

Cite this: *Biomater. Sci.*, 2022, **10**, 3410Received 16th September 2021,  
Accepted 13th April 2022

DOI: 10.1039/d1bm01452h

rsc.li/biomaterials-science

# Pre-clinical non-viral vectors exploited for *in vivo* CRISPR/Cas9 gene editing: an overview

Nadia Rouatbi, Tasneem McGlynn and Khuloud T. Al-Jamal \*

Clustered regulatory interspaced short palindromic repeats or CRISPR/Cas9 has emerged as a potent and versatile tool for efficient genome editing. This technology has been exploited for several applications including disease modelling, cell therapy, diagnosis, and treatment of many diseases including cancer. The *in vivo* application of CRISPR/Cas9 is hindered by poor stability, pharmacokinetic profile, and the limited ability of the CRISPR payloads to cross biological barriers. Although viral vectors have been implemented as delivery tools for efficient *in vivo* gene editing, their application is associated with high immunogenicity and toxicity, limiting their clinical translation. Hence, there is a need to explore new delivery methods that can guarantee safe and efficient delivery of the CRISPR/Cas9 components to target cells. In this review, we first provide a brief history and principles of nuclease-mediated gene editing, we then focus on the different CRISPR/Cas9 formats outlining their potentials and limitations. Finally, we discuss the alternative non-viral delivery strategies currently adopted for *in vivo* CRISPR/Cas9 gene editing.

## 1. Introduction

In the past decades, great breakthroughs have been made in the development of molecular therapies to treat genetic disorders, neuropathies, cancer, and viral infections. These therapeutic approaches utilise molecular probes (*i.e.*, small interfering RNAs (siRNAs), antisense oligonucleotides (ASOs), and messenger RNAs (mRNAs)) acting at the genetic level to delete, replace, or alter the expression of specific genes, with the final aim of offering a more efficient and durable treatment.<sup>1</sup> With the US Food and Drug Administration (FDA) approval of Mipomersen sodium (Kynamro™) (2013),<sup>2</sup> a second-generation ASO based therapy for the treatment of Transthyretin amyloidosis (ATTR), substantial efforts have been made towards the designing of vectors that can improve the bio-availability of these molecular probes and promote their clinical translation. This, in turn, stimulated the development of different genetic medicines that are currently being used in the clinic<sup>3,4</sup> including the most recently approved vaccines<sup>5–8</sup> against COVID-19.

Recently, considerable attention has shifted towards a new generation of molecular probes characterized by site-specific nucleases capable of performing precise gene editing. Among these machineries, the first to be discovered were Zinc Finger

Nucleases (ZFNs)<sup>9</sup> followed by Transcription Activator-Like Effector Nucleases (TALEN),<sup>10</sup> and more recently the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and its CRISPR associated Cas protein (CRISPR/Cas) (Fig. 1).<sup>11</sup>

As shown in Fig. 1, mechanistically, these nucleases create double-strand breaks (DSBs) in the locus of interest, which are subsequently repaired by host cellular machinery *via* either non-homologous end-joining (NHEJ) or homology-directed repair (HDR),<sup>12</sup> resulting in the deletion, correction and modification of the target gene.

From an early stage, CRISPR/Cas has quickly gained momentum as a versatile tool for relatively easy and fast gene editing in a multitude of cell types,<sup>12,13</sup> and organisms.<sup>14,15</sup> Its application has been exploited for different purposes, including the generation of genetically engineered mouse models,<sup>16</sup> identifying genes underlying drug resistance<sup>17</sup> and providing a better understanding of cancer biology.<sup>18</sup> Furthermore, there has been great enthusiasm in developing the CRISPR/Cas toolbox for use in clinical medicine, as a potential screening and therapeutic tool. While its application *in vitro* is relatively easy, its use in humans comes with multiple hurdles, which may include potential off-target effects, instability in the bloodstream and immunogenicity.<sup>19</sup> To mitigate these limitations, many different strategies have been employed. One such method is to bypass these obstacles entirely and perform *ex vivo* gene editing, whereby autologous or allogenic cells are edited *in vitro* before being infused into patients.<sup>20</sup> The safety and feasibility of this method is evinced by the explosive increase in the number of clinical trials that utilise this tech-

Institute of Pharmaceutical Science, Faculty of Life Sciences & Medicine,  
King's College London, Franklin-Wilkins Building, 150 Stamford Street,  
London SE1 9NH, UK. E-mail: khuloud.al-jamal@kcl.ac.uk





**Fig. 1** Schematic diagram of nuclease-driven gene editing technologies. Zinc Finger Nucleases (ZFN) were the first nuclease-driven gene editing technology to be discovered, followed by Transcription Activator-Like Effector Nucleases (TALEN), and most recently by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). The three types of nucleases can induce double-strand breaks that are repaired by either one of two DNA repair processes: non-homologous end joining (NHEJ) or homology-directed repair (HDR). The first, NHEJ, is an error prone mechanism that causes insertions and deletions (indels) which results in target gene disruption. HDR, is a more accurate process, by which, in the presence of a donor DNA, precise DNA modifications can be introduced to repair a certain gene. Scheme adapted from Karim Shalaby and Esmée Dragt by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>

nique for the treatment of different types of malignancies, sickle cells disease and  $\beta$ -thalassemia, which have been reviewed recently.<sup>20–23</sup> In cases where *ex vivo* gene editing is problematic, CRISPR/Cas may be delivered *in vivo* via viral and non-viral vectors. While viral vectors have been demonstrated to be highly efficient at *in vivo* gene editing in multiple disease models, such as Duchenne muscular dystrophy (DMD),<sup>24</sup> cardiovascular diseases<sup>25</sup> and various malignancies,<sup>26</sup> their

application in humans raises concerns associated with safety and immunogenicity. For this reason, much effort has been invested in designing non-viral carriers for *in vivo* gene editing.

Besides being stable, biocompatible, and efficient, non-viral vectors should guarantee the following: (1) physiochemical stability of the cargos to ensure protection from enzymatic degradation in the extracellular, cellular, and intracellular



compartments, (2) facilitate cellular internalization and endosomal escape of the cargos, (3) limit off-target effects, and (4) reduce immunogenic side effects.

These features could be achieved only by the incorporation of chemical modifications of the different CRISPR/Cas cargoes as well as by exploiting several types of biomaterials to formulate efficient and safe delivery systems.

Among non-viral vectors, the most commonly utilised are based on lipid, polymeric, organic or hybrid nanoparticles, as well as cell-penetrating peptides. These vectors have proven to efficiently and safely deliver CRISPR/Cas components *in vivo* to different target genes in multiple tissues such as eyes,<sup>27</sup> adipocytes,<sup>28</sup> muscles,<sup>29</sup> brain,<sup>30</sup> liver,<sup>31</sup> as well as superficial<sup>32</sup> and internal<sup>33</sup> malignancies. Herein, we provide an overview of the CRISPR/Cas9 technology, followed by an outline of different physical and viral methods used for CRISPR/Cas9 delivery. We will then, describe the latest advances in the implementation of non-viral approaches for *in vivo* gene editing while discussing the landscape and challenges for clinical translation.

## 2. CRISPR

CRISPR was first observed in *Escherichia coli* in 1987 by Nakata *et al.*,<sup>34</sup> and subsequently identified in an increasing number of prokaryotic organisms including bacteria and archaea.<sup>35</sup> It was not until 2007 that its function in bacteria as an adaptive immune system was elucidated<sup>36</sup> and it was further adapted as a gene editing tool.

The bacterial genomic construct of CRISPR arrays features two key elements, the first of which is the CRISPR cassette. Cassettes consist of conserved, repeating sequences of DNA, typically 21 to 48 base pairs in length with unique non-repeating DNA sequences known as spacers interspersed between them.<sup>35</sup> Spacers of individual cassettes are similar in length, often between 26–72 base pairs. They are derived from DNA of previous infective agents such as bacteriophages, invasive plasmids and other mobile genetic elements.<sup>37</sup> Through the incorporation of pathogenic-DNA into their genome, bacteria and archaea have evolved a mechanism of heritable acquired immunity, which aids in recognition and defence against future pathogens. The second key element of CRISPR arrays are CRISPR-associated (Cas) genes, which encode numerous, highly diverse Cas proteins.<sup>37,38</sup>

Mechanistically, CRISPR immunity utilises a self-non-self-discrimination principle<sup>37</sup> which can be broadly divided into three phases: (1) immunization, (2) generation of CRISPR RNA (crRNA), and (3) interference.<sup>39</sup> In phase 1, foreign nucleic acids from invading pathogens are selected, processed and integrated into the CRISPR array as spacer sequences.<sup>40</sup> This provides a ‘memory’ of prior infections that allows the host to defend against future infections by the same pathogen. In phase 2, CRISPR arrays are transcribed to generate precursor crRNA (pre-crRNA). Pre-crRNAs are further processed *via* endonucleolytic cleavage into mature crRNAs, short sequences of approximately 20 nucleotides that are complementary to the

foreign DNA (or RNA).<sup>37,40,41</sup> The 5′ end of the crRNAs encodes the spacer sequence, whilst the 3′ end encodes part of the CRISPR-array repeat sequence. When re-infection occurs (phase 3), crRNAs are loaded onto effector modules, structures that consist of either a single Cas protein or a complex of Cas proteins. Guided by crRNAs, the effector modules cleave complementary sequences (proto-spacers) in the pathogenic genome. Invading DNA is then identified *via* recognition of protospacer adjacent motifs (PAMs). These are 2–6 base pair nucleotides sequences that are typically found 3–4 base pairs downstream of protospacer sequences in the pathogenic genome.<sup>42</sup>

Two classes of CRISPR systems have been established (1 and 2), with class 1 systems divided into types I, III and IV, and class 2 systems divided into types II, V and VI.<sup>38</sup> The different CRISPR systems are classified based upon the structural composition of their effector modules, which are responsible for the processing and transcription of CRISPR arrays, as well as interrogation and cleavage of foreign DNA or RNA.<sup>37,43</sup> In class 1, effector modules contain multiple Cas proteins, whereas class 2 systems employ a singular Cas protein.<sup>43</sup> The type II CRISPR system (CRISPR/Cas9) is the simplest and most commonly used for genome editing, as it requires the fewest components to induce DSBs.<sup>44</sup>

In CRISPR/Cas9, a second RNA sequence, trans-activating CRISPR RNA (tracrRNA), a non-coding RNA sequence, is transcribed from a genomic locus upstream of the CRISPR locus. In type II systems, this is essential for the maturation of pre-crRNA, as well as for binding of crRNA with Cas9 protein.<sup>44</sup> tracrRNA forms a complex with pre-crRNA by base-pairing with the repeat sequence present in the pre-crRNA. This results in a double-stranded RNA, which is subsequently cleaved by RNase II enzyme. The end result is a functional single guide RNA (sgRNA) complex containing a single spacer sequence.<sup>45</sup> As shown in Fig. 1, the sgRNA guides the Cas9 protein to the target site, where the Cas9 nuclease upon recognition of the PAM sequence (5′-NGG-3′) downstream of the target site, induces DNA cleavage. Unlike its predecessors, ZFNs and TALENs, target recognition in CRISPR/Cas9 is not directed by protein DNA-binding domains, but by the 20-nucleotide sgRNA sequence complementary to the target DNA.<sup>46</sup> This offers many advantages over ZFNs and TALENs. Namely, by eliminating the need to engineer individual protein domains for each DNA target, CRISPR/Cas9 has better scalability for large-scale genomic manipulation.<sup>47</sup> Though CRISPR/Cas9 holds great promise in performing simple and precise gene editing, its safe and efficient translation for *in vivo* applications is still limited, due to multiple factors that will be outlined later in this review (Table 1).

## 3. CRISPR/Cas9 gene editing formats

As shown in Fig. 2, there are 3 common formats into which CRISPR/Cas9 technology can be incorporated: plasmid DNA (pDNA), messenger RNA (mRNA), and ribonucleoprotein



**Table 1** Comparison of different gene editing format characteristics

	Plasmid	mRNA	RNP
Cas9 expression rapidity	Low	Moderate	High
Editing efficiency	Moderate/high	Moderate	Moderate/high
Stability	High	Moderate	Moderate
Persistence in cells	High	Moderate	Low
Immunogenicity	High	Moderate	Moderate
Cytotoxicity	Moderate	Low	Low
Insertional mutagenesis	High	—	—
Production speed	High	Moderate	Low
Cost	Low	Moderate	High

(RNP) complexes. The choice of which CRISPR editing format to use relies on several factors, including but not limited to transfection efficiency, the required speed/duration of expression, and safety.

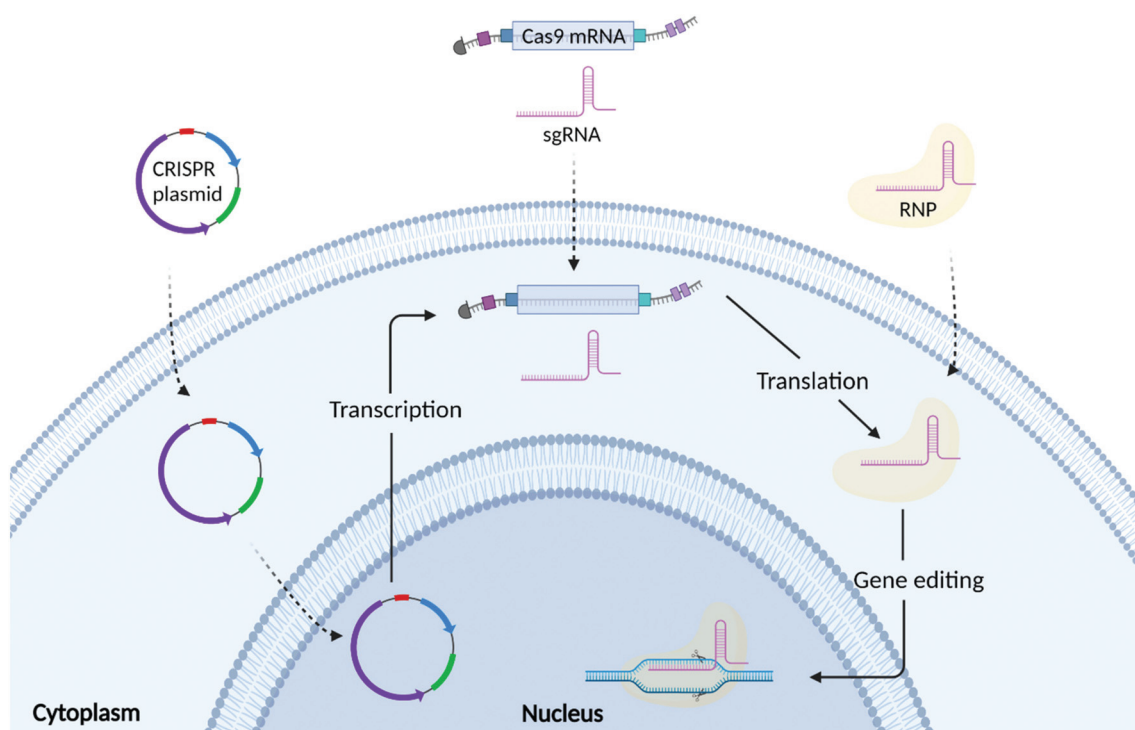
### 3.1 Plasmid mediated CRISPR/Cas9 gene editing

