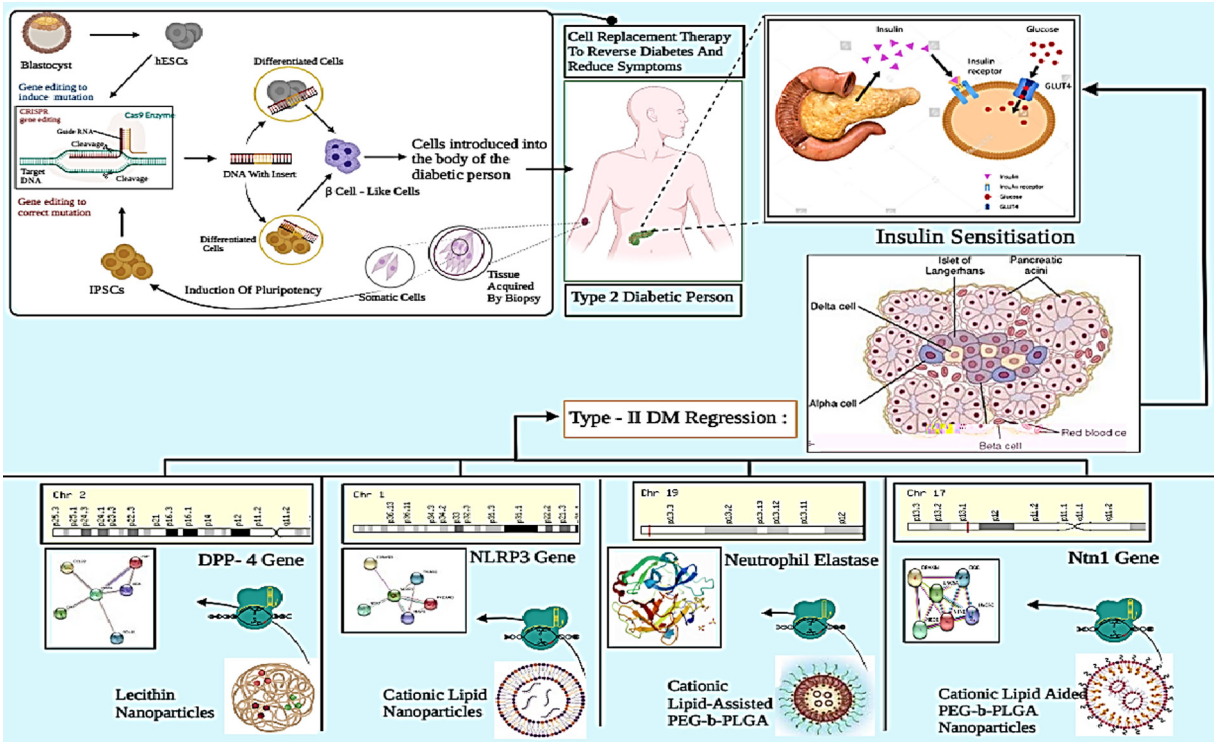

Fig. 6. Application of Nano CRIPSR synergy for Type 2 Diabetes Treatment. Lipid-assisted nanoparticles were used to deliver CRISPR-Cas 9 to neutralize Ntn1, elastase, NLRP3, and DPP-4 gene accordingly to cause type 2 DM regression.

the mutation of APP, presenilin-1 (PSEN1), and presenilin-2 (PSEN2) for abnormal Aβ metabolism [82]. Despite a plethora of knowledge about the molecular basis of AD gathered over the last three decades, effective disease-modifying drugs have been ineffective owing to the toxicity caused by reducing beta-amyloid formation, and aggregation [83]. The negatively charged nucleic acid cargo is simply complexed with positively charged peptides to produce nano-complexes. They possess certain limitations owing to their inability to deliver to the brain as they cannot penetrate the blood–brain barrier (BBB) via the systemic pathway and are actively eliminated from blood circulation by the reticuloendothelial system (RES) [84]. Consequently, intrathecal and intracerebroventricular injections are frequently employed for the same. The research presented here illustrates the deployment of non-viral Cas9 nano-complexes to be exploited in synergy with the CRISPR-Cas9 system as a therapeutic solution against AD (Fig. 7).

Park *et al.* employed nano-complexes containing R7L10 peptide in combination with the Cas9-sgRNA ribonucleoprotein, which was injected directly into the hippocampi for targeting the BACE1 gene in a murine model. The nano-CRISPR-complex successfully attenuated its BACE1 gene expression without causing significant off-target mutations and improved cognitive dysfunction [81]. Using DNA nano-clews to deliver the Cas9/sgRNA complex could also be a viable option. Traditional DNA nanostructure relies on base-pairing, which is difficult and time-consuming. In a similar study, Sun *et al.* first described DNA nano-clews as nanosized DNA cages containing polyethyleneimine to exert a positive charge enabling improved endosomal escape and cell absorption and thus offering enhanced stability due to the higher charge density [46]. Nano-clews containing sgRNA/Cas9 complex were targeted by enhanced green fluorescent protein (EGFP) being injected locally into the tumors-bearing mice causing a 25 % reduction in EGFP expression [46]. This study paved the way for the use of nano-clews in Alzheimer's disease, however, local injection remains a nuisance. Apart from its benefits, nano-clews may cause immunogenic reactions, which need to be investigated further. Similarly, Mout *et al.* created nano-assemblies by mixing positively charged arginine-functionalized gold nanoparticles with a glutamate peptide tag added to the *N*-terminus of negatively charged Cas9 protein to deliver the Cas9/sgRNA complex for targeting the human AAPS1 gene. A nuclear localization signal was added to the Cas9 C-terminus to improve the nuclear targeting with a 90 % delivery efficiency and 30 % gene silencing efficiency when integrating with the cell membrane via cholesterol for endocytosis causing improved cognitive dysfunction [85].