Both Cas9 protein and sgRNA can be encoded into single or multiple plasmids.<sup>32</sup> As shown in Fig. 2, plasmid DNA requires nuclear translocation and transcription to Cas9 mRNA (mCas9) prior to Cas9 protein translation.<sup>41</sup> Plasmids are easy to design and do not require high manufacturing costs making them an appealing approach for gene editing. Compared to mRNA and RNP, plasmids exhibit greater stability, offering a prolonged expression of the CRISPR com-

ponents.<sup>48</sup> This, however, could increase the likelihood of off-target effects and immunogenic reactions.<sup>49</sup> Plasmid DNA is also prone to random integration into host-cell DNA (insertional mutagenesis), which can lead to unintended mutations of genes and subsequent phenotypic changes.<sup>50</sup> Plasmid DNA is physically larger than both the mRNA and RNP formats, which presents an additional challenge in the formulation of delivery vectors that can effectively encapsulate them.<sup>51</sup>

### 3.2 RNA mediated CRISPR/Cas9 gene editing

RNA based transfection methods utilise a strategy whereby mRNA encoding for Cas9 protein is delivered alongside a sgRNA, avoiding the need for the transcription step, but still requiring translation of the Cas9 mRNA into its functional protein. Compared to DNA, mRNA turnover in the cell is faster, limiting the persistence of the protein, thus reducing potential off-target effects. However, mCas9RNA can be subject to enzymatic degradation resulting in poor stability. For this reason, Cas9 mRNA is often synthesized incorporating an Anti-Reverse Cap Analog (ARCA) at the 5' end and a poly(A) tail at the 3' end.<sup>52</sup> These modifications can ensure more efficient mRNA translation as well as increasing mRNA stability in the cytosol.<sup>53</sup> Another stumbling block, is represented by the recognition of the mCas9 by Toll-like receptors (TLRs), which can activate a cascade of events triggering moderate to severe immune responses.<sup>54</sup> Uridine depletion and incorporation of



**Fig. 2** Schematic representation of CRISPR/Cas9 delivery to the cell using three different formats: plasmid DNA, RNA (Cas9 mRNA/sgRNA) and ribonucleic protein (RNP) complexes. Once the plasmid enters the cells it is translocated from the cytoplasm to the nucleus, where transcription into Cas9 mRNA and sgRNA happens. Then, once into the cytoplasm, the Cas9 mRNA is translated into Cas9 protein that complexes with the sgRNA forming the RNP. Afterwards, the RNP are internalized into the nucleus to target a specific DNA sequence and induce gene insertions or deletions. Created with BioRender.com.





pseudo bases during Cas9 mRNA synthesis have been used to further increase mRNA stability as well as reduce immunogenicity.<sup>52,54</sup> Genome editing research mainly employs a chemically synthesized sgRNA containing a fusion loop between the tracrRNA and crRNA.<sup>55</sup> Further chemical modifications have also been implemented for sgRNA synthesis with the final aim of increasing their stability, RNP complexation ability, shelf-life, thermostability and reducing their immunogenicity.<sup>56</sup> Although these chemical modifications can increase the stability of both Cas9 mRNA and sgRNA, it is of the utmost importance that the RNA is formulated with a delivery system that can both protect it from enzymatic degradation as well as guarantee its efficient cellular uptake and endosomal escape.<sup>57,58</sup>

### 3.3 RNP mediated CRISPR/Cas9 gene editing

RNP mediated gene editing involves the delivery of Cas9 protein and sgRNA to the cell. Compared to pDNA and mRNA, delivery of RNP bypasses both transcription and translation and only requires the formation of the RNP complex and its subsequent translocation to the nucleus.<sup>42</sup> As the RNP is delivered directly to the cell, it guarantees a high gene editing efficiency while limiting the probability of potential off-target effects given their shorter half-life.<sup>12,59</sup> However, Cas9 protein is susceptible to enzymatic degradation once injected into the bloodstream. Furthermore, its large size limits trafficking through the cell membrane, requiring the need for formulation with a complex delivery vector.<sup>60</sup> The delivery system should also help to mask the presence of the Cas9 protein, which can elicit immune responses. Several independent studies have reported the presence of antibodies against Cas9 protein in both humans and mice.<sup>61,62</sup> In depth *in vitro* and *in vivo* studies showed that pre-exposure to Cas9 was responsible for the enrichment of different lineage of Cas9 reactive T cells.<sup>63–65</sup> In immunized mice, it was observed that CRISPR/Cas9 gene edited liver tissues elicited a strong CD8<sup>+</sup> T cell response, which induced a rapid clearance of gene edited hepatocytes, without compromising the regeneration of the liver through proliferation of unedited cells.<sup>62</sup> Additionally, whilst Cas9 protein can be easily obtained from several suppliers, the protein purification process is costly and laborious.

## 4. The need for a non-viral delivery vector

*In vivo* delivery of all three of these CRISPR/Ca9 formats presents both general and specific problems requiring delivery vectors that can guarantee stability and specificity for target cells. Whilst many CRISPR/Cas9 delivery strategies have been developed,<sup>66</sup> they are typically physical interventions<sup>51</sup> (e.g., electroporation,<sup>67</sup> hydrodynamic injection,<sup>17</sup> microinjection) or virally vectored delivery<sup>68</sup> (e.g., adeno-associated virus (AAVs),<sup>69</sup> lentivirus (LVs)<sup>70</sup>). Although there has been some success with these delivery methods, there are still questions

to address concerning their safety and clinical applicability. Physical approaches often rely on techniques that are not translatable for *in vivo* use. For example, the microinjection technique involves microscopic-guided injection of the gene editing cargo into the target cells and is technically difficult to perform, often resulting in mechanical damage.<sup>71</sup> Electroporation is another example of a physical delivery approach, in which an electrical field is applied to temporarily induce pores in the membrane of target cells, facilitating the permeability of the gene editing cargos into the cytoplasm.<sup>34</sup> The need for large voltages to achieve the desired levels of gene transfer can induce significant cell apoptosis, limiting its application both *in vitro* and *in vivo*. Hydrodynamic injection *via* the tail vein has been used successfully to induce *in vivo* gene editing of hepatocytes in animal models,<sup>72,73</sup> but is limited to preclinical murine models.<sup>74</sup> In contrast, saphenous vein injection was used for the delivery of both siRNA and pDNA to muscle cells.<sup>75</sup> This process has been successful in gene transfer in both small animals and primates,<sup>76,77</sup> however, the need for a surgical procedure represents a limiting factor for safe and easy clinical translation.

Viral vectors have been extensively used to deliver CRISPR/Cas9 constructs resulting in efficient *in vitro* and *in vivo* gene editing.<sup>78</sup> Despite this, their potential clinical translation is hampered by several concerns. LVs are single-stranded RNA viruses capable of efficiently infecting both dividing and non-dividing cells. However, LVs can integrate their genome into host cells, potentially resulting in persistent expression of CRISPR/Cas9 components, the generation of unwanted off-target effect, insertional mutagenesis and persistent immune reactions.<sup>78</sup> AAVs are single-stranded DNA vectors, with a reduced packaging capacity compared to LVs (4.7 kb *vs.* 8 kb), this often necessitates the need to use multiple vectors, one for the Cas9 protein and one for the sgRNA. AAVs do not induce persistent genomic integration, but instead deliver the genetic material in an episomal form, which reduces unwanted off-target effects and potential immunogenic responses.<sup>79,80</sup> While AAVs have been extensively utilised, there is evidence to suggest that their application for *in vivo* gene editing might still be limited due to the presence of multifunctional immunological responses against CRISPR-AAVs.<sup>61,62</sup> Mice receiving CRISPR-AAVs not only showed humoral and cell-mediated immunity against Cas9, but also anti AAV capsid-specific antibodies could be detected.<sup>61</sup> Epitope mapping further confirmed the presence of B cell epitopes in the three different isoforms of viral proteins VP1/2/3, with higher prevalence of VP3 which is expressed on the surface of the capsids. These findings are in line with other research studies investigating the immune response to AAVs in humans and which have reported the manifestation of both cell-mediated and humoral immunity against different AAVs.<sup>81,82</sup> Combined, these *in vivo* immunogenicity findings have raised concerns about the feasibility of the clinical translation of AAV mediated gene editing.

By exploiting different biomaterials, non-viral vectors offer a powerful and safer alternative in delivering genetic payloads,



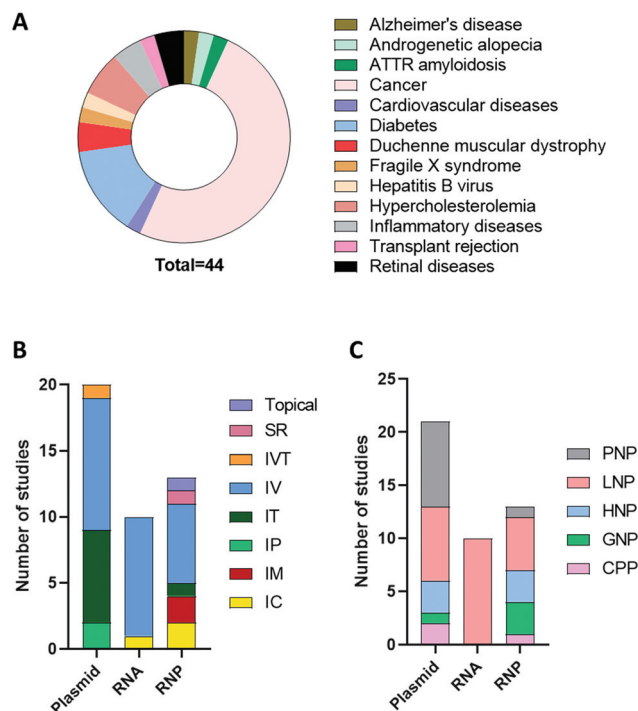
drugs, and proteins. While the *in vivo* gene editing efficiency of non-viral approaches is arguably lower than viral vectors, there is an increased interest in their implementation due to their perceived benefits and safety profile. Successful non-viral vectors should, minimally, sequester the nucleic acids from enzymatic degradation and mask them from recognition by TLR receptors on immune cells,<sup>83</sup> increase their bioavailability in specific organs, promote cellular internalization and aid in endosomal escape. In addition, the use of nanocarriers may also have many unique benefits over viral vectors. For example, the nanocarrier may be designed to carry therapeutic cargos of different physico-chemical characteristics, ranging from small drugs to large biologicals such as CRISPR plasmids and RNP. This affords the opportunity to simultaneously deliver multiple therapeutic modalities which may synergise with the therapeutic outcome of gene-editing.

## 5. Non-viral vectors for *in vivo* gene editing

An advanced literature search was carried out on PubMed for publications published prior to September 2021, the following search terms were utilised: “CRISPR”, “CRISPR/Cas9”, “CRISPR-Cas9”, “RNP”, “plasmid”, “RNA”, “Cas9 mRNA”, “sgRNA”, “*in vivo* gene editing”, “*in vivo* delivery”, “non-viral delivery”, “non-viral vector”, “nanoparticles”, “nanocomplex”. Articles that did not include *in vivo* investigation or included non-clinically translatable routes of administrations (*i.e.*, hydrodynamic injection) were excluded. The studies extracted explored the delivery of CRISPR/Cas9 as a potential therapeutic approach when used as a single agent or in combination with other modalities in animal disease models. As can be seen in Fig. 3A, cancer was the most investigated disease (47.5%), followed by diabetes (15%) and hypercholesterolemia (7.5%). In these studies, the most common platform for CRISPR/Cas9 delivery was plasmid, followed by RNP and RNA. As shown in Fig. 3B and C, different routes of administration and delivery systems were employed for each CRISPR/Cas9 format. In the sections below, a critical overview of the delivery systems utilised for *in vivo* gene editing will be provided. For each study, the key information, including the nature of the nanoparticles, target genes, animal model and findings are summarized in Tables 2–4.

### 5.1 Lipid-based nanoparticles

In the past decades, lipid nanoparticles (LPNs) have been used to deliver a plethora of cargos, including small molecules, drugs<sup>117</sup> and nucleic acids.<sup>118</sup> Recently, their application for CRISPR research has shown great progress in enhancing the stability of the CRISPR machinery as well as improving its bioavailability *in vivo*. Lipid nanoparticles are typically formulated through rapid mixing of a water phase containing the negatively charged nucleic acids and a positively charged organic phase made of different lipids: a sterol (*e.g.* cholesterol), a helper lipid (*e.g.* 1,2-distearoyl-sn-glycero-3-phosphocholine



**Fig. 3** Overview of the studies exploring *in vivo* non-viral delivery of CRISPR/Cas9. Representative pie chart of the diseases for which CRISPR/Cas9 *in vivo* non-viral gene editing have been investigated (A). Representative bar charts of the number of studies delivering CRISPR/Cas9 in the form of plasmid, RNA or RNP and the corresponding route of administration and vectors utilized (B and C). *Abbreviations*: SR = sub-retinal, IVT = intravitreal, IV = intravenous, IT = intratumoural, IP = intra-peritoneal, IM = intramuscular, IC = intracranial, PNP = polymeric nanoparticles (NP), LNP = lipid NP, HNP = hybrid NP, GNP = gold NP, CPP = cell penetrating peptide.

(DSPE)), a cationic lipid (*i.e.*, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)) and a polyethylene glycol (PEG)-lipid (*e.g.*  $\alpha$ -3'-[1,2-di(myristyloxy)propanoxy]carbonylamino}propyl)- $\omega$ -methoxy, polyoxymethylene (PEG2000-C-DMG)). Cationic lipids electrostatically complex with the anionic nucleic acids to form the nanoparticles and guarantee high encapsulation efficiency of the cargoes.<sup>58</sup> Cholesterol and helper lipids confer structural stability to the lipid bilayer and enhance transfection efficacy *via* membrane fusion and endosomal escape.<sup>119</sup> PEGylated lipids are arrayed on the outer layer to reduce LNPs aggregation and opsonization in the bloodstream.<sup>120</sup>

A number of studies have employed LNPs for the delivery of CRISPR plasmids to disrupt different genes (Nf1, CD80, CD86, CD40, Plk1, BCR-ABL, DNMT1) and have significantly enhanced the therapeutic outcomes in animal models for type 1 and 2 diabetes (T1D, T2D),<sup>88,90,95</sup> melanoma,<sup>87</sup> chronic leukaemia<sup>91</sup> and ovarian cancer.<sup>121</sup> While promising, plasmid delivery, like viral vectors, still carries a risk of genomic integration, as well as immunogenicity, this has shifted the attention towards developing RNA-based gene editing (Cas9 mRNA/sgRNA) approaches.



Table 2 Examples of non-viral delivery of CRISPR/Cas9 plasmid *in vivo*.

Disease model	Target	Vector	Mouse model	Dose	Admin. route	Findings	Ref.
Osteosarcoma	VEGFA	PPCNP	BALB/c nude	0.75 mg kg <sup>-1</sup> of plasmid	IV	<ul style="list-style-type: none"> <li>- Significantly higher indel formation in osteosarcoma tissues and lung metastasis compared to liver tissues was confirmed.</li> <li>- IHC showed reduced expression of VEGFA and other relevant markers for proliferation, metastasis and invasion.</li> <li>- Successful gene editing of VEGFA resulted in significant reduction of the tumour size as well as reduction in the number of lung metastasis.</li> <li>- IHC analysis showed significant reduction in MTH1 expression following CRISPR treatment.</li> <li>- Successful gene editing resulted in significant reduction of proliferative marker, Ki-67, and significantly inhibited tumour growth.</li> <li>- Safety of the complex was confirmed by the low (0.4%) mutation frequencies in potential off-target sites.</li> </ul>	84
Ovarian cancer	MTH1	RRPHC	BALB/c nude	10 injections of 5 µg of plasmid once every 3 days for 30 days	IP	<ul style="list-style-type: none"> <li>- Successful gene editing resulted in significant reduction of proliferative marker, Ki-67, and significantly inhibited tumour growth.</li> <li>- Safety of the complex was confirmed by the low (0.4%) mutation frequencies in potential off-target sites.</li> </ul>	85
Melanoma	Plk1	TAT-GN	BALB/c nude	10 µg pCas9 + 10 µg psgPlk1 once every other day for 15 days	IT	<ul style="list-style-type: none"> <li>- Successful reduction of Plk1 protein expression as detected by western blot (WB).</li> <li>- Plk1 knockdown resulted in significant suppression of tumour growth compared to control groups, without inducing any relevant toxicity.</li> </ul>	86
Melanoma	Plk1	PLNP	BALB/c	10 µg plasmid once daily for 15 days	IT	<ul style="list-style-type: none"> <li>- Successful PLK1 knockdown was confirmed by WB.</li> <li>- Treatment with PLNP-CRISPR significantly inhibited tumour growth by 67% of the PBS group and 50% the PLNP-siRNA group.</li> </ul>	87
Type 2 diabetes	Ntn1	CLAN	C57BL/6	1 or 2 mg kg <sup>-1</sup> pCas9/sgNtn1 every other day for 14 days	IV	<ul style="list-style-type: none"> <li>- Incorporation of the CD68 promoter ensured specific macrophages/monocytes targeting, as confirmed by the Cas9 expression detected with WB.</li> <li>- Ntn1 gene editing was confirmed by the surveyor assay, with indels frequency of 8.8% and 18.1% for the 1 mg kg<sup>-1</sup> and 2 mg kg<sup>-1</sup> dose respectively and subsequent reduction of the Ntn1 protein expression as detected by WB.</li> <li>- CLAN treatment with 2 mg kg<sup>-1</sup> dose produced glucose-response profile similar to glyburide positive control, further improving insulin sensitivity.</li> </ul>	88
Cervical cancer	Plk1	P-HNP	BALB/c nude	20 µg pCas9 + 20 µg psgPlk1 on alternate days for 10 days	IT	<ul style="list-style-type: none"> <li>- Treatment with P-HNP-pCas9-psgPlk1 significantly inhibited tumour growth by 71%, which improved the overall survival rate by 60%.</li> </ul>	89
Type 2 diabetes	NE	CLAN	C57BL/6	2 mg kg <sup>-1</sup> pCas9/sgNE once every other day for 14 days	IV	<ul style="list-style-type: none"> <li>- Deep sequencing confirmed 35% Plk1 gene editing around the target site, which resulted in 67% reduction in the Plk1 protein expression as detected by WB.</li> <li>- High uptake of CLAN by Kupffer cells and neutrophils of liver (83.9% and 94.6% respectively).</li> <li>- T7E1 assay on neutrophils sorted from eWAT and the liver revealed indel frequencies of 19.5% and 27.3% respectively. Minimal (&lt;1%) indel frequency detected in potential off-target sites.</li> <li>- WB analysis confirmed significant reduction in NE protein expression in both eWAT and liver.</li> <li>- CLAN treatment improved glucose tolerance and enhanced insulin sensitivity with efficiency comparable to that of positive controls metformin and Sivelestat.</li> </ul>	90
Melanoma	Plk1	Lipid-coated TAT-GN	BALB/c nude	10 µg pCas9-sgPlk1 every other day for 20 days [±Irradiation]	IT	<ul style="list-style-type: none"> <li>- CLAN treatment limited neutrophils infiltration and reduced the concentration of pro-inflammatory factors (TNF-α, CCL2, CXCL1, IL-1β) in the serum.</li> <li>- Enhanced gene KO mediated by the lipid-coated TAT-GN was confirmed <i>in vitro</i> using WB.</li> <li>- Treatment with lipid-coated TAT-GN + irradiation was significantly more efficient in reducing tumour growth than non-coated TAT-GN + irradiation.</li> </ul>	32



Table 2 (Contd.)

Disease model	Target	Vector	Mouse model	Dose	Admin. route	Findings	Ref.
Leukaemia	BCR-ABL	CLAN	NOD/SCID	1.6 mg kg <sup>-1</sup> CLAN (pCas9/gBCR-ABL) once every other day for 14 days	IV	<ul style="list-style-type: none"> <li>- Indel frequency at target locus was 19.3% and 16.1% in tumour cells sorted from blood and bone marrow respectively of CLAN treated animals.</li> <li>- CLAN treatment ensured significant reduction of the BCR-ABL protein expression in sorted tumour cells.</li> <li>- CLAN treatment significantly increased the survival rate compared to the control groups. Further therapeutic improvement was confirmed by the reduction in white blood cell levels as well as reduced splenomegaly.</li> <li>- R8-dGR conjugated LNP significantly inhibited tumour growth more efficiently than naked LNP.</li> </ul>	91
Pancreatic cancer	HIF1A	R8-dGR-Lip	BALB/c nude	<p>Antitumour effect: 10 µg of pCas9 + 10 µg of psgHIF1A 3 times a week for 3 weeks</p> <p>Antimetastatic effect: 10 µg of pCas9 + 10 µg of psgHIF1A and 2.5 mg kg<sup>-1</sup> PTX every 3 days for 5 times</p>	IV	<ul style="list-style-type: none"> <li>- Efficient <i>in vivo</i> gene editing was confirmed by HIF1A mRNA analysis and protein analysis <i>via</i> WB.</li> </ul>	92
Type 2 diabetes	Fabp4	ATS-9R oligoplexes	C57BL/6j	0.35 mg kg <sup>-1</sup> of pCas9-sgFabp4 twice a week for 6 weeks	IP	<ul style="list-style-type: none"> <li>- The best survival rate was observed in the R8-dGR conjugated LNP group. Codelivery of PTX further increased survival time and showed enhanced antiproliferative effect indicated by increased apoptosis and necrosis of tumour cells.</li> <li>- Reduction of metastasis was confirmed by IHC analysis of the MMP-9 expression in liver and lungs tissues.</li> <li>- Fabp4 mRNA levels in mature adipocytes was significantly downregulated following treatment.</li> <li>- Blood glucose levels reduced with repeated treatment.</li> <li>- Mean body weights in treatment group decreased by 20%.</li> <li>- Serum levels of free fatty acid, TG, ALT, and AST reduced by up to ~50%. IHC further confirmed reduced liver steatosis of treated animals.</li> </ul>	28
Breast cancer	Len2	tNLG	Nude	1 mg kg <sup>-1</sup> pCas9-sgLen2 once a week for 4 weeks	IV	<ul style="list-style-type: none"> <li>- Significant reduction of tumour growth was observed in tNLG-sgLen2 treated group.</li> <li>- qRT-PCR confirmed that treatment ensured ~81% Len2 gene editing. IHC analysis further confirmed significant reduction in Len2 and Ki67 protein expression in tumour tissues.</li> </ul>	93
Hepatocellular carcinoma	Survivin	LBP	BALB/c nude	20 µg pCas9-sgSurvivin every 2 days for 35 days [±Sorafenib]	IV	<ul style="list-style-type: none"> <li>- <i>In vivo</i> gene editing efficiency of LBP treated animals (26.4%) was confirmed by Sanger sequencing. This resulted in significant reduction of Survivin protein expression as confirmed by IHC.</li> <li>- Concomitant <i>in vivo</i> gene editing and chemotherapy significantly induced reduction in tumour growth and the number of tumour nodules.</li> </ul>	94
Type 1 diabetes	CD80	CLAN	NOD/LtJ	2 mg kg <sup>-1</sup> pCas9 + sgCD80 (or sgCD56, sgCD40) + 0.25 mg kg <sup>-1</sup> autoimmune diabetes-relevant peptide twice a week for 5 weeks	IV	<ul style="list-style-type: none"> <li>- Successful gene editing of the three target loci of DCs in the lymph nodes, blood and spleen was confirmed <i>via</i> T7E1 assay, with indel frequency between 3.5 and 10%. Antibody staining showed significant reduction in the expression of CD80, CD56 and CD40.</li> <li>- Diabetes development was significantly inhibited in the group receiving concomitant CLAN and peptide treatment. In this group histopathology analysis revealed decreased infiltration of pro-inflammatory cells and reduced insulinitis in the pancreas.</li> </ul>	95



Table 2 (Contd.)

Disease model	Target	Vector	Mouse model	Dose	Admin. route	Findings	Ref.
X-linked juvenile retinoschisis	RS1	SMNP	BALB/c	Single-injection of 50 ng pCas9-sgRNA + 50 ng donor RS1-GFP plasmid.	IVT	- Optical coherence tomography and fundus camera confirmed RS1-GFP gene integration at day 18 post IVT. - PCR amplification and Sanger sequencing further confirmed the integration of the RS1 gene in the excised treated eyes. - No histological abnormalities in the retinas were observed following gene knock-in.	27
Colon carcinoma	Cdk5	mPEG-CDM	BALB/c	20 µg pCas9-sgCdk5 ± 4 mg kg <sup>-1</sup> PTX once every 3 days for 12 days.	IV	- WB analysis confirmed successful Cdk5 KO, which resulted in the reduction of PDL-1 protein expression as hypothesized by the authors. - Concomitant Cdk5 KO and PTX treatment significantly reduced tumour growth compared to single gene editing or PTX treatment only (85.8% vs. 69.3% vs. 51.7% respectively). This success was also reflected in the survival study (36 vs. 30 vs. 26 days). - As for the melanoma model dual gene editing and chemotherapy outperformed single treatments in inhibiting the tumour growth (88% vs. 58% vs. 72.8% respectively). - mRNA analysis and WB confirmed successful Cdk5 gene KO, which resulted in downregulation of the PD-L1 expression. - <i>In vivo</i> gene KO resulted in significant suppression of the tumour growth compared to the other control groups. This was also reflected in extending the overall survival from 26 days for negative control group (PBS), to 32 days for anti-PD-L1 treatment to 40 days for the aPBAE NP.	96
Melanoma	Cdk5	mPEG-CDM	C57BL/6	20 µg pCas9-sgCdk5 ± 4 mg kg <sup>-1</sup> PTX once every 3 days for 12 days.	IV	- As for the melanoma model dual gene editing and chemotherapy outperformed single treatments in inhibiting the tumour growth (88% vs. 58% vs. 72.8% respectively). - mRNA analysis and WB confirmed successful Cdk5 gene KO, which resulted in downregulation of the PD-L1 expression. - <i>In vivo</i> gene KO resulted in significant suppression of the tumour growth compared to the other control groups. This was also reflected in extending the overall survival from 26 days for negative control group (PBS), to 32 days for anti-PD-L1 treatment to 40 days for the aPBAE NP.	97
Melanoma	Cdk5	aPBAE NP	C57BL/6	5 µg pCas9-sgCdk5 once every 3 days for 12 days.	IT	- Similar to the effects observed with the melanoma model, the aPBAE NP outperformed anti-PD-L1 treatment in reducing tumour growth progression. - KOaPBAE NP group showed superior inhibition of lung metastasis. Fewer tumour nodules were observed in the lungs of the treatment group (5 ± 3 nodules) compared to anti-PD-L1 antibody (25 ± 5 nodules) and PBS control (36 ± 4 nodules) controls. - Animals injected with PAR-Lipo exhibited greater tumour reduction compared to Lipo2000 positive control. - PAR-Lipo also induced stronger downregulation of cancer cell proliferation and metastasis as determined by Ki67 staining assay.	97
Triple negative breast cancer	Cdk5	aPBAE NP	BALB/c	5 µg pCas9-sgCdk5 once every 3 days for 12 days.	IT	- Similar to the effects observed with the melanoma model, the aPBAE NP outperformed anti-PD-L1 treatment in reducing tumour growth progression. - KOaPBAE NP group showed superior inhibition of lung metastasis. Fewer tumour nodules were observed in the lungs of the treatment group (5 ± 3 nodules) compared to anti-PD-L1 antibody (25 ± 5 nodules) and PBS control (36 ± 4 nodules) controls. - Animals injected with PAR-Lipo exhibited greater tumour reduction compared to Lipo2000 positive control. - PAR-Lipo also induced stronger downregulation of cancer cell proliferation and metastasis as determined by Ki67 staining assay.	97
Breast cancer	CDC6	PAR-Lipo	Nude	1.25 mg kg <sup>-1</sup> pSpCas9-sgCDC6	IV	- GDC6 downregulation was confirmed with WB in MCF-7 tumour tissues. - Treatment of animals with PBAE alone or in the presence of HPPCs revealed significant reduction in tumour growth compared to saline control. - IHC showed significant reduction in HPV16E7 expression following gene KO compared to saline group.	98
Cervical cancer	HPV16E7	HPPCs PBAE	Nude	10 µg of pCas9/4 doses every 3 days.	IT	- Treatment of animals with PBAE alone or in the presence of HPPCs revealed significant reduction in tumour growth compared to saline control. - IHC showed significant reduction in HPV16E7 expression following gene KO compared to saline group.	99

ALT = alanine aminotransferase, aPBAE NP = 1-(3-aminopropyl)-4-methylpiperazine (AMP)-modified poly-beta-amino ester (PBAE) nanoparticle, AST = aspartate transaminase, ATS-9R oligoplex = adipose tissue targeting sequence (ATS) peptide conjugated to D-form of 9-mer arginine (9R), BCR-ABL = fusion gene of breakpoint cluster region protein (BCR) and tyrosine protein kinase ABL1 (ABL1) genes, CCL2 = chemokine (C-C motif) ligand 2, CD80/86/40 = cluster of differentiation 80/86/40, CDC6 = cell division control protein 6 homolog, Cdk5 = cyclin dependent kinase 5, CHO-PGEA = cholesterol (CHO)-terminated ethanalamine-aminated poly(glycidyl methacrylate), CTGF = connective tissue growth factor, CXCL1 = chemokine (C-X-C motif) ligand 1, Fabp4 = fatty acid-binding protein 4, Fbn1 = fibrillin-1, GFP = green fluorescent protein, H&E = haematoxylin and eosin histological stains, HIF1A = hypoxia-inducible factor 1-alpha, HP16E7 = human papilloma virus type 16 E7 oncogene, HPPCs PBAE = poly(amide-amine)-poly(beta-amino ester) hyperbranched copolymer, HPV16 = high-risk human papillomavirus subtype 16, IHC = immunohistochemical analysis, IL-1β = interleukin 1-beta, IL-6 = interleukin 6, IP = intraperitoneal, IT = intratumoural, IV = intravenous, IVT = intravitreal, KO = knock out, LBP = lactose-derived branched cationic biopolymer, Lcn2 = lipocalin 2, MMP-2 = matrix metalloproteinase 2, MMP-9 = matrix metalloproteinase 9, mPEG-CDM = methoxy poly(ethylene glycol) (mPEG) carboxydimehyl-maleic anhydride (CDM) nanoparticle, MTH1 = Muft homolog 1, NE = neutrophil elastase, Ntn1 = netrin-1, CLAN = cationic-lipid assisted PEG-*b*-PLGA nanoparticle, PBS = phosphate buffered saline, PD-L1 = programmed death ligand 1, PPCNP = PEG-PEI-Cholesterol nanoparticle, P-HNP = PEGylated helical polypeptide nanoparticle, Plk1 = polo-like kinase 1, PLNP = poly(ethylene-glycol)-phospholipid modified cationic lipid nanoparticle, PTX = paclitaxel, R8-dGR-Lip = R8-dGR-PEG-peptide modified cationic liposome, RRPHC = artificial virus composed of fluorinated polymer (PF33)/CRISPR/Cas9 core coated with RGD-R8-PEG-HA polymer, RS1 = retinoschisin 1, SMNP = supramolecular nanoparticle, TAT-GN = HIV-1-transactivator of transcription peptide (TAT peptide) modified cationic gold nanoparticle, TG = triglycerides, tLNG = noncationic deformable tumour-targeted nanolipogel system, TNF-α = tumour necrosis factor alpha, VEGF = vascular endothelial growth factor.