In a similar investigation, Lee *et al* revealed the utilization of gold nanoparticles complexed with donor DNA, cationic poly(*N*-(2-aminoethyl-2-aminoethylaspartamide) (PAspDET) to deliver Cas9-sgRNA. The cationic polymer causes endosomal rupture allowing CRISPR-Gold to enter the cytoplasm via endocytosis. The release of donor DNA and Cas9-sgRNA is aided by cytoplasmic glutathione for targeting the CXCR4 gene with 3–4 % HDR efficiency in the murine model as a therapeutic solution against AD. Based on the aforementioned findings, the delivery of CRISPR-Cas9 by cationic nano-systems and nano clews to the brain to treat AD used either local or intravascular routes of administration. Although oral delivery is convenient, patient-friendly, and non-invasive, it is extremely difficult due to the various hurdles that the delivery system must overcome to transport its gene-editing cargo to the blood [86]. The intranasal method has gotten considerable interest as it allows non-invasive and efficient bypass of BBB causing nose-to-brain delivery as one way to quickly advance CRISPR-Cas9 therapies in AD into clinical research [86].

Furthermore, intraperitoneal, and subcutaneous injections are other routes that may be deployed, but given their pharmacokinetic limitations, they would likely be used only as a last option. Even though the synergy of nano-CRISPR posed varied advantages in the management of AD; further investigation targeting AD needs to be done to obtain a better and more vivid idea of nano-CRISPR synergy in a clinical setting.

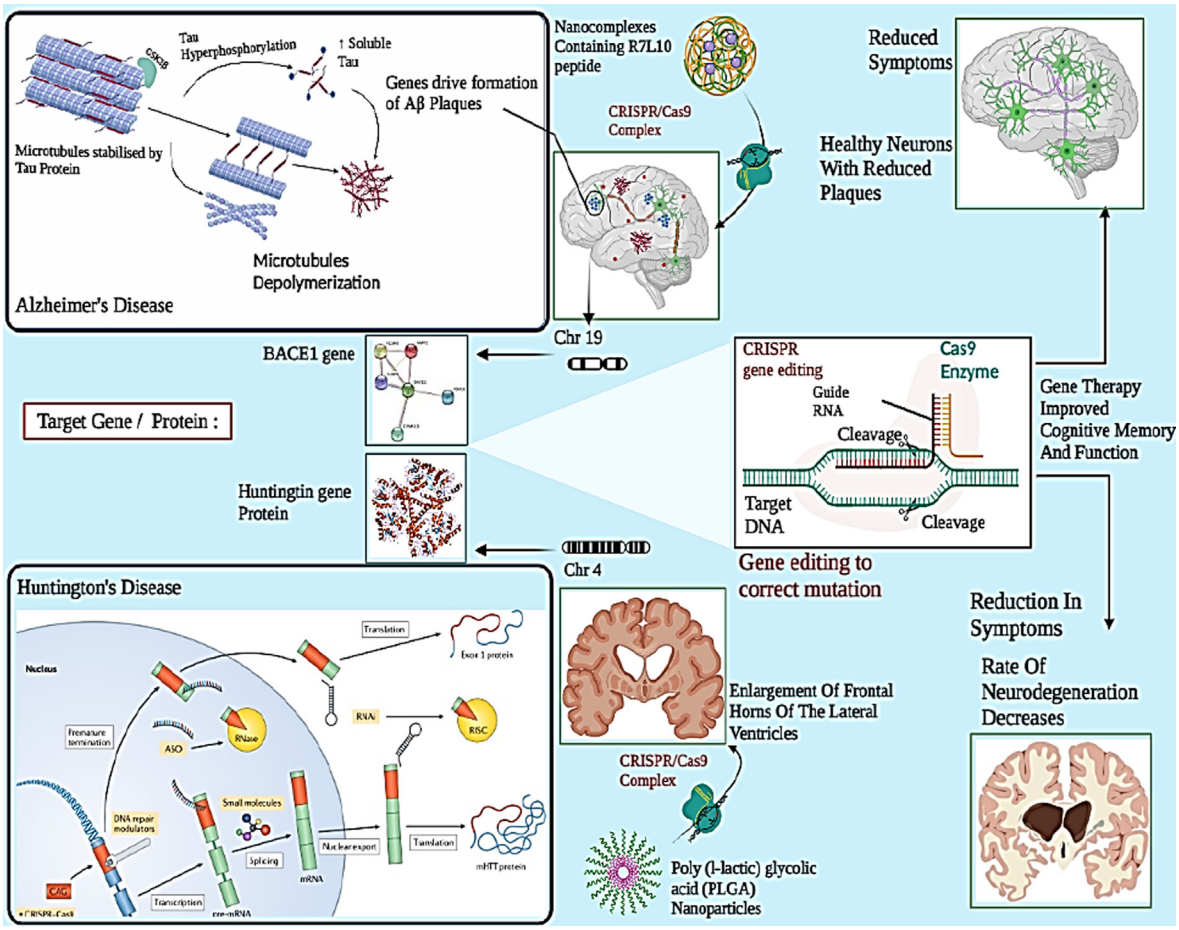

Fig. 7. Application of Nano CRIPSR synergy for the regression of the neurological disorder.