Table 3 Examples of non-viral delivery of CRISPR/Cas9 RNAs *in vivo*.

Disease model	Target	Vector	Mouse model	Dose	Admin. route	Findings	Ref.
Hepatitis B virus	HBV DNA	TT3-LLN	C57BL/6	0.56 mg kg <sup>-1</sup> Cas9 mRNA + 0.25 mg kg <sup>-1</sup> sgRNA 6 hours post Cas9 mRNA injection	IV	- HBV viral loads were significantly reduced (analysis carried on liver and serum HBV antigens as well as RNA and DNA levels). - DNA sequencing confirmed indel formation at the sgRNA target site.	100
Hypercholesterolemia	Pcsk9	LNP	C57BL/6	1.2 mg kg <sup>-1</sup> Cas9 mRNA + 0.5 mg kg <sup>-1</sup> sgPcsk9	IV	- <i>In vivo</i> gene editing was confirmed by T7E1 assay of liver tissues and further screening of Pcsk9 protein in the serum which was undetectable 5 days post-injection. - Efficient Pcsk9 gene editing resulted in 30–45% reduction of total cholesterol.	101
Inflammatory diseases	ICAM-2	LNP (7C2, 7C3)	C57BL/6J	2 mg kg <sup>-1</sup> total RNA on day 0 and 2 (3/1 mCas9/sgICAM-2 w/w)	IV	- 7C3 LNP outperformed 7C2 in inducing ICAM-2 gene editing of endothelial splenic cells.	102
Type 2 diabetes	NLRP3	CLAN	C57BL/6	0.5, 2 and 2 mg kg <sup>-1</sup> Cas9 mRNA/sgNLRP3	IV	- <i>In vivo</i> gene editing of peritoneal macrophages was shown to be dose and time-dependent with the highest indel frequency (~47%) obtained at 24 h. This was also reflected in the proteomics data showing ~57% reduction in NLRP3 protein expression compared to PBS group. - Treatment of T2D animals with CLAN, induced an improved glucose tolerance and insulin sensitivity comparable to positive control group treated with glyburide.	103
Cardiovascular disease	Pcsk9	BAMEA-O16B	C57BL/6	0.6 mg kg <sup>-1</sup> Cas9 mRNA + 0.8 mg kg <sup>-1</sup> sgRNA	IV	- BAMEA-O16B treatment resulted in efficient Pcsk9 gene KO as demonstrated by 80% reduction in Pcsk9 serum levels (ELISA). - H&E staining revealed no obvious hepatocellular injury after treatment. - Efficient CD40 KO in dendritic cells confirmed with T7E1 and WB.	104
Transplant rejection	CD40	CLAN	BALB/c (graft donor) C57BL/6 (graft recipient)	3 mg kg <sup>-1</sup> Cas9 mRNA/sgCD40 once every other day, 3 times prior to transplant and 3 times post-transplant	IV	- CD40 disruption significantly inhibited T cell activation resulting in a lower graft rejection. This effect was more efficient than Rapamycin positive control.	105
Glioma tumour	Plk1	LNP	C57BL/6J 6J0laHsd	0.05 mg kg <sup>-1</sup> total RNA (mCas9/sgRNA 3/1 w/w)	IC	- NGS confirmed efficient gene editing (~68%) of Plk1 locus in tumour cells. - Plk1 KO, significantly reduced tumour growth and increased the average survival time from 32.5 days to >48 days; with 30% treated mice surviving for 60 days.	33
Ovarian cancer	Plk1	Anti-EGFR coated LNP	C57BL/6J	0.75 mg kg <sup>-1</sup> total RNA (mCas9/sgRNA 3/1 w/w), 2 injections 7 days apart.	IP	- NGS analysis confirmed 82% of editing in the PLK1 locus. Survival was increased by ~80%.	33
Familial hypercholesterolaemia	Pcsk9	SORT-LNP	C57BL/6J	2.5 mg kg <sup>-1</sup> total RNA (mCas9/sgPcsk9 4/1 w/w), 3 doses every other day.	IV	- Tumour growth was significantly inhibited, and the overall survival was increased by ~80%.	31
ATTR amyloidosis	Ttr	LNP (INT01)	CD-1	0.3, 1 and 3 mg kg <sup>-1</sup> total RNA (mCas9/sgTtr)	IV	- High indel formation at Pcsk9 locus in liver tissues. - KO of Pcsk9 gene induced ~100% reduction of Pcsk9 protein in the liver (WB) and serum (ELISA). - Liver editing levels were dose dependent. More than 97% reduction of serum Ttr and ~70% editing in the liver was observed at the highest dose. - Editing rates and serum reduction of Ttr remained stable for at least 12 months.	106

ALT = alanine aminotransferase, AST = aspartate transaminase, ATTR = transthyretin amyloidosis, BAMEA-O16B = irreducible lipid nanoparticle with integrated disulphide bonds, CD40 = cluster of differentiation protein 40, CLAN = cationic-lipid assisted PEG-*b*-PLGA nanoparticle, H&E = haematoxylin and eosin histological stains, HBV = hepatitis B virus, ICAM-2 = intracellular adhesion molecule 2, IL-1 $\beta$  = interleukin 1-beta, IT = intratumoural, IV = intravenous, KO = knockout, LNP = lipid nanoparticle, MCP-1 = monocyte chemoattractant protein 1, NLRP3 = NLR family pyrin domain containing 3, Pcsk9 = proprotein convertase subtilisin kexin type 9, Plk1 = polo-like kinase 1, SORT-LNP = selective organ targeting lipid nanoparticle, TNF- $\alpha$  = tumour necrosis factor alpha, TT3-LLN = N1,N3,N5-tris(2-aminoethyl) benzene-1,3,5-tricarboxamide derived lipid-like nanoparticle, Ttr = transthyretin.

Table 4 Examples of non-viral delivery of CRISPR/Cas9 RNP *in vivo*.

Disease model	Target	Vector	Mouse model	Dose	Admin. route	Findings	Ref.
Fragile X syndrome	mGluR5	CRISPR-Gold	Fmr1	50 pmol Cas9 and 50 pmol sg mGluR5	IC	- TIDE analysis identified 14.6% indel formation at the target site, and no significant off-target effects were detected. - mGluR5 mRNA and protein levels were reduced by ~50%. - Reduced hyper locomotor activities were observed in CRISPR-Gold treated animals.	30
Glioma	Plk1	LHNP	Athymic nude	1 mg LHNP (containing Cas9 protein/sgPlk1) 3 times a week for 3 weeks	IV	- WB analysis showed that Plk1 expression was significantly inhibited in both subcutaneous (~30%) and intracranial (~60%) U87 model. - LHNP treatment significantly reduced the tumour volume of subcutaneous tumours.	107
Macrophage-related diseases	PTEN	ArgNP	BALB/c	0.9 nmol Cas9E20 + 0.9 nmol sgPTEN for 4 consecutive days	IV	- LHNP treatment significantly prolonged the overall animal survival compared to animals treated with PBS (40 vs. 29 days). - ICP-MS analysis confirmed higher uptake of ArgNP by macrophages rather than B and T cells (liver and spleen). This was also reflected by T7EI assay showing higher PTEN indel formation in the macrophages (8% vs. 4% for B/T cells).	108
Alzheimer's disease	Bace1	R7L10-NC	5XFAD	10 $\mu$ L of nanocomplex containing 14.3 $\mu$ M of RNP, and R7L10 peptide (1/3 sgRNA/R7L10 w/w)	IC	- T7EI assay showed effective gene editing, which was confirmed by WB, showing 70% reduction in Bace1 expression in CA3 hippocampal region. - IHC revealed a significant reduction of A $\beta$ plaque accumulation and decreased A $\beta$ 42 secretion.	109
Type 2 diabetes	DPP-4	NL	db/db	1.6 $\mu$ gCas9 + 0.55 $\mu$ g sgDPP-4 once daily for 28 days	IV	- Improved spatial working memory. - NL treatment resulted in reduced levels of DPP-4 mRNA and protein levels in the liver.	110
Androgenetic alopecia	SRD5A2	US-activated MB-NL	C57BL/6	1 ml of 5% glucose solution containing 0.75 $\mu$ M Cas9 and 1.25 $\mu$ M sgrNA daily for 7 weeks [ $\pm$ US]	Topical	- GLP-1 serum concentration of NL treated animals (21%) increased to levels comparable to that of positive control Sitagliptin (24%). As a consequence, glucose tolerance increased, and insulin resistance was reduced. - US-activated MB-NL treated animals showed up to 90% hair regeneration.	111
Nasopharyngeal carcinoma	Nrf2	NTA-NP	nu/nu	1.5 mg kg <sup>-1</sup> Cas9/sgRNA every 2 days for 9 days. [ $\pm$ 1 mg kg <sup>-1</sup> Ce6, $\pm$ NIR]	IV	- US activated treatment induced efficient gene editing (71.6%), which resulted in significant reduction of SRD5A2 mRNA and protein levels. - As a result of SRD5A2 suppression VEGF expression increased 2-fold in US treated skin (ELISA). - The inclusion of targeting peptide and NIR ensured a more efficient knockout of the Nrf2 gene (31.2% vs. 15.4%, T7EI), which resulted in reduced protein expression (IFC, WB). - NIR mediated <i>in vivo</i> gene editing with concomitant Ce6 reduced tumour growth by 87.7% compared to NTA/NIR/Ce6 treatment alone (41.8%) which resulted in significant extension of the overall animal survival.	112
Duchenne muscular dystrophy	DMD	DLNP	$\Delta$ Ex44 DMD	1 mg kg <sup>-1</sup> sgDMD, once weekly for 3 weeks. (Cas9/sgRNA MR 1/3)	IM	- Efficient gene editing (5.7%) was confirmed by T7EI assay in the treatment group.	113
Hypercholesterolemia	Pcsk9	DLNP	C57BL/6	2.5 mg kg <sup>-1</sup> sgPcsk9, once weekly for 3 weeks. (Cas9/sgRNA MR 1/3)	IV	- Treatment with DLNP restored dystrophin protein expression as confirmed by IHC and WB (+4.2%). - Pcsk9 protein levels were reduced in both serum and liver tissue.	113



Table 4 (Contd.)

Disease model	Target	Vector	Mouse model	Dose	Admin. route	Findings	Ref.
Melanoma	PD-L1	VLN	C57BL/6	10 µg Cas9 every 3 days for 19 days. (Cas9/sgRNA MR 1/1) [±0.55 mg kg <sup>-1</sup> Axitinib]	IV	- WB revealed efficient (45.1% PD-L1 knockout) in significant tumour growth suppression and increase of the survival time. - The tumour growth inhibition and increase in survival was further improved with combinatorial Axitinib. This combinatorial treatment ensured significant increase in CD8 <sup>+</sup> cells infiltration and reduced T <sub>regs</sub> population. - HDR frequency in animals treated with GNP was significantly higher than naked RNP + donor DNA treated group (5.4% vs. 0.3%). - Tissue immunofluorescence showed relevant dystrophin levels comparable to wild-type animals. - GNP treatment improved animals' strength and agility even without CTX.	114
Duchenne muscular dystrophy	DMD	GNP	C57BL/10ScSn-Dmdmdx/J	120 µg Cas9 + 30 µg sgDMD + 6.75 pmol of donor DNA. [±CTX]	IM	- HDR frequency in animals treated with GNP was significantly higher than naked RNP + donor DNA treated group (5.4% vs. 0.3%). - Tissue immunofluorescence showed relevant dystrophin levels comparable to wild-type animals. - GNP treatment improved animals' strength and agility even without CTX.	29
Osteosarcoma	EGFP	NC	Nude	40 µg of Cas9 (Cas9/sgRNA MR 1/1)	IT	- Immunofluorescence staining of U20S.EGFP subcutaneous tumours showed reduction of ~25% of EGFP expression at 10 days post intratumoural injection of NC compared to untreated group or group treated with negative sgRNA.	115
Retinal diseases	Td - Tomato	SMOF NP	Ai14 mice	4 µg of RNP (Cas9/sgRNA MR 1/1)	SR	- Confocal laser scanning microscopy images of the retinas excised 13–14 days post gene editing showed strong tdTomato signal confirming successful targeting of the Ai14 stop cassette.	116

ArgNP = Arginine-functionalized gold nanoparticles, Aβ = amyloid beta, Bace1 = beta-secretase 1, CTX = cardiotoxin, DLNP = modified dendrimer lipid nanoparticles, DMD = dystrophin, DPP-4 = dipeptidyl peptidase 4, EGFP = Enhanced Green Fluorescent Protein, GLP-1 = glucagon-like peptide 1, GNP = gold nanoparticle, IC = intracranial, ICP-MS = inductively coupled plasma mass spectrometry, IHC = immunohistochemical analysis, IM = intramuscular injection, IT = intratumoural injection, IV = intravenous, LHNP = liposome-templated hydrogel nanoparticles, mGluR5 = metabotropic glutamate receptor 5, MR = molar ratio, NC = Self-assembled nanoclews, NL = lecithin-based liposomal nanocarrier particle, Nr1f2 = nuclear factor erythroid 2-related factor 2, NTA-NP = NTA-disulfanediylidipropionate-poly(ethylene glycol)-*b*-poly(caprolactone) nanoparticle, Pcsk9 = proprotein convertase subtilisin kexin type 9, PD-L1 = programmed death ligand 1, Plk1 = Polo-like kinase 1, PTEN = phosphatase and tensin homolog, R7L10-NC = R7L10-peptide-based nanocomplex, SMOF NP = PH-responsivesilica-metal-organic framework hybrid nanoparticle, SR = subretinal injection, SRD5A2 = steroid 5 alpha-reductase 2, Tregs = regulatory T cells, US-activated MB-NL = ultrasound-activated microbubble nanoliposome, VEGF = vascular endothelial growth factor, VLN = virus-like nanoparticle.



Cationic lipid assisted nanoparticles (CLAN) encapsulating mCas9/sgRNA were utilised to knockout NLRP3 in macrophages and alleviate LPS-induced septic shock, monosodium urate crystal-induced peritonitis and high-fat diet-induced T2D.<sup>103</sup> In another study, the same group used CLAN to deliver mCas9/sgCD40 to knockout CD40 in dendritic cells (DCs) to limit allogenic immune response caused by antigen presentation to T cells responsible for transplants rejection.<sup>105</sup> Others have employed the use of different types of LNPs for the treatment of hypercholesterolemia,<sup>31,101</sup> transthyretin amyloidosis,<sup>106</sup> glioblastoma and ovarian cancer.<sup>33</sup> As reported in Tables 2 and 3, CLANs have been used in multiple studies for the *in vivo* delivery of CRISPR plasmids and RNAs. CLANs were originally designed for siRNA delivery<sup>122</sup> and later adapted for the delivery of CRISPR plasmids<sup>88,95</sup> and RNAs, they are fabricated by a double emulsion method and comprise an inner core of cationic lipid *N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-(2-cholesteryloxycarbonyl aminoethyl) ammonium bromide (BHEM-Chol), and an external layer of polyethylene glycol-*block*-poly(*D,L*-lactic-*co*-glycolic acid) (PEG-*b*-PLGA). Both surface charge and PEGylation can have effects on the uptake of the LNPs by specific subsets of cells. For example, Xu, C. *et al.* in their screenings found that a higher surface charge and moderate PEG density could ensure a better cellular internalization by macrophages.<sup>103</sup>

A significant step forward in the formulation of LNPs was the substitution of permanently cationic lipids with ionizable lipids. Ionizable lipids can electrostatically bind nucleic acids at low pH, ensuring high encapsulation efficiency and stability of the complex. At physiological pH, the ionizable lipid confers a near neutral charge on the LNP, which reduces interactions with serum proteins in the bloodstream. Within the acidic lumen of the endosome, the lipid head becomes charged interacting with the negatively charged membrane, while the non-polar tail facilitates fusion and endosomal escape, releasing the nucleic acid content to the cytosol.<sup>123</sup> Progress in lipid design and LNPs formulation led to the FDA approval of ONPATTRO™ in 2018, a siRNA-based drug, formulated by the incorporation of an ionizable lipid 4-(dimethylamino)-butanoic acid, (10Z,13Z)-1-(9Z,12Z)-9,12-octadecadien-1-yl-10,13-nonadecadien-1-yl ester (DLin-MC3-DMA).<sup>124</sup>

Many groups have designed and incorporated different ionizable lipids into their formulations to further improve transfection efficiency, reduce toxicity and increase the bio-availability of the encapsulated cargos.<sup>58</sup> For example, to allay concerns associated with the bioaccumulation of lipids following systemic delivery of LNPs, Intellia Therapeutics developed a branched ionizable lipid (LP01), that due to the inclusion of liable ester linkages, is rapidly cleared from the liver when compared to LNPs formulated with DLin-MC3-DMA (6 h vs. 24 h).<sup>106</sup> Rosenblum *et al.* formulated LNPs with a novel ionizable lipid (L8) which, unlike DLin-MC3-DMA, possesses an ethanolamine linker group between the linoleic fatty acid chain and the ionizable amine head group in place of the ester linker moiety.<sup>33</sup> In their experiments, it was shown that although mCas9/sgRNA LNPs formulated with L8 or DLin-

MC3-DMA shared similar physicochemical properties and encapsulation efficiency, L8-LNPs were significantly superior at reducing green fluorescent protein (GFP) expression *in vitro*. Using confocal microscopy, Ramishetti *et al.* in their previous studies designed different ionizable lipids for the delivery of siRNA and demonstrated that LNPs formulated with L8 were better internalized by cells than other lipids.<sup>125</sup> It could be speculated that this improved internalization capacity is responsible for the improved gene editing efficiency. Many groups synthesised libraries of lipids with the aim of enhancing the properties of ionizable lipids to deliver nucleic acids *in vivo*. For example, the group of Anderson screened a range of ionisable lipids for the delivery of CRISPR RNAs to knockout proprotein convertase subtilisin/kexin type 9 (Pcsk9), resulting in the development of branched lipopeptide 3,6-bis(4-(bis(2-hydroxydodecyl)amino)butyl)piperazine-2,5-dione (cKK-E12).<sup>101,126</sup> In more recent studies they showed that LNPs formulated with ckk-E12 enhanced serum stability and albumin corona formation which promoted LNP uptake by hepatocytes.<sup>101,127</sup>

In the majority of the studies identified in Tables 2 and 3, intravenous (IV) injection was utilised as the route of administration. Following IV injection, it is reported that LNPs are mainly internalised by hepatic tissues.<sup>128</sup> Cheng and co-workers reported a Selective Organ Targeting (SORT) strategy where nanoparticles are engineered to target specific organs, by adjusting the percentage of a 5<sup>th</sup> lipid added into the traditional four component mix.<sup>31</sup> Cheng *et al.* demonstrated that by increasing the percentage of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), a permanently cationic lipid, from 20% DOTAP to 50%, SORT LNP could be redirected from the liver to the lungs. With a similar logic they incorporated into the formulation 10 to 30% of 1,2-dioleoyl-*sn*-glycero-3-phosphate (18PA), a permanently negative lipid, and observed that the delivery of luciferase mRNA (mLuc) was selectively confined to the spleen. Then they studied the effect of incorporating an additional ionizable lipid such 1,2-dioleoyl-3-dimethylammonium propane (DODAP). Interestingly in this case they did not observe a change in the biodistribution profile, but instead showed that addition of 20% DODAP resulted in 10-fold higher liver delivery as compared to SORT LNP with lower or higher % of DODAP. They used this model to deliver Cas9 mRNA/sgRNA against Cre gene after intravenous administration in Cg-Gt(ROSA)26Sortm9(CAGtdTomato)Hze/J mice. Optical imaging of *ex vivo* organs ten days post injection confirmed tdTomato expression, as a result of successful Cre recombination, in both liver and lungs consistent with their hypothesis. As a therapeutic proof of concept, they utilized 20% DODAP SORT LNP to target Pcsk9. Following three IV injections, they confirmed efficient gene editing and achieved 100% reduction in Pcsk9 serum levels.

Many studies attempted to perform gene editing in for cancer therapy. Rosenblum *et al.* have developed anti-EGFR-antibody functionalised LNPs, following peritoneal administration (IP), LNPs were demonstrated to accumulate in OV8 ovarian cancer.<sup>33</sup> The same group utilised direct intracranial



administration to perform gene editing of E00 GBM tumours and reduce the off-target effects.<sup>33</sup> To enhance the tumour accumulation of the CRISPR/Cas9 plasmid and reduce off target effects, Li *et al.* formulated cationic liposomes conjugated to R8-dGR (Cys-RRRRRRRRdGR), a cell penetrating peptide displaying a dual affinity to integrin  $\alpha_v\beta_3$  and neuropilin-1 (NRP-1) receptors, both highly expressed on pancreatic cancer cells.<sup>92</sup> Liposomes consisting of cholesterol, DOTAP and DSPE-PEG<sub>2000</sub>-R8-dGR were formulated by film hydration method. The chemo drug paclitaxel (PTX) and CRISPR plasmid targeting hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) were encapsulated into the liposomes using the post-insertion method. *In vivo* biodistribution studies confirmed higher accumulation of the R8-dGR-liposomes into BxPC-3 xenografts compared to the non-functionalized ones. These results were also reflected in the therapy studies where R8-dGR liposomes outperformed untargeted liposomes in inhibiting the tumour growth. Moreover, it was encouraging to see that applying gene editing and chemotherapy modalities outperformed single therapeutic approaches in suppressing metastasis and prolonging animal survival.

While in the studies described above different groups have used either a passive or active targeted LNP administered parentally, Ryu *et al.* have developed a unique multicompartiment ultrasound-guided lipid-based vector for topical use in the treatment of alopecia.<sup>111</sup> First RNP targeting steroid type II 5-alpha-reductase (SRD5A2) were complexed and encapsulated into nanoliposomes (NL) following lyophilization, rehydration and then extrusion. Next, lipidic microbubbles (MB) were generated by Sulphur hexafluoride (SF<sub>6</sub>) gas bubbling. NL were then conjugated onto the MB following pyridyl disulfide reaction chemistry. The microbubbles conjugated nanoliposome (MB-NL) system, when applied with concomitant ultrasound, showed promising results in enhancing the formation of the hair follicle and hair growth compared to the control groups (Table 4).

## 5.2 Polymer-based nanoparticles

Polymeric nanoparticles (PNPs) hold the promise of being a potential alternative for safer non-viral delivery of genetic cargos. PNPs are commonly synthesized using cationic polymers that can electrostatically bind the negatively charged phosphate moieties of the DNA/RNA backbone. Once complexed, PNPs can ensure that the nucleic acids are protected from nucleases found in the body, reduce immunogenicity and improve cellular uptake of the genetic cargos.<sup>129</sup> Common polymers used in the synthesis of polymer-based nanoparticles include poly(lactic-co-glycolic acid) (PLGA), polyethyleneimine (PEI), poly-beta-amino ester (PBAE), poly L-lysine (PLL), chitosan and poly(amidoamine) (PAMAM).<sup>130</sup>

Zhang *et al.* formulated polymeric nanoparticles using cholesterol (CHO)-terminated ethanolamine-aminated poly (glycidyl methacrylate) (CHO-PGEA) rich in hydroxyl groups to deliver a plasmid cassette encoding Cas9 and sgRNA against fibrillin-1 (Fbn1), which mutations are responsible for Marfan syndrome carrying several complications including aortic

aneurysms.<sup>131</sup> CHO-PGEA was selected based on previous findings suggesting that the presence of hydroxyl groups in both lipid<sup>132</sup> and polymer<sup>133</sup> based transfection reagents improved their safety, biocompatibility and transfection efficiency. Du *et al.* showed how the formulated nanoparticles significantly reduced the protein adsorption and weakened the haemolytic properties as compared to polyethyleneimine (PEI)-CRISPR conjugates. Because accumulation of nanoparticles is difficult to achieve in the aorta, Du *et al.* treated the animals daily for 7 days with a pressor dose of Angiotensin II (Ang II) prior to the administration of the nanoparticles. They hypothesized that this process would increase vascular wall pressure, enhancing the vascular permeability, thus facilitating nanoparticles accumulation in the aorta. This was confirmed by *ex vivo* optical images showing that, following intravenous administration, Sulfo-Cyanine5 (Cy5) labelled CHO-PGEA NPs accumulated in the aorta with a higher significance in animals pretreated with Ang II. In further studies, the authors confirmed successful gene editing of Fbn1, with no signs of toxicity.

Qi *et al.* formulated lactose-derived branched cationic biopolymers (LBP) delivering plasmid encoding for Cas9 and sgRNA against Survivin to treat orthotopic hepatocellular carcinoma (HCC).<sup>94</sup> The authors hypothesized that lactose is likely to be a highly efficient carrier for HCC therapy, due to the great affinity between asialoglycoprotein receptor (ASGPr), expressed prevalently on parenchymal liver cells and hepatic cancer cells and the galactose residues present in the lactose. First, lactose was reacted with cystamine to produce amine lactose (L-NH<sub>2</sub>). Then, an open-ring reaction between L-NH<sub>2</sub> and triglycidyl iso-cyanurate was performed to obtain LBP. The highly cationic LBP were then electrostatically complexed with the LBP. In the formulation process, cysteamine was used to introduce disulphide linkages that can be easily degraded in the reductive intra-cellular space. In fact, in the cytoplasm and nucleus, the high concentrations of redox molecules, such as glutathione, facilitate the cleavage of the disulphide bonds and the release of the nucleic acids.<sup>134</sup> To confirm their hypothesis on LBP affinity to liver cells *via* galactose and ASGPr interaction, the authors intravenously administered LBP or negative control PEI labelled with Cyanine7-N-hydroxysuccinimide ester (Cy7-NHS). Optical imaging of liver tissues four hours post injection showed significantly higher Cy7 signal in the animals injected with LBP as compared to those injected with PEI. Therapy studies revealed that animals receiving LBP had reduced tumour growth compared to untreated controls. Efficacy of Survivin gene editing was further enhanced by co-administration of sorafenib, a multi-kinase inhibitor clinically used for the treatment of HCC.

In another study, Tu *et al.* utilised PEG-coated PEI-PLGA PNPs for the co-encapsulation of CRISPR/Cas9-Cdk5 plasmid and paclitaxel to trigger immunogenic cell death in tumour models for melanoma and colon carcinoma.<sup>96</sup> Given the hydrophobicity of PXT, the drug was firstly encapsulated into PEI-PLGA. Then, the CRISPR/Cas9-Cdk5 plasmid was incubated with PXT-PEI-PLGA nanoparticles (NP<sub>PXT</sub>) to facilitate electrostatic condensation of the nucleic acids onto the posi-



tively charged nanoparticles. The PXT-CRISPR nanoparticles ( $NP_{PXT-Cdk5}$ ), were then coated with PEG conjugated to maleic acid amide linker (PEG-CDM) to protect the plasmid from enzymatic degradation and reduce opsonization and phagocytosis of the PNPs. Once the PEG-CDM coated  $NP_{PXT-Cdk5}$ , reach the acidic tumour microenvironment the CDM linker is cleaved, and the cationic  $NP_{PXT-Cdk5}$  are taken up by cancer cells through endocytosis. IV injection of these nanoparticles caused efficient Cdk5 gene KO, which resulted in the reduction of the programmed death-ligand 1 (PD-L1). Immunohistochemical (IHC) analysis of tumour tissues identified the presence of different damage-associated molecular patterns (DAMPs) in the groups treated with paclitaxel, confirming PTX triggered immunogenic cell death. Animals receiving combination treatment showed significantly reduced tumour growth as compared to untreated controls and animals treated with either gene editing or chemotherapy. Cdk5 targeting using PNPs was also adopted by Deng *et al.* for the treatment of melanoma.<sup>97</sup> To address toxicity concerns caused by the polymer PEI, Deng *et al.* utilized the biodegradable cationic polymer poly( $\beta$ -amino esters) (PBAEs). Intravenous injection of these PNPs resulted in successful gene editing leading to significantly reduced tumour growth compared to anti-PD-L1 antibody treatment controls in both B16F10 and 4T1 tumour models.

Conjugation of tumour-specific aptamers to NPs is often utilized to increase tumour accumulation of the NPs with the final aim of improving treatment efficacy and concomitant reduction of overall toxicity. Liang *et al.* designed PEG-PEI-Cholesterol (PPC) NPs encapsulating CRISPR plasmid targeting VEGFA and conjugated with an osteosarcoma cell-specific aptamer.<sup>84</sup> These NPs showed an improved tumour-specific biodistribution compared to their non-functionalized counterpart. The delivery system could induce significant VEGFA KO, which resulted in reductions in tumour growth and reduced lung metastasis.

To encapsulate pCas9/sgrRNA and donor Retinoschisin 1 (RS1)-GFP-plasmid, Chou *et al.* developed supramolecular nanoparticles (SMNP) generated through the self-complexation of three different polymers, namely  $\beta$ -cyclodextrin (CD)-grafted branched polyethyleneimine (CD-PEI), adamantane (Ad)-grafted poly-amidoamine dendrimer (Ad-PAMAM), and Ad-grafted poly(ethylene glycol) (Ad-PEG), as an application for X-linked juvenile retinoschisis treatment.<sup>27</sup> Following a high-throughput approach based on two microfluidic systems<sup>135</sup> the authors screened different formulations *in vitro* in B16 cells and identified the best formulation which was then intravitreally injected into the mice eyes. SMNP treatment resulted in successful RS1/GFP expression at 18 post injection as detected by optical coherence tomography, Sanger sequencing and IHC analysis, with no evidence of histological abnormalities.