4.3.2. Huntington's disease (HD)

HD is a neurodegenerative disease caused by an autosomal dominant gene mutation in a single gene that codes for the protein huntingtin causing cognitive, motor, and psychiatric symptoms [87]. The drugs available for HD have not only been overused but are also overpriced. Davidson *et al.* demonstrated in the murine model and RCT that CRISPR-Cas could provide the same benefits with a single treatment that permanently inactivates the faulty gene with surprising efficiency [87]. But the primary limitation of the CRIPSR-Cas complex remains its effective delivery. Nanoparticle-based drug carriers are a promising technology for transferring RNA, protein, and template to targeted cells in HD therapy because of their ability to carry large sizes when used as a vehicle. Thus, there is an urgent need for efficient therapeutics utilizing nano- CRISPR in a synergistic manner [88] (Fig. 7). R-Cas9 targeting recurring RNAs like CAGN repeats suggests that it could be utilized to treat HD.

Yang *et al.* used CRISPR-Cas9-mediated inactivation for the persistent repression of endogenous mHTT expression in a murine model for effectively depleting HTT aggregates and attenuating early neuropathology. CRISPR-Cas9-mediated gene editing with non–allele specificity has been utilized for efficient and effective eradication of polyglutamine expansion–mediated neurotoxicity in the adult brain and thus potentially leading to therapeutic strategies for the treatment of HD [89].

Based on the studies it could be inferred that CRISPR-Cas 9 based targeting of HTT gene upon delivered via lipid nano system would facilitate the management of HD. However, further investigation into its immunogenicity, off-target cutting and biocompatibility needs to be done.

4.4. Nano-CRISPR in genetic diseases

4.4.1. Fragile X Syndrome (FXS)

FXS is usually the highest inherited cause of intellectual disability and a genetically determined reason for autism. Current drug treatments against FXS include psychostimulants, antidepressants, and antipsychotics which are rendered ineffective owing to the underlying etiology [90]. However, creating FXS drugs based on classical small molecules has been difficult due to the limited number of proven FXS therapeutic targets. Thus, utilizing nano-CRISPR in synergy would be an ideal option under this scenario.

Lee *et al.* introduced intracranial injection of CRISPR–Gold as a non-viral delivery vehicle for CRISPR–Cas9 RNP to downregulate the metabotropic glutamate receptor 5 (mGluR5) genes using Cas9 and Cpf1 ribonucleoproteins with undetectable levels of toxicity in neural cells in a murine model [91]. Thus, providing an idea platform for the management of FXS management (Fig. 8). Cas9 RNPs coupled with NLS signals alter genes in the adult brain when injected intracranially for curative gene editing in the brain has yet to be realized [92]. Trinucleotide expansion produced by mutations in the FMR1 gene leads to hypermethylation and gene silencing causing FXS. One potential treatment technique that has been evaluated utilizes both, candidate pharmaceuticals and cell-based screening for reactivating the silenced FMR1 gene. However, no compounds have yet been discovered that successfully restart the silenced FMR1 gene, necessitating a high-throughput indepen-

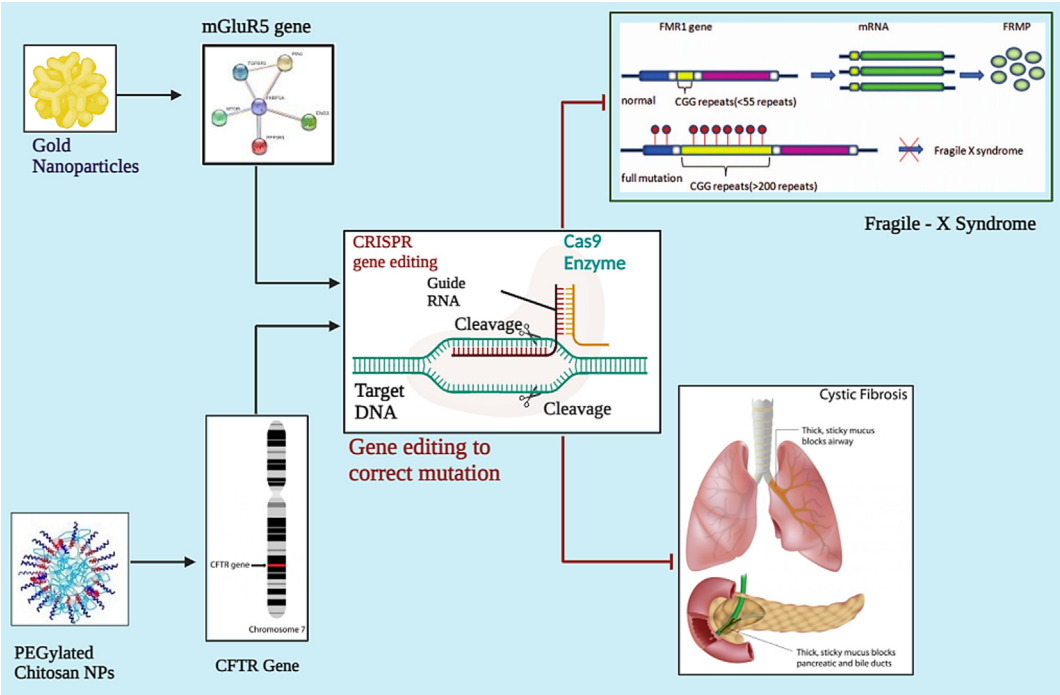

Fig. 8. Schematic illustration providing an understanding of targeting mGluR5 and CFTR via AuNPs and Polymer assisted nano-systems in genetic disease.

dent screening. Moreover, adding a Nano luciferase (Nluc) gene into the endogenous human FMR1 gene utilizing CRISPR-Cas9 establishes a robust FMR1-Nluc reporter hiPSC9 (human induced pluripotent stem cell) line to test such drugs and assess their potential genetic reactivation strategies [93].

CHO-PGEA: Cholesterol (CHO)-terminated ethanolamine-aminated poly (glycidyl methacrylate) with rich hydroxyl groups; PEG: polyethylene glycol.