### 5.3 Cell-penetrating peptides

Cell-penetrating peptides (CPPs) are short lengths of amino acids (typically 5–30 amino acid residues in length) that can be cationic, amphipathic, or non-polar. They can enhance trans-

port of proteins, drugs, nucleic acids and imaging agents directly across the cell membrane or following clatherin-mediated endocytosis.<sup>54</sup> CPPs have been safely used *in vivo* in several pre-clinical and clinical research studies for imaging and therapeutic purposes.<sup>136</sup>

Wang *et al.* formulated PEGylated helical polypeptide nanoparticles (P-HNPs) which consisted of poly( $\gamma$ -4-((2-(piperidin-1-yl)ethyl)amino-methyl)benzyl-L-glutamate) (PPABLG) surrounded by PEG-PEI encapsulating Plk1 targeting CRISPR plasmid.<sup>89</sup> PPABLG was chosen based on previous findings by the Cheng group,<sup>137</sup> who proposed a core of  $\alpha$ -helical polypeptide with side-chains featuring a cationic terminus to facilitate effective gene transfer. Due to the non-natural amino acid sequence in the PPABLG backbone and the long hydrophobic side chains, P-HNPs featured improved stability against nucleases. Following three intratumoural injections of P-HNPs, successful *in vivo* PLK1 gene knockout was confirmed by Western Blot. Reduction in Plk1 expression significantly inhibited HeLa xenograft growth and improved overall animal survival.

To increase CRISPR/Cas9 gene editing specificity to target cells and reduce potential off-target effects Chung *et al.* formulated oligoplexes consisting of adipose tissue targeting sequence (ATS) peptide (CKGGRAKDC) conjugated to a 9-mer of D-arginine (9R),<sup>28</sup> previously reported to specifically bind Prohibitin protein expressed on the membrane of adipose tissues.<sup>138</sup> CRISPR plasmid for dead Cas9 (dCas9) and sgrRNA against fatty acid binding protein 4 (Fabp4) were complexed with ATS-9R to form nanocomplexes as a treatment for obesity induced metabolic syndrome. Peritoneal injection of these nanocomplexes in high fat diet (HFD) mouse models resulted in reduced inflammation in adipose tissues as well as reduction in blood glucose levels and body weight.

Owing to the amphiphilic nature, R7L10 peptide (NH<sub>2</sub>-RRRRRRRLLLLLLLLL-COOH) has been widely used to encapsulate hydrophobic drugs into the core of self-assembling peptide-micelles.<sup>139</sup> R7L10 micelles possess a highly cationic surface charge making them good candidates for nucleic acid encapsulation and delivery. Park *et al.* used R7L10 peptide to formulate nanocomplexes carrying RNP to target beta-secretase 1 (Bace1) and tyrosine hydroxylase (Th) in post-mitotic neurons.<sup>109</sup> The complexation of R7L10 peptide and the RNP was facilitated by electrostatic interactions between the negatively charged RNP and cationic R7L10. As reported in Table 4, gene knockout in the brain was confirmed and was able to induce measurable therapeutic improvement in Alzheimer's disease mouse models such as significant plaque reductions and improved spatial working memory.

### 5.4 Gold-based nanoparticles

Gold nanoparticles (GNP) are inorganic vectors which due to their tuneable size, shape and large surface area can be utilized to load different cargos *via* ionic or covalent coupling, electrostatic interactions or physical adsorption.<sup>140</sup> Notably GNP benefit from surface plasmon resonance properties making them suitable for thermal and photodynamic therapeutic applications.<sup>141</sup>



In the context of *in vivo* CRISPR delivery, GNP were utilized to deliver large cargoes such as plasmids and RNP. Following the loading of the CRISPR cargoes onto the GNP, these were functionalized through different approaches to enhance their cellular and nuclear internalization. Based on previous reports suggesting that HIV-1-transactivator of transcription (TAT) peptide can promote nuclear localization of GNPs and improve pDNA *in vitro* transfection,<sup>142</sup> Jiang group loaded TAT peptide onto gold nanoclusters (GNs) generating positively charged TAT-GNs.<sup>86</sup> This binary complex was then electrostatically complexed with Cas9 protein and sgPlk-1 plasmid. To protect the cargoes from enzymatic degradation and facilitate the circulation of the nanoclusters in the bloodstream, TAT-GNs-Cas9-sgPlk-1 complexes were coated with a cationic lipid shell consisting of DOTAP, DOPE and cholesterol and a PEG layer (DSPE-PEG). This vehicle could induce significant reduction in Plk-1 expression as well as ensuring significant suppression of melanoma tumours following a course of multiple intratumoural injections.

To enhance membrane fusion of GNPs and facilitate translocation of the RNP directly into the cytosol bypassing endosomes, Lee *et al.* formulated arginine coated self-assembling gold nanocomplexes.<sup>108</sup> Following systemic administration of these nanocomplexes, significant accumulation of gold was observed in macrophages of liver and spleen, which resulted in efficient gene editing of phosphatase and tensin homolog (PTEN) gene. The authors speculate that the preferential accumulation of the nanocomplexes in macrophages might be due to the large particles size (285 nm) and the absence of PEGylation. These two factors can facilitate the passive targeting of macrophages as described by Chan's group in their studies investigating the macrophages' uptake of GNs in correlations with the nanoparticles size, PEGylation and protein corona adsorption.<sup>143</sup>

Lee *et al.* designed CRISPR-gold nanoparticles which showed efficient transfection in different cell lines.<sup>29,30,57</sup> The carrier utilized in this study was composed of an internal gold core which was firstly coated with a 5' thiol modified single stranded oligonucleotide, containing a region complementary to the donor DNA. Then, the donor DNA was hybridized with DNA-SH, followed by adsorption of RNP, forming a CRISPR-gold complex. Next, the CRISPR-gold complex was coated with a cationic endosomal disruptive polymer Poly-*N*-[*N*-(2-aminoethyl)-2-aminoethyl] aspartamide (Pasp(DET)). Pasp(DET) features selective membrane destabilizing properties within the acidic endosomal compartment enabling efficient endosomal escape of the cargoes.<sup>144,145</sup> Once in the cytoplasm, glutathione cleaves the thiol linker of DNA-SH allowing the release of the donor DNA and RNP. The cationic charge of the polymer triggered endosomal disruption causing the release of the CRISPR components. Lee *et al.* utilized these nanoparticles for the correction of a point mutation in the dystrophin gene for the treatment of Duchenne Muscular Dystrophy (DMD).<sup>29</sup> Intramuscular injection of the designed nanoparticles could ensure 5.4% editing of the dystrophin gene which was reflected in improved strength and agility of the treated

animals. These nanoparticles were then utilized by the same group in another study to deliver RNP targeting the metabotropic glutamate receptor 5 (mGluR5) gene.<sup>30</sup> The study showed that intracranial injection of the nanocomplex induced successful mGluR5 knockout and subsequently reduced phenotypic repetitive behaviours associated with Fragile X Syndrome. *In vitro* biocompatibility testing also concluded that CRISPR-gold showed no adverse effects on neuronal membrane health or neuronal excitability. However, *in vivo* testing only administered very small quantities (picomoles) of gold to mice brain tissues. Furthermore, the study acknowledged that with multiple injections of CRISPR-Gold, the gold nanoparticle core could potentially accumulate in tissues of the brain and cause toxicity. This is a significant limitation when it comes to clinical translation.

### 5.5 Hybrid nanoparticles

Hybrid nanoparticles (HNP) are formulated combining multiple types of materials, with the aim of enhancing the properties or creating synergy between the individual components. HNP, with different compositions, were utilized in four studies for the delivery of CRISPR plasmid and RNP.

Wang *et al.* generated hybrid nanoparticles combining gold, targeting peptides and lipids.<sup>32</sup> Plk-1 targeting plasmids were electrostatically complexed with the TAT-GNs and coated with a combination of lipids (DOTAP, DOPE, cholesterol, PEG2000-DSPE). The therapeutic effect of TAT-GNs mediated Plk-1 gene editing of subcutaneous melanoma tumour model was further enhanced by concomitant application of irradiation, resulting in greater tumour eradication.

Li *et al.* developed hybrid nanoparticles featuring a polymeric fluorinated core encapsulating CRISPR plasmid, which was then coated with a multifunctional shell composed of PEG functionalized hyaluronan (HA) and linked to RGD peptide octa-arginine conjugates (RGD-R8).<sup>85</sup> The authors hypothesized that the incorporation of RGD-R8 tandem peptide, into the RGD-R8-PEG-HA core-shell resulted in an artificial virus-like structure (RRPHC) that could guarantee specific targeting to integrin  $\alpha_v\beta_3$  often overexpressed on tumour vascular endothelial cells. Once in the tumour, HA could be partially degraded by the hyaluronidase overexpressed in the intracellular compartments of the cells, releasing the plasmid complex. RRPHC, injected intraperitoneally, were able to successfully knockout the MTH1 gene of human ovarian cancer in a metastatic model, which resulted in a significant reduction of tumour nodules compared to control groups.

Guo *et al.* formulated a tumour targeted non-cationic, deformable nanolipogel (tNLG) to encapsulate CRISPR plasmids.<sup>93</sup> The nanolipogel was based on a combination of non-cationic lipid and polymeric components. The use of non-cationic components bypassed reliance on electrostatic interactions to form the complex, whilst also avoiding cationic charge-induced toxicities. The lipid bilayer of tNLG was comprised of two lipids and a naturally occurring biopolymer (alginate). This created a polysaccharide network between the alginate hydrogel and lipid bilayers, which ensured encapsulation





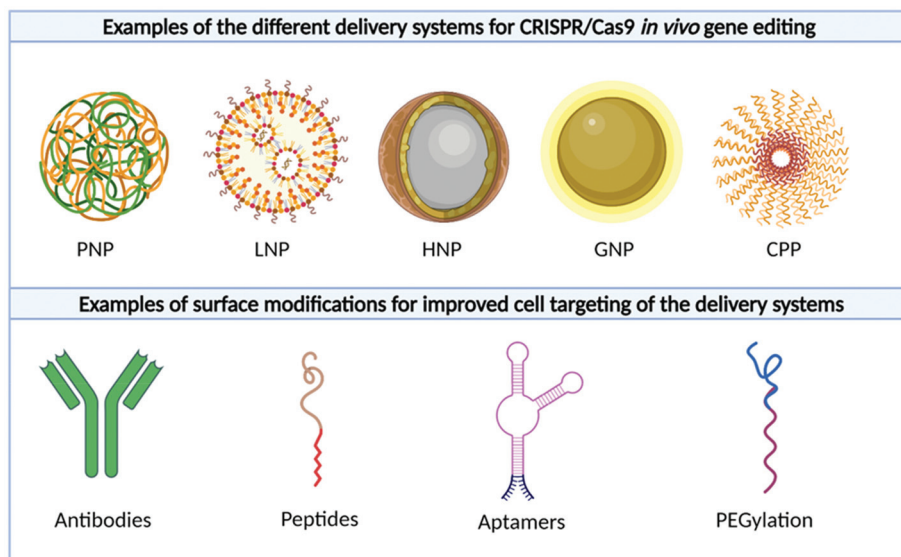
and retention of the CRISPR plasmids within the nanoparticles. Intracellular Adhesion Molecule 1 (ICAM-1) antibodies were then conjugated to the surface of the nanoparticles to allow for tissue-specific targeting of triple-negative breast cancer (TNBC) tissues. ICAM-1 tumour specificity was confirmed both *in vitro* and *in vivo*. Higher uptake of ICAM-1 tNLG was observed in orthotopic TNBC model compared to TNLG alone. Systemic delivery of these hybrid nanoparticles ensured high Lcn2 gene knockout, resulting in significant tumour growth reduction.

Liu *et al.* created a virus like nanoparticles (VLN) for the delivery of RNP in a melanoma disease model. Co-delivery of RNP with the anticancer drug Axitinib was also investigated.<sup>114</sup> The core of VLN consisted of surface-thiolated mesoporous silica nanoparticles (MSN). For co-delivery studies, Axitinib, a small molecule inhibitor of tyrosine kinase, was loaded into the pores of the nanoparticles. The pores were then 'locked' by conjugation with the RNP *via* disulphide bonds. The complex was coated with a lipid layer in order to protect RNP from enzymatic degradation. The mechanism of RNP release from the VLN core was based on the glutathione cleavage of the disulphide bonds between RNP and the MSN core. Since glutathione tends to be aberrantly overexpressed by tumour cells, the authors hypothesized that this approach could guarantee a tumour-specific release of the RNP. This phenomenon was confirmed by *in vitro* Förster resonance energy transfer (FRET) analysis, which demonstrated that RNP detachment from VLN occurred exclusively in the presence of glutathione. *In vivo* co-treatment with RNP and Axitinib showed the most efficient inhibition of tumour growth compared to mono treatment controls.

## 6. Discussion

The CRISPR/Cas9 system sparked a revolution in the field of gene editing, representing a powerful tool to be exploited in different disciplines from basic science to disease modelling and development of clinical and pre-clinical therapeutic applications. The sharp increase in popularity of this technology relies on the simple design, versatility in potentially targeting any gene, and its relatively low cost. Though promising, several factors must be considered before the CRISPR/Cas9 technology can be adopted for clinical applications.

Whilst it is relatively simple to perform *in vitro* gene editing, the *in vivo* translation is limited by a multitude of factors. Firstly, a key obstacle is the inability to administer any of the naked CRISPR formats directly into the body. Once injected, CRISPR cargos are quickly degraded by endogenous endonucleases and proteases found in the bloodstream. Secondly, due to their large size, anionic charge and hydrophilic nature, CRISPR formats have limited ability to cross the cell membrane and traffic into the intracellular compartments, thus impeding specific and efficient gene editing. The delivery of naked genetic material (pDNA, RNA) can rapidly induce an innate immune response *via* the pattern recognition receptors (e.g. TLR3, TLR7/8), which can then result in the acquisition of adaptive immunity.<sup>146</sup> Adaptive immunity could also be elicited by the delivery of Cas9 protein which is commonly derived from *Streptococcus pyogenes*, and *Staphylococcus aureus*, bacteria that frequently infect humans.<sup>65</sup> There is, therefore, the need for a delivery system that could guarantee protection of the CRISPR/Cas9 components, efficiently deliver it to the target cells, and attenuate its immunogenic pro-



**Fig. 4** Examples of different delivery systems and surface modifications adopted for CRISPR/Cas9 *in vivo* gene editing. Representative schematic illustration of polymeric nanoparticles (PNP), lipid nanoparticles (LNP), hybrid nanoparticles (HNP), gold nanoparticles (GNP) and cell penetrating peptides (CPP) utilized for the delivery of CRISPR/Cas9 cargos. Surface modification of these nanoparticles includes incorporation of antibodies, peptides, aptamers, and PEGylation. Created with BioRender.com.



properties. While viral vectors have been widely used for *in vivo* CRISPR/Cas9 gene editing, their human translation is intrinsically limited by genotoxicity, mutagenesis, and immunogenicity concerns.

Different organic and inorganic materials have been employed to formulate nanoparticles with controlled size, shape and surface charge enabling efficient encapsulation of the different CRISPR/Cas9 cargos (Fig. 4). Among these, lipid-based NPs represent the major class of non-viral vectors that have been utilized to overcome the main challenges of CRISPR/Cas9 *in vivo* application. Cationic lipids can electrostatically complex with the anionic nucleic acids and RNP forming nanoparticles characterized by an external phospholipid bilayer and an aqueous core in which the genetic material lies, thus protecting the payloads from enzymatic degradation.

Nanoparticles, once injected, can be easily opsonized, and cleared through the reticuloendothelial system (RES), hindering the delivery of the cargos to the target cells. Functionalization of the nanoparticles with hydrophilic polymers, such as PEG, is often implemented to reduce the adsorption of serum proteins onto the NPs. This can prolong the NPs stability in the physiological fluids as well as reducing its clearance by the RES.

Once the target site has been reached, the NPs must guarantee the internalization of the cargos into the intracellular compartments. Due to their fusogenic properties, lipids are often incorporated not only in lipid-based nanocarriers but also as a coating for other types of carriers such as polymeric and gold nanoparticles, to facilitate the endocytosis of the NPs into the intracellular compartments.

Once internalized by the endosome, the NPs must remain intact and facilitate endosomal escape prior to lysosomal digestion, which otherwise will eventually degrade the genetic material. Depending on the composition of the NPs, endosomal escape could be achieved through different approaches. In the acidic environment of the endosome, pH-responsive lipids protonate and fuse with the endosomal membrane destabilizing it, enabling the release of the cargos into the cytosol.<sup>147</sup> Cationic polymers like PAMAM and PEI, can also induce endosomal escape *via* the proton sponge effect.<sup>148,149</sup> Cell-penetrating peptides can be utilized alone or to coat NPs to enhance endosomal membrane disruption or facilitate the formation of micropores that enables the release of the cargos.<sup>150</sup>

NPs are often conjugated with peptides or antibodies that can specifically bind to receptors overexpressed on the target cells (*i.e.*, EGFR or  $\alpha_v\beta_3$  on cancer cells). This approach can enhance the uptake of the nanoparticles in the organ of interest and limit unwanted off-target effects on healthy tissues.

As highlighted in this review, several types of non-viral vectors have been successfully developed to mediate the delivery of CRISPR/Cas9 *in vivo* with therapeutic efficacy. While promising, each technology holds specific limitations that should be accurately addressed to increase its efficacy, safety and suitability for clinical translation. A noteworthy Phase I

clinical study sponsored by Intellia Therapeutics, has been evaluating the safety and pharmacodynamic of NTLA-2001, a lipid-based nanoparticle system encapsulating Cas9 mRNA and sgRNA targeting TTR protein in patients affected by hereditary ATTR amyloidosis with polyneuropathy.<sup>151</sup> A dose-dependent response was observed 28 days post single administration, with maximal reduction in TTR expression (87%) in the group that received a dose of 0.3 mg per kilogram. This study represents the first proof of concept of the feasibility of *in vivo* non-viral CRISPR/Cas9 mediated gene editing in humans. While further examination is still required, these early positive outcomes are highly encouraging, further incentivising pre-clinical and clinical research for a safer and efficient *in vivo* non-viral CRISPR/Cas9 gene editing.

## Conflicts of interest

The authors have declared that no competing interest exists.

## Acknowledgements

Authors would like to thank Dr Adam A Waters for proof reading the manuscript. Khuloud T. Al-Jamal acknowledges funding from the Brain Tumour Charity (GN-000398).

## References

- 1 J. A. Kulkarni, D. Witzigmann, S. B. Thomson, S. Chen, B. R. Leavitt, P. R. Cullis, *et al.*, The current landscape of nucleic acid therapeutics, *Nat. Nanotechnol.*, 2021, **16**(6), 630–643.
- 2 P. Hair, F. Cameron and K. McKeage, Mipomersen sodium: first global approval, *Drugs*, 2013, **73**(5), 487–493.
- 3 U. Patel, M. Boucher, L. de Léséleuc and S. Visintini, in *Voretigene Neparvovec: An Emerging Gene Therapy for the Treatment of Inherited Blindness*, Canadian Agency for Drugs and Technologies in Health, Ottawa (ON), 2016.
- 4 M. Caillaud, M. El Madani and L. Massaad-Massade, Small interfering RNA from the lab discovery to patients' recovery, *J. Controlled Release*, 2020, **321**, 616–628.
- 5 F. P. Polack, S. J. Thomas, N. Kitchin, J. Absalon, A. Gurtman, S. Lockhart, *et al.*, Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine, *N. Engl. J. Med.*, 2020, **383**(27), 2603–2615.
- 6 L. R. Baden, H. M. El Sahly, B. Essink, K. Kotloff, S. Frey, R. Novak, *et al.*, Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine, *N. Engl. J. Med.*, 2021, **384**(5), 403–416.
- 7 N. Lurie, M. Saville, R. Hatchett and J. Halton, Developing Covid-19 Vaccines at Pandemic Speed, *N. Engl. J. Med.*, 2020, **382**(21), 1969–1973.
- 8 J. Sadoff, G. Gray, A. Vandebosch, V. Cárdenas, G. Shukarev, B. Grinsztejn, *et al.*, Safety and Efficacy of Single-Dose Ad26.COV2.S Vaccine against Covid-19, *N. Engl. J. Med.*, 2021, **384**(23), 2187–2201.



- 9 J. C. Miller, M. C. Holmes, J. Wang, D. Y. Guschin, Y.-L. Lee, I. Rupniewski, *et al.*, An improved zinc-finger nuclease architecture for highly specific genome editing, *Nat. Biotechnol.*, 2007, **25**(7), 778–785.
- 10 J. P. Guilinger, V. Pattanayak, D. Reyon, S. Q. Tsai, J. D. Sander, J. K. Joung, *et al.*, Broad specificity profiling of TALENs results in engineered nucleases with improved DNA-cleavage specificity, *Nat. Methods*, 2014, **11**(4), 429–435.
- 11 S. Chandrasegaran and D. Carroll, Origins of Programmable Nucleases for Genome Engineering, *J. Mol. Biol.*, 2016, **428**(5 Pt B), 963–989.
- 12 F. A. Ran, P. D. Hsu, J. Wright, V. Agarwala, D. A. Scott and F. Zhang, Genome engineering using the CRISPR-Cas9 system, *Nat. Protoc.*, 2013, **8**(11), 2281–2308.
- 13 L. Cong, F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, *et al.*, Multiplex genome engineering using CRISPR/Cas systems, *Science*, 2013, **339**(6121), 819–823.
- 14 A. E. Friedland, Y. B. Tzur, K. M. Esvelt, M. P. Colaiácovo, G. M. Church and J. A. Calarco, Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system, *Nat. Methods*, 2013, **10**(8), 741–743.
- 15 N. Chang, C. Sun, L. Gao, D. Zhu, X. Xu, X. Zhu, *et al.*, Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos, *Cell Res.*, 2013, **23**(4), 465–472.
- 16 H. Wang, H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng, F. Zhang, *et al.*, One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering, *Cell*, 2013, **153**(4), 910–918.
- 17 C. Q. Song, Y. Li, H. Mou, J. Moore, A. Park, Y. Pomyen, *et al.*, Genome-Wide CRISPR Screen Identifies Regulators of Mitogen-Activated Protein Kinase as Suppressors of Liver Tumors in Mice, *Gastroenterology*, 2017, **152**(5), 1161–1173.
- 18 F. J. Sánchez-Rivera and T. Jacks, Applications of the CRISPR–Cas9 system in cancer biology, *Nat. Rev. Cancer*, 2015, **15**(7), 387–393.
- 19 T. Wan, D. Niu, C. Wu, F.-J. Xu, G. Church and Y. Ping, Material solutions for delivery of CRISPR/Cas-based genome editing tools: Current status and future outlook, *Mater. Today*, 2019, **26**, 40–66.
- 20 Y. Li, Z. Glass, M. Huang, Z.-Y. Chen and Q. Xu, Ex vivo cell-based CRISPR/Cas9 genome editing for therapeutic applications, *Biomaterials*, 2020, **234**, 119711.
- 21 X. Ou, Q. Ma, W. Yin, X. Ma and Z. He, CRISPR/Cas9 Gene-Editing in Cancer Immunotherapy: Promoting the Present Revolution in Cancer Therapy and Exploring More, *Front. Cell Dev. Biol.*, 2021, **9**, 674467.
- 22 K. Khalaf, K. Janowicz, M. Dyszkiewicz-Konwińska, G. Hutchings, C. Dompe, L. Moncrieff, *et al.*, CRISPR/Cas9 in Cancer Immunotherapy: Animal Models and Human Clinical Trials, *Genes*, 2020, **11**(8), 921.
- 23 H. Frangoul, D. Altshuler, M. D. Cappellini, Y.-S. Chen, J. Domm, B. K. Eustace, *et al.*, CRISPR-Cas9 gene editing for sickle cell disease and  $\beta$ -thalassemia, *N. Engl. J. Med.*, 2021, **384**(3), 252–260.
- 24 C. E. Nelson, C. H. Hakim, D. G. Ousterout, P. I. Thakore, E. A. Moreb, R. M. C. Rivera, *et al.*, In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy, *Science*, 2016, **351**(6271), 403–407.
- 25 X. Wang, A. Raghavan, T. Chen, L. Qiao, Y. Zhang, Q. Ding, *et al.*, CRISPR-Cas9 Targeting of PCSK9 in Human Hepatocytes In Vivo—Brief Report, *Arterioscler., Thromb., Vasc. Biol.*, 2016, **36**(5), 783–786.
- 26 X. Song, C. Liu, N. Wang, H. Huang, S. He, C. Gong, *et al.*, Delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy, *Adv. Drug Delivery Rev.*, 2021, **168**, 158–180.
- 27 S. J. Chou, P. Yang, Q. Ban, Y. P. Yang, M. L. Wang, C. S. Chien, *et al.*, Dual Supramolecular Nanoparticle Vectors Enable CRISPR/Cas9-Mediated Knockin of Retinoschisin 1 Gene—A Potential Nonviral Therapeutic Solution for X-Linked Juvenile Retinoschisis, *Adv. Sci.*, 2020, **7**(10), 1903432.
- 28 J. Y. Chung, Q. U. Ain, Y. Song, S. B. Yong and Y. H. Kim, Targeted delivery of CRISPR interference system against Fbp4 to white adipocytes ameliorates obesity, inflammation, hepatic steatosis, and insulin resistance, *Genome Res.*, 2019, **29**(9), 1442–1452.
- 29 K. Lee, M. Conboy, H. M. Park, F. Jiang, H. J. Kim, M. A. Dewitt, *et al.*, Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair, *Nat. Biomed. Eng.*, 2017, **1**, 889–901.
- 30 B. Lee, K. Lee, S. Panda, R. Gonzales-Rojas, A. Chong, V. Bugay, *et al.*, Nanoparticle delivery of CRISPR into the brain rescues a mouse model of fragile X syndrome from exaggerated repetitive behaviours, *Nat. Biomed. Eng.*, 2018, **2**(7), 497–507.
- 31 Q. Cheng, T. Wei, L. Farbiak, L. T. Johnson, S. A. Dilliard and D. J. Siegwart, Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR-Cas gene editing, *Nat. Nanotechnol.*, 2020, **15**(4), 313–320.
- 32 P. Wang, L. Zhang, W. Zheng, L. Cong, Z. Guo, Y. Xie, *et al.*, Thermo-triggered Release of CRISPR-Cas9 System by Lipid-Encapsulated Gold Nanoparticles for Tumor Therapy, *Angew. Chem., Int. Ed.*, 2018, **57**(6), 1491–1496.
- 33 D. Rosenblum, A. Gutkin, R. Kedmi, S. Ramishetti, N. Veiga, A. M. Jacobi, *et al.*, CRISPR-Cas9 genome editing using targeted lipid nanoparticles for cancer therapy, *Sci. Adv.*, 2020, **6**(47), eabc9450.
- 34 Y. Ishino, H. Shinagawa, K. Makino, M. Amemura and A. Nakata, Nucleotide sequence of the *iap* gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product, *J. Bacteriol.*, 1987, **169**(12), 5429–5433.
- 35 Y. Ishino, M. Krupovic and P. Forterre, History of CRISPR-Cas from Encounter with a Mysterious Repeated Sequence to Genome Editing Technology, *J. Bacteriol.*, 2018, **200**(7), e00580-17.