4.4.2. Cystic fibrosis

Cystic fibrosis (CF) is a progressive and persistent disease caused due to mutations in both alleles of the CFTR gene that encodes an apical membrane Cl- /HCO3 channel. It has an impact on >70,000 people globally, essentially entails the failure of secretory epithelial cells that causes blockages in the lungs and pancreatic ducts [97]. Although CF is one of the most researched genetic disorders, current CF treatment focuses on symptom management rather than correction of the genetic abnormality. Thus, the need for new therapeutics and nano-CRISPR complex is an ideal solution under this scenario. CRISPR-Cas9 genome editing was utilized for the correction of CFTR locus using HDR in clonally expanded organoids cultivated in intestinal stem cells from CF patients for its expression and full functioning [98]. But to solve the issue of delivery of CRISPR, the nano-based formulation was used in conjugation with the CRISPR-Cas complex (Fig. 8).CF patients have much more mucus and are more glycosylated, and acidic than healthy people, creating a tough barrier for drug delivery *in vivo* [99]. *Zhang et al.* utilized a previously described modified mucus model to deliver CRISPR-Cas complex using PEG by functionalizing with polyethyleneimine (PEI) and poly-L-lysine for enhanced diffusion rate of nanoparticulate gene carriers in CF mucus [100]. mPEG was coupled to chitosan utilizing PEGylated chitosan as a carrier to bypass the viscous and sticky mucus barrier in the airway for pulmonary gene editing. Even though the diffusion rate was not assessed in the research, both mPEG-OC/DNA and mPEG-C/DNA nanocomplexes demonstrated a higher transport capacity through mucus than the mucoadhesive chitosan group [100]. As a result, this PEGylated chitosan complex coupled with CRISPR-Cas could be utilized for the treatment of CF.

Thus, based on the aforementioned studies it can be inferred that the combination of nano systems assisted targeted CRISPR-Cas delivery serves a boon in the sector of genetic disease. However, further studies need to be done to explore its limitation on immunogenicity, retention capacity, efficiency, off-target cutting, biocompatibility and toxicity.

4.5. Nano-CRISPR in bacterial infection

The overuse of antibiotics has greatly accelerated the spread of multi-drug resistance (MDR) pathogens over the past several decades [5]. These bacteria often develop extreme pathogenicity and infect communities, healthcare units, and hospitals. The majority of these pathogens originate in human commensal bacteria, causing opportunistic infections in immunocompromised patients. The pathogens include *Staphylococcus aureus* (Methicillin-resistant), *Enterobacteriaceae* (Carbapenem-resistant), *Acinetobacter baumannii,* and *Pseudomonas aeruginosa* (Multidrug-resistant) and *Enterococcus*, and *Staphylococcus aureus* (Vancomycin-resistant) [101]. Thus, therapeutic choices have become more limited owing to the aforementioned pathogens, and using more potent drugs will only lead to more virulent or resistant strains with enhanced toxicity when administered to patients.

CRISPR-Cas complex has recently been used as antimicrobial and genetic editing in a couple of years against all of these aforementioned pathogens but the major hurdle with those studies is their effective delivery [31]. Therefore, utilizing a combination of nanocarriers with CRISPR would be an ideal scenario as it would not only overcome the limitations of viral vectors but also overcome the limitations of gene therapy owing to the targeted delivery of nanocarriers or nano-conjugates. Table 3 summarizes the influence of nano-CRISPR synergy in infections. Thus, highlighting the antimicrobial role of the CRISPR Cas complex along with its impact on genetic editing in AMR pathogens (Fig. 9).

Table 3
Application of Nanocarrier-CRISPR-Cas9 against infections.

NP	Vector characterization	Infection	Target gene/ Protein	Efficiency	Preclinical study	Interaction	Reference
SORT-lipid NPs	mRNA/sgRNA	liver, lung, and spleen infection	PTEN	N/A	*in vitro and in vivo*	N/A	[102]
Polymer NPs	plasmid DNA, antisense oligonucleotide, small interfering RNA (siRNA)	Methicillin-resistant *Staphylococcus aureus* infection	mecA	60 %	*in vitro*	electrostatic interaction	[103]
AuNCs	SpCas9	Oncovirus infection	E6 oncogene	N/A	*In vitro*	electrostatic interaction	[104]
magneto-electric NPs	Cas9/gRNA	NeuroHIV/AIDS	HIV-LTR	>60 %	In-vitro	electrostatic interaction	[105]

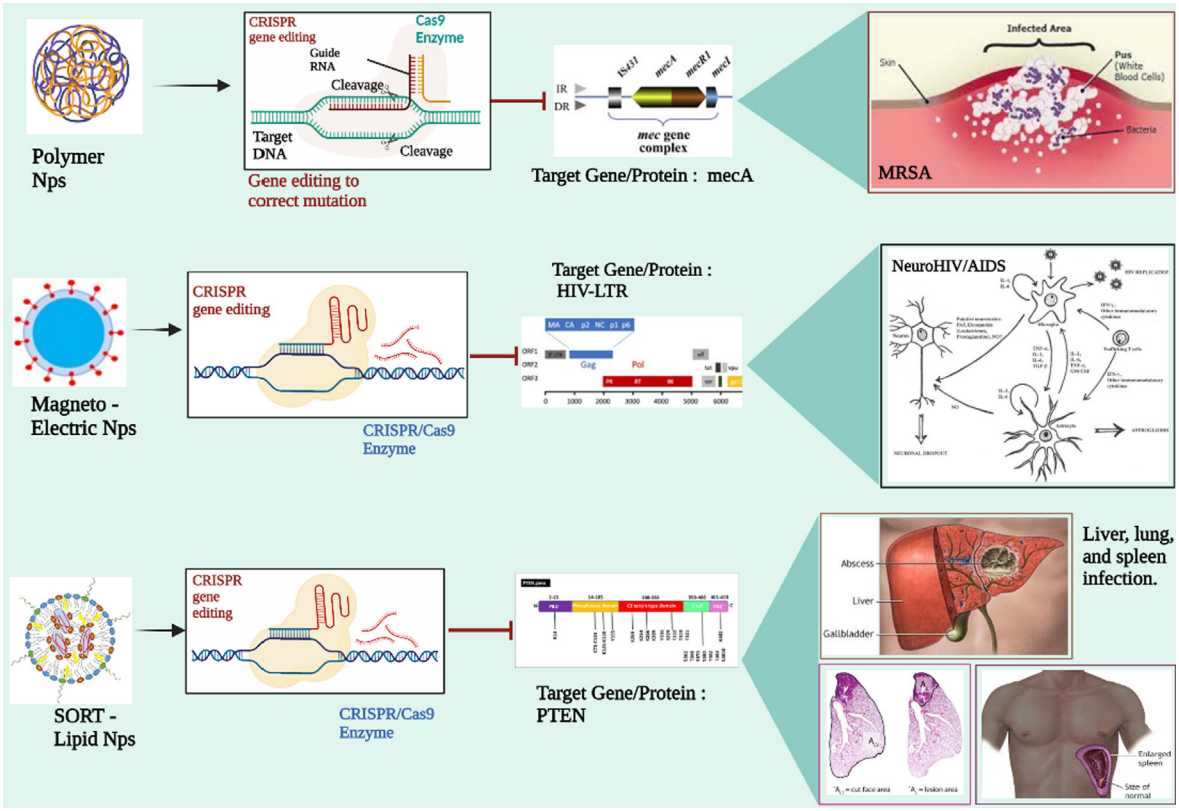

Fig. 9. Application of Nano CRIPSR synergy for inhibition of bacterial infection.

Non-viral vectors for delivering the CRISPR system, primarily deploy nanoparticle-based delivery utilizing lipid nanoparticles, polymeric nanoparticles, and gold nanoparticles (AuNPs), to overcome the shortcomings of viral vectors [106]. Cas9 mRNA can be protected by nanoparticles via chemical modifications that improve its stability. One of the most widely explored nanoparticle systems for CRISPR delivery is the lipid nanoparticles as several lipid-based carriers for gene therapy have been approved for clinical trials [107]. Because of their low immunogenicity and great biocompatibility, polymeric NPs are a significant approach for CRISPR delivery. For rapid cellular uptake and distribution inside cells, polymeric nanoparticles can be combined with cell-penetrating peptides on the surface with nuclear localization signal peptides. Polymeric nanoparticles have delivered CRISPR-Cas9 plasmids using carboxylated branching poly (β-amino ester) nanoparticles for increased hydrogen bonding and hydrophobic effects. At low RNP concentrations, the Cas9 RNP delivery polymer set off a large amounts of gene editing *in vitro* and *in vivo* [107]. Kaushik *et al.* were the first to combine Cas9/gRNA in a nano-formulation that used magneto-electric nanoparticles to hinder infection caused by HIV-1 infection in the region of microglial cells across the BBB. An intracellular Cas9/gRNA release via optimized magnetic field MENPs facilitated HIV inhibition. This CNS-based delivery of Cas9-mediated MENPs over BBB will have clinical utility as a tailored nanomedicine for neuro HIV/AIDS management in the future.

An amphiphilic cationic lipids polymer, polyethylene glycol-poly lactic-glycolic acid (PEG-*b*-PLGA) has been employed to load Cas9 (mRNA or plasmids) for intracellular delivery to macrophages post intravenous injection to alter the macrophage-specific promoter [81]. Liu *et al.* used lipid nanoparticles an amphiphilic molecule to aid in the encapsulation of CRISPR plasmid DNA and mRNA for directing and protecting RNA from crossing the cell membrane. BAMEA-O16B is a lipid nanoparticle that forms disulfide bonds for delivering Cas9/sgRNA(mRNA) to knock down green fluorescent protein (GFP) expression in human embryonic kidney cells with an efficiency of 90 % [81]. While fashioning nanoparticles for gene delivery, it can be challenging to target certain organs. So, Cheng

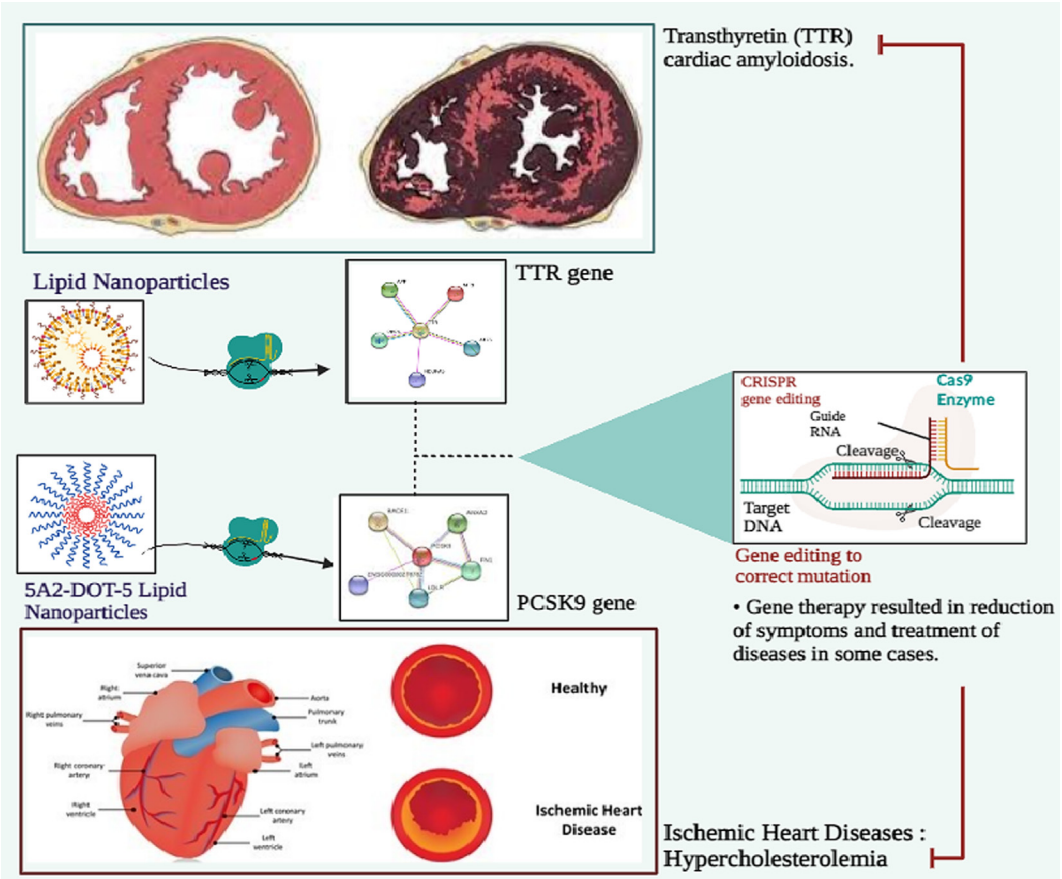

Fig. 10. Application of Nano CRIPSR synergy for the regression of cardiovascular diseases. Lipid nano-system assisted delivery of CRISPR-Cas 9 to neutralize TTR, PCSK9 gene accordingly for the management of CVD.

et al. devised the selective organ targeting (SORT) technique, which involved modifying lipid NPs with varying percentages of SORT molecules to accurately transport Cas9 mRNA/sgRNA and Cas9 ribonucleoprotein to the liver, lung, and spleen to combat infection [102]. Nguyen *et al.* recently used a new platform that combines polymeric NPs and CRISPR Cas complex causing modified homology-directed repair which could be utilized for pathogen inhibition [108].

Polymeric NPs fashioned from anionic poly-L-glutamic acid (PGA) helped stabilize RNPs by protecting excess positively charged Cas9 protein residues, for improved gene editing effectiveness, cell viability, and fewer off-target effects. The knockout of the dTomato gene was accomplished by CRISPR-Cas9 plasmid delivered via polymeric hybrid poly-L-arginine hydrochloride dextran sulfate (PARG/DEXS)3 capsules in HEK293-T cells [109]. Furthermore, another study revealed CRISPR-Cas9 components macrophage-specific gene editing administered by cationic lipid-assisted PEG-*b*-PLGA nanoparticles (CLANs) functionalized with PEGylation for pathogen suppression by reducing non-specific contacts and immune recognition [67]. Similarly, a perfluorobutanamide-modified oligo-PEI and polypeptide RGD-R8-modified hyaluronic acid were utilized for a multifunctional nuclear-targeted nuclear-shell structure to load CRISPR/Cas9 plasmids for accomplishing endosomal escape and nuclear delivery by knocking out the target gene successfully. Owing to their unique tunable properties, precise alteration, and relative safety compared to lipid and polymer nanocarriers, AuNPs are regarded as a good alternative to polymeric NPs for CRISPR RNP complex distribution. Shahbazi *et al.* developed an AuNP-based CRISPR nano-formulation on the surface of AuNPs (AuNP/CRISPR) for efficient penetration of CD34+ hematopoietic stem cells (HSPCs) and modified CCR5 and γ-globin promoter gene loci without causing any side effects [110].

Based on the above-mentioned studies, it could clearly be concluded that polymer assisted as well as lipid assisted nano-system provides a beacon of hope in the sector of MDR pathogens as it successfully delivers CRISPR-Cas systems to neutralize AMR mecA or pTEN via HDR or NHDR. This causes a drastic decrease in bacterial infection. Even though a plethora of studies needs to be conducted to compare the biocompatibility, immune-compatibility and off-target cutting of this combinatorial technology, yet provides a landmark that needs to be investigated and properly used. Future studies focusing on nano-CRISPR based detection device and sensors needs to be explored.

4.6. Nano-CRISPR in cardiovascular disease (CVD)

CVD poses a critical threat to human health and contributes as a major cause of death in many advanced countries, accounting for 32 % mortality with 17.9 million instances worldwide due to either a genetic aberration or an amalgamation of inherited heterozygous mutations [111]. Nano-carrier CRISPR-Cas complex has been used for the understanding of downregulating genes and molecular mechanisms to develop gene therapy against *in vivo* CVD models [1] (Fig. 10). Amongst the most promising drug targets for hypercholesterolemia treatment is the PCSK9 gene. Wei *et al.* injected 5A2-DOT-5 LNPs encapsulating Cas9/sgPCSK9 RNPs in the murine model via tail vein injection to downregulate the PCSK9 levels for therapeutic targeting of CVDs [96].

Transthyretin (TTR) cardiac amyloidosis is an infiltrative heart muscle disease caused by abnormal pre-albumin (transthyretin)

protein deposition heritable TTR gene mutations and the collection of wild-type transthyretin [112]. Since the majority of transthyretin is produced by the liver, it is an ideal candidate for somatic genome editing. Finn *et al.* used a combination therapy of a lipid nanoparticle comprising Cas9 mRNA and gRNA for the first time to downregulate the TTR gene *in vivo* [113]. Thus, substantiating a potential platform for genome editing as a therapeutic solution against transthyretin (TTR) cardiac amyloidosis. However, the exact molecular mechanism for the same is yet to be found. Since the aorta is completely coated with an endothelial barrier delivering the CRISPR-Cas9 system to vasculatures for *in vivo* gene editing of genetic vascular diseases, is a major challenge [113]. Zhang *et al.,* for the first time, employed the combination therapy comprising a CHO-PGEA based nanocarrier (cholesterol (CHO)-terminated ethanolamine-aminated poly (glycidyl methacrylate) with rich hydroxyl groups to deliver a plasmid-based pCas9-sgFbn1 system for the knockout of exon 10 in the Fbn1 gene. Thus, triggering Smad2/3 phosphorylation and causing upregulated expression of Mmp-2 and Ctgf via Fbn1 signals. A dose of angiotensin II (Ang II) is administered for the aortic enrichment of CHO-PGEA/Cas9-sgFbn1 in *in vivo* murine model. Hence, the combination of CHO-PGEA/pCas9-sgFbn1 nano-systems and Ang II infusion produces gene editing *in vivo* in the aorta. In a similar investigation, Zang *et al.* instigated downregulated Pcsk9 gene responsible for LDL-C titers in a murine model utilizing the cationic HIV-1-transactivating transcriptor (TAT) peptide-modified gold nanoclusters-carrying CRISPR-Cas9 system thus indicating a new therapeutic approach for CVD treatment [112]. Thus, it could be concluded that nano-CRISPR synergy provides novel gene therapy-based avenues for the management of CVD by precision targeting of either TTR gene or PCSK9 gene through lipid assisted smart nanoparticles.

4.7. Muscular dystrophies (MDs) and Nano-CRISPR

MDs are chronic inherited genetic diseases prevalent among 20 % cases per 100,000 causing multi-organ diseases by primarily affecting the skeletal muscles that degenerate over time causing loss of independence, disability, and premature death [114]. Steroid supplementation and morpholino antisense oligomer injection are the only available treatments against MDs [115]. Since, neither of the aforementioned treatment establishes a mechanism for correcting mutations in the endogenous DMD gene permanently, thus, utilizing the CRISPR-Cas 9 system as a potent genome-editing tool yields relatively new promises against DMD and DM [116,117]. To improve their clinical uses, Cas9/sgRNA ribonucleoprotein complexes were coupled with non-viral delivery vectors. The challenges stem from the large size of Cas 9 and the difficulty of keeping the RNP complex from degradation during the formulation and delivery procedure [96]. Lee *et al.* used the combination of CRISPR and nanocarriers in MDs to restore dystrophin expression in a murine model by inducing *in vivo* HDR [118]. GNP was coated with thiol-terminated DNA to effectively hybridize the donor DNA and induce its release into the cytoplasm via disulfide-bond cleavage. The Cas9 protein/sgRNA was adsorbed onto the NPs for endosomal rupture via HDR causing restoration of the dystrophin gene without any toxicity [119].

In another similar investigation, conducted by Wei *et al.*, lipid NPs were utilized for accomplishing tissue-specific gene editing by delivering Cas9/sgRNA RNP complexes to muscle, brain, liver, and lungs in a murine model post-systemic injection for restoration of the dystrophin expression by altering molecular components, lipid ratios and disulfide reduction mechanisms [96]. The Cas9 protein was fused with GFP1-10 and GFP11 via auto-assembly between two polypeptides (residues 11 chains) to resolve the issues persisting with CRIPSR-Cas editing. Cas9 could then be assembled into exosomes automatically using split GFP complementation [120].

A similar approach was utilized to load chemically induced Cas9 protein. Since rapamycin can cause a dimerization relationship between the FK506 binding protein (FKBP12) and the FKBP-rapamycin binding domain (FRB), so when Cas9 fuses to FRB and VSVG blends to FKBP12, the Cas9 protein can be selectively packed into EVs. This Nano-MEDIC induces genome editing in a range of human cell types, especially pluripotent stem cells. (iPSCs). Cas9 is delivered with great efficiency by Nano-MEDIC to the scissor acceptor and donor site of iPSCs generated from Duchenne muscular dystrophy patients (DMD) in a murine model with an efficiency of above 90 % [121].

Thus, based on the studies it could be inferred that nano-CRISPR synergy provides a platform of theragnostic impact of DMD wherein the nano systems deliver CRISPR-Cas systems as molecular scissors to target and neutralize dystrophin or FKBP12 gene accordingly (Fig. 11).

4.8. Nano-CRISPR in Auto-immune disorder

B cells have a variety of effector functions and their dysfunction causes the development of diseases like autoimmune and inflammatory ailments [122] wherein B cell-based intervention could be an effective strategy for treating such B cell diseases. CRISPR-Cas9 gene-editing has been utilized to edit, delete or modify genes delivered by nanocarriers to the B cells *in vivo* [1]. A combination therapy, for the first time, deployed NPs and CRISPR-Cas complex in rheumatoid arthritis. A library of nanoparticles (NPs) was developed and evaluated for the most suitable NP for *in vivo* B cell targeting using different polyethylene glycol (PEG) concentrations and zeta potentials. The selected NP could deliver the CRISPR-Cas9 system in a B220-dependent manner to target B cells wherein Cas9 expression could be induced in the cell environment against rheumatoid arthritis in a murine model [67].

5. Conclusion and perspective

Nano-CRISPR is a novel therapeutic strategy that overcomes the limitation of the CRISPR-Cas complex without encountering toxicity. The synergy possesses varied biomedical applications in cancer, neurological diseases, diabetes, autoimmune disorder, muscular dystrophy, bacterial infections (AMR), and cardiovascular diseases in *in-vivo* and *in-vitro* studies. Numerous strategies which include environmental (pH, stimuli, and light), chemical, and physical (ultrasound and mechanical) have been maneuvered to enhance the delivery efficiency of nanocarriers for improved CRISPR-Cas genome editing in the aforementioned appliances. Some regulatory issues need to be addressed along with studies focusing on the completion of clinical trials warranted before the utilization of this approach in a clinical setting. Although the exploitation of CRISPR-Cas systems *in vitro* laboratory engineering has various advantages but realizing the potential of CRISPR-Cas9 techniques will necessitate overcoming certain obstacles, off-target cutting being one such concern within the system. Cas nickases and mutants that minimize non-specific DNA binding have been developed to specifically address this issue. They are, however, still in their infancy and a work in progress. The use of various predictive software sgRNA design tools have come into being from extensive work to study the binding and mismatch of sgRNA, but studies about them are still in the early stages of progress. Moreover, Immunity to the CRISPR-Cas technology and the carrier used to deliver it may be addressed by using a CRISPR-Cas system protein supply that is less immunogenic since the Cas protein is only available in the target cell for a short duration. Also, nanomaterials

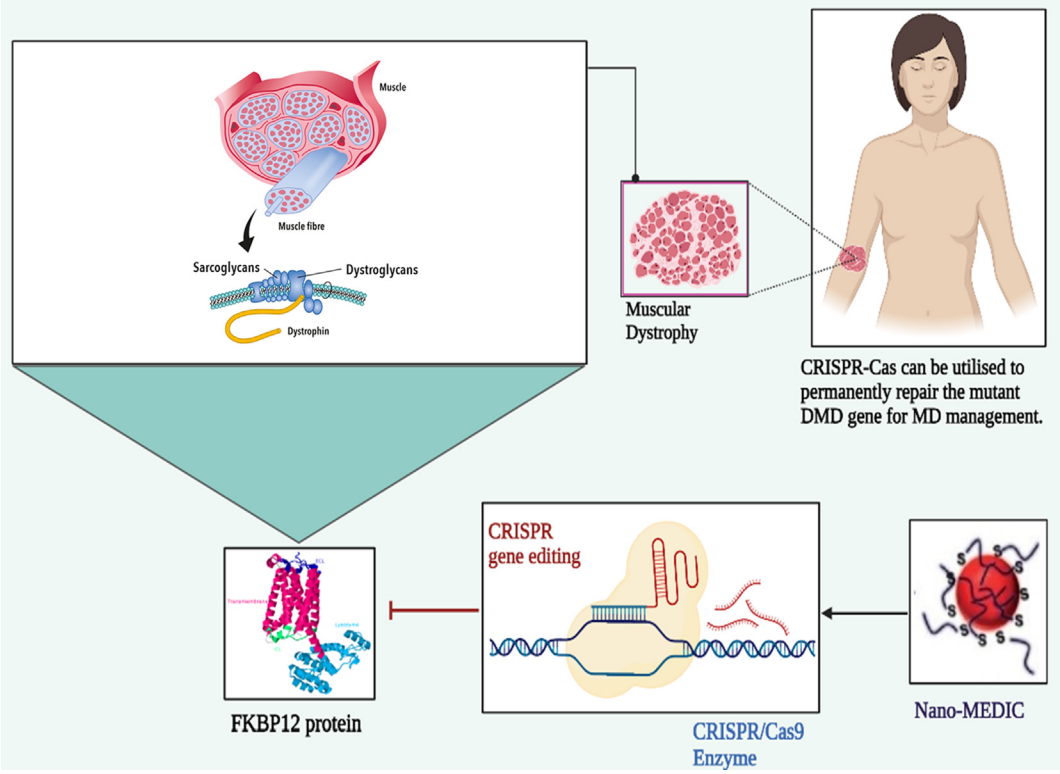

Fig. 11. Schematic model depicting the Application of Nano-CRISPR synergy for the management of Muscular Dystrophy by altering the FKBP12 gene.

designed to enhance transport efficacy, a high-capacity and non-immunogenic freight are required for effective genetic modification in a biocompatible, efficient manner, and these features are essential for non-viral administration. Additionally, if CRISPR-Cas complexes can be programmed to target specific genes of importance, the efficiency with which antimicrobial resistance (AMR) is achieved may be dramatically enhanced. Such development might have ramifications for managing resistance pools and helping antibiotics maintain or restore their antibacterial effectiveness. This rapid screening of genetic material might assist in the identification of genes implicated in medication tolerance to neurodegeneration. As a consequence, scientists will be able to develop effective treatments for both hereditary and neurological disorders. Furthermore, if a damaged gene is difficult to fix due to its genomic setting, a pseudogene that could be activated to replace the damaged gene may appear. Nevertheless, if the disease is triggered by a protein with aberrant features (like misfolding and tissue accumulation), the protein's synthesis may be inhibited at numerous points along its expression route.

CRISPR-Cas9 gene editing by nanotechnology is a new golden age in the realm of medical science. The ultimate goal of nanocarrier design is to examine the basic working strategy of CRISPR-Cas9 using nanoparticles, cells, or tissues, and the application of laboratory findings to the clinics. Even though the majority of existing CRISPR-Cas9 nano-carriers never meet all the standards of clinical trials, the outlook remains optimistic. Gradually, any limits will be solved and tackled owing to the perseverance of scientists from distinct areas. We predict that future improvements in nanotechnology-based vehicles will increase the output of CRISPR-Cas9-based treatment and expand its applicability.

Data availability

All data included in this study are available upon request from the corresponding author.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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