- 36 R. Barrangou, C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, *et al.*, CRISPR provides acquired resistance against viruses in prokaryotes, *Science*, 2007, **315**(5819), 1709–1712.
- 37 K. S. Makarova and E. V. Koonin, Annotation and Classification of CRISPR-Cas Systems, *Methods Mol. Biol.*, 2015, **1311**, 47–75.
- 38 A. V. Anzalone, L. W. Koblan and D. R. Liu, Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors, *Nat. Biotechnol.*, 2020, **38**(7), 824–844.
- 39 F. Jiang and J. A. Doudna, CRISPR-Cas9 Structures and Mechanisms, *Annu. Rev. Biophys.*, 2017, **46**, 505–529.
- 40 B. Wiedenheft, S. H. Sternberg and J. A. Doudna, RNA-guided genetic silencing systems in bacteria and archaea, *Nature*, 2012, **482**(7385), 331–338.
- 41 P. D. Hsu, E. S. Lander and F. Zhang, Development and applications of CRISPR-Cas9 for genome engineering, *Cell*, 2014, **157**(6), 1262–1278.
- 42 D. Gleditsch, P. Pausch, H. Muller-Esparza, A. Ozcan, X. Guo, G. Bange, *et al.*, PAM identification by CRISPR-Cas effector complexes: diversified mechanisms and structures, *RNA Biol.*, 2019, **16**(4), 504–517.
- 43 K. S. Makarova, Y. I. Wolf and E. V. Koonin, Classification and Nomenclature of CRISPR-Cas Systems: Where from Here?, *CRISPRJ.*, 2018, **1**(5), 325–336.
- 44 S. Shmakov, A. Smargon, D. Scott, D. Cox, N. Pyzocha, W. Yan, *et al.*, Diversity and evolution of class 2 CRISPR-Cas systems, *Nat. Rev. Microbiol.*, 2017, **15**(3), 169–182.
- 45 K. Chylinski, A. Le Rhun and E. Charpentier, The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems, *RNA Biol.*, 2013, **10**(5), 726–737.
- 46 A. Mir, J. F. Alterman, M. R. Hassler, A. J. Debacker, E. Hudgens, D. Echeverria, *et al.*, Heavily and fully modified RNAs guide efficient SpyCas9-mediated genome editing, *Nat. Commun.*, 2018, **9**(1), 2641.
- 47 T. Gaj, C. A. Gersbach and C. F. Barbas 3rd., ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering, *Trends Biotechnol.*, 2013, **31**(7), 397–405.
- 48 Z. Glass, M. Lee, Y. Li and Q. Xu, Engineering the Delivery System for CRISPR-Based Genome Editing, *Trends Biotechnol.*, 2018, **36**(2), 173–185.
- 49 Y. Fu, J. A. Foden, C. Khayter, M. L. Maeder, D. Reyon, J. K. Joung, *et al.*, High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells, *Nat. Biotechnol.*, 2013, **31**(9), 822–826.
- 50 N. Rivera-Torres, K. Banas, P. Bialk, K. M. Bloh and E. B. Kmiec, Insertional Mutagenesis by CRISPR/Cas9 Ribonucleoprotein Gene Editing in Cells Targeted for Point Mutation Repair Directed by Short Single-Stranded DNA Oligonucleotides, *PLoS One*, 2017, **12**(1), e0169350.
- 51 A. K. Fajrial, Q. Q. He, N. I. Wirusanti, J. E. Slansky and X. Ding, A review of emerging physical transfection methods for CRISPR/Cas9-mediated gene editing, *Theranostics*, 2020, **10**(12), 5532–5549.
- 52 S. Vaidyanathan, K. T. Azizian, A. Haque, J. M. Henderson, A. Hendel, S. Shore, *et al.*, Uridine Depletion and Chemical Modification Increase Cas9 mRNA Activity and Reduce Immunogenicity without HPLC Purification, *Mol. Ther.–Nucleic Acids*, 2018, **12**, 530–542.
- 53 H. Zhang and X. Xia, RNA cancer vaccines: developing mRNA nanovaccine with self-adjuvant property for cancer immunotherapy, *Hum. Vaccines Immunother.*, 2021, 1–4.
- 54 J. Devoldere, H. Dewitte, S. C. De Smedt and K. Remaut, Evading innate immunity in nonviral mRNA delivery: don't shoot the messenger, *Drug Discovery Today*, 2016, **21**(1), 11–25.
- 55 G. Mullally, K. van Aelst, M. M. Naqvi, F. M. Diffin, T. Karvelis, G. Gasiunas, *et al.*, 5' modifications to CRISPR-Cas9 gRNA can change the dynamics and size of R-loops and inhibit DNA cleavage, *Nucleic Acids Res.*, 2020, **48**(12), 6811–6823.
- 56 J. Filippova, A. Matveeva, E. Zhuravlev and G. Stepanov, Guide RNA modification as a way to improve CRISPR/Cas9-based genome-editing systems, *Biochimie*, 2019, **167**, 49–60.
- 57 S. Aghamiri, S. Talaei, A. A. Ghavidel, F. Zandsalimi, S. Masoumi, N. H. Hafshejani, *et al.*, Nanoparticles-mediated CRISPR/Cas9 delivery: Recent advances in cancer treatment, *J. Drug Delivery Sci. Technol.*, 2020, **56**, 101533.
- 58 M. L. Guevara, F. Persano and S. Persano, Advances in Lipid Nanoparticles for mRNA-Based Cancer Immunotherapy, *Front. Chem.*, 2020, **8**, 589959.
- 59 C.-F. Xu, G.-J. Chen, Y.-L. Luo, Y. Zhang, G. Zhao, Z.-D. Lu, *et al.*, Rational designs of in vivo CRISPR-Cas delivery systems, *Adv. Drug Delivery Rev.*, 2021, **168**, 3–29.
- 60 B. Sun, H. Chen and X. Gao, Versatile modification of the CRISPR/Cas9 ribonucleoprotein system to facilitate in vivo application, *J. Controlled Release*, 2021, **337**, 698–717.
- 61 W. L. Chew, M. Tabebordbar, J. K. Cheng, P. Mali, E. Y. Wu, A. H. Ng, *et al.*, A multifunctional AAV-CRISPR-Cas9 and its host response, *Nat. Methods*, 2016, **13**(10), 868–874.
- 62 A. Li, M. R. Tanner, C. M. Lee, A. E. Hurley, M. De Giorgi, K. E. Jarrett, *et al.*, AAV-CRISPR Gene Editing Is Negated by Pre-existing Immunity to Cas9, *Mol. Ther.*, 2020, **28**(6), 1432–1441.
- 63 J. M. Crudele and J. S. Chamberlain, Cas9 immunity creates challenges for CRISPR gene editing therapies, *Nat. Commun.*, 2018, **9**(1), 3497.
- 64 C. T. Charlesworth, P. S. Deshpande, D. P. Dever, J. Camarena, V. T. Lemgart, M. K. Cromer, *et al.*, Identification of preexisting adaptive immunity to Cas9 proteins in humans, *Nat. Med.*, 2019, **25**(2), 249–254.
- 65 D. L. Wagner, L. Amini, D. J. Wendering, L.-M. Burkhardt, L. Akyüz, P. Reinke, *et al.*, High prevalence of *Streptococcus pyogenes* Cas9-reactive T cells within the adult human population, *Nat. Med.*, 2019, **25**(2), 242–248.





- 66 C. Liu, L. Zhang, H. Liu and K. Cheng, Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications, *J. Controlled Release*, 2017, **266**, 17–26.
- 67 M. Hashimoto and T. Takemoto, Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing, *Sci. Rep.*, 2015, **5**, 11315.
- 68 C. L. Xu, M. Z. C. Ruan, V. B. Mahajan and S. H. Tsang, Viral Delivery Systems for CRISPR, *Viruses*, 2019, **11**(1), 28.
- 69 Y. Yang, L. Wang, P. Bell, D. McMenamin, Z. He, J. White, *et al.*, A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice, *Nat. Biotechnol.*, 2016, **34**(3), 334–338.
- 70 L. B. Ryø, E. A. Thomsen and J. G. Mikkelsen, Production and Validation of Lentiviral Vectors for CRISPR/Cas9 Delivery, *Methods Mol. Biol.*, 2019, **1961**, 93–109.
- 71 C. A. Lino, J. C. Harper, J. P. Carney and J. A. Timlin, Delivering CRISPR: a review of the challenges and approaches, *Drug Delivery*, 2018, **25**(1), 1234–1257.
- 72 H. Yin, W. Xue, S. Chen, R. L. Bogorad, E. Benedetti, M. Grompe, *et al.*, Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype, *Nat. Biotechnol.*, 2014, **32**(6), 551–553.
- 73 M. J. Kim and N. Ahituv, The hydrodynamic tail vein assay as a tool for the study of liver promoters and enhancers, *Methods Mol. Biol.*, 2013, **1015**, 279–289.
- 74 B. Bonamassa, L. Hai and D. Liu, Hydrodynamic Gene Delivery and Its Applications in Pharmaceutical Research, *Pharm. Res.*, 2011, **28**(4), 694–701.
- 75 J. E. Hagstrom, J. Hegge, G. Zhang, M. Noble, V. Budker, D. L. Lewis, *et al.*, A Facile Nonviral Method for Delivering Genes and siRNAs to Skeletal Muscle of Mammalian Limbs, *Mol. Ther.*, 2004, **10**(2), 386–398.
- 76 J. O. Hegge, C. I. Wooddell, G. Zhang, J. E. Hagstrom, S. Braun, T. Huss, *et al.*, Evaluation of hydrodynamic limb vein injections in nonhuman primates, *Hum. Gene Ther.*, 2010, **21**(7), 829–842.
- 77 H. Herweijer and J. A. Wolff, Gene therapy progress and prospects: Hydrodynamic gene delivery, *Gene Ther.*, 2007, **14**(2), 99–107.
- 78 J. E. Rittiner, M. Moncalvo, O. Chiba-Falek and B. Kantor, Gene-Editing Technologies Paired With Viral Vectors for Translational Research Into Neurodegenerative Diseases, *Front. Mol. Neurosci.*, 2020, **13**, 148.
- 79 R. Goswami, G. Subramanian, L. Silayeva, I. Newkirk, D. Doctor, K. Chawla, *et al.*, Gene Therapy Leaves a Vicious Cycle, *Front. Oncol.*, 2019, **9**, 297.
- 80 X. Chen and M. A. Goncalves, Engineered Viruses as Genome Editing Devices, *Mol. Ther.*, 2016, **24**(3), 447–457.
- 81 G. Ronzitti, D.-A. Gross and F. Mingozzi, Human Immune Responses to Adeno-Associated Virus (AAV) Vectors, *Front. Immunol.*, 2020, **11**, 670.
- 82 G. J. Xu, T. Kula, Q. Xu, M. Z. Li, S. D. Vernon, T. Ndung'u, *et al.*, Viral immunology. Comprehensive serological profiling of human populations using a synthetic human virome, *Science*, 2015, **348**(6239), aaa0698.
- 83 K. A. Hajj and K. A. Whitehead, Tools for translation: non-viral materials for therapeutic mRNA delivery, *Nat. Rev. Mater.*, 2017, **2**(10), 17056.
- 84 C. Liang, F. Li, L. Wang, Z.-K. Zhang, C. Wang, B. He, *et al.*, Tumor cell-targeted delivery of CRISPR/Cas9 by aptamer-functionalized lipopolymer for therapeutic genome editing of VEGFA in osteosarcoma, *Biomaterials*, 2017, **147**, 68–85.
- 85 L. Li, L. Song, X. Liu, X. Yang, X. Li, T. He, *et al.*, Artificial Virus Delivers CRISPR-Cas9 System for Genome Editing of Cells in Mice, *ACS Nano*, 2017, **11**(1), 95–111.
- 86 P. Wang, L. Zhang, Y. Xie, N. Wang, R. Tang, W. Zheng and X. Jiang, Genome editing for cancer therapy: delivery of Cas9 protein/sgRNA plasmid via a gold nanocluster/lipid core-shell nanocarrier, *Adv. Sci.*, 2017, **4**(11), 1700175.
- 87 L. Zhang, P. Wang, Q. Feng, N. Wang, Z. Chen, Y. Huang, *et al.*, Lipid nanoparticle-mediated efficient delivery of CRISPR/Cas9 for tumor therapy, *NPG Asia Mater.*, 2017, **9**(10), e441.
- 88 Y.-L. Luo, C.-F. Xu, H.-J. Li, Z.-T. Cao, J. Liu, J.-L. Wang, *et al.*, Macrophage-specific in vivo gene editing using cationic lipid-assisted polymeric nanoparticles, *ACS Nano*, 2018, **12**(2), 994–1005.
- 89 H. X. Wang, Z. Song, Y. H. Lao, X. Xu, J. Gong, D. Cheng, *et al.*, Nonviral gene editing via CRISPR/Cas9 delivery by membrane-disruptive and endosomolytic helical polypeptide, *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**(19), 4903–4908.
- 90 Y. Liu, Z. T. Cao, C. F. Xu, Z. D. Lu, Y. L. Luo and J. Wang, Optimization of lipid-assisted nanoparticle for disturbing neutrophils-related inflammation, *Biomaterials*, 2018, **172**, 92–104.
- 91 Y. Liu, G. Zhao, C.-F. Xu, Y.-L. Luo, Z.-D. Lu and J. Wang, Systemic delivery of CRISPR/Cas9 with PEG-PLGA nanoparticles for chronic myeloid leukemia targeted therapy, *Biomater. Sci.*, 2018, **6**(6), 1592–1603.
- 92 M. Li, H. Xie, Y. Liu, C. Xia, X. Cun, Y. Long, *et al.*, Knockdown of hypoxia-inducible factor-1 alpha by tumor targeted delivery of CRISPR/Cas9 system suppressed the metastasis of pancreatic cancer, *J. Controlled Release*, 2019, **304**, 204–215.
- 93 P. Guo, J. Yang, J. Huang, D. T. Auguste and M. A. Moses, Therapeutic genome editing of triple-negative breast tumors using a noncationic and deformable nanolipogel, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**(37), 18295–18303.
- 94 Y. Qi, Y. Liu, B. Yu, Y. Hu, N. Zhang, Y. Zheng, *et al.*, A Lactose-Derived CRISPR/Cas9 Delivery System for Efficient Genome Editing In Vivo to Treat Orthotopic Hepatocellular Carcinoma, *Adv. Sci.*, 2020, **7**(17), 2001424.
- 95 Y.-L. Luo, L.-F. Liang, Y.-J. Gan, J. Liu, Y. Zhang, Y.-N. Fan, *et al.*, An All-in-One Nanomedicine Consisting of CRISPR-Cas9 and an Autoantigen Peptide for Restoring Specific Immune Tolerance, *ACS Appl. Mater. Interfaces*, 2020, **12**(43), 48259–48271.
- 96 K. Tu, H. Deng, L. Kong, Y. Wang, T. Yang, Q. Hu, *et al.*, Reshaping Tumor Immune Microenvironment through



- Acidity-Responsive Nanoparticles Featured with CRISPR/Cas9-Mediated Programmed Death-Ligand 1 Attenuation and Chemotherapeutics-Induced Immunogenic Cell Death, *ACS Appl. Mater. Interfaces*, 2020, **12**(14), 16018–16030.
- 97 H. Deng, S. Tan, X. Gao, C. Zou, C. Xu, K. Tu, *et al.*, Cdk5 knocking out mediated by CRISPR-Cas9 genome editing for PD-L1 attenuation and enhanced antitumor immunity, *Acta Pharm. Sin. B*, 2020, **10**(2), 358–373.
- 98 H. Yin, X. Yuan, L. Luo, Y. Lu, B. Qin, J. Zhang, *et al.*, Appropriate Delivery of the CRISPR/Cas9 System through the Nonlysosomal Route: Application for Therapeutic Gene Editing, *Adv. Sci.*, 2020, **7**(14), 1903381.
- 99 X. Gao, Z. Jin, X. Tan, C. Zhang, C. Zou, W. Zhang, *et al.*, Hyperbranched poly( $\beta$ -amino ester) based polyplex nanoparticles for delivery of CRISPR/Cas9 system and treatment of HPV infection associated cervical cancer, *J. Controlled Release*, 2020, **321**, 654–668.
- 100 C. Jiang, M. Mei, B. Li, X. Zhu, W. Zu, Y. Tian, *et al.*, A non-viral CRISPR/Cas9 delivery system for therapeutically targeting HBV DNA and psc9 in vivo, *Cell Res.*, 2017, **27**(3), 440–443.
- 101 H. Yin, C. Q. Song, S. Suresh, Q. Wu, S. Walsh, L. H. Rhym, *et al.*, Structure-guided chemical modification of guide RNA enables potent non-viral in vivo genome editing, *Nat. Biotechnol.*, 2017, **35**(12), 1179–1187.
- 102 C. D. Sago, M. P. Lokugamage, K. Paunovska, D. A. Vanover, C. M. Monaco, N. N. Shah, *et al.*, High-throughput in vivo screen of functional mRNA delivery identifies nanoparticles for endothelial cell gene editing, *Proc. Natl. Acad. Sci. U. S. A.*, 2018, **115**(42), E9944–E52.
- 103 C. Xu, Z. Lu, Y. Luo, Y. Liu, Z. Cao, S. Shen, *et al.*, Targeting of NLRP3 inflammasome with gene editing for the amelioration of inflammatory diseases, *Nat. Commun.*, 2018, **9**(1), 4092.
- 104 J. Liu, J. Chang, Y. Jiang, X. Meng, T. Sun, L. Mao, *et al.*, Fast and Efficient CRISPR/Cas9 Genome Editing In Vivo Enabled by Bioreducible Lipid and Messenger RNA Nanoparticles, *Adv. Mater.*, 2019, **31**(33), e1902575.
- 105 Y. Zhang, S. Shen, G. Zhao, C.-F. Xu, H.-B. Zhang, Y.-L. Luo, *et al.*, In situ repurposing of dendritic cells with CRISPR/Cas9-based nanomedicine to induce transplant tolerance, *Biomaterials*, 2019, **217**, 119302.
- 106 J. D. Finn, A. R. Smith, M. C. Patel, L. Shaw, M. R. Youniss, J. van Heteren, *et al.*, A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing, *Cell Rep.*, 2018, **22**(9), 2227–2235.
- 107 Z. Chen, F. Liu, Y. Chen, J. Liu, X. Wang, A. T. Chen, *et al.*, Targeted Delivery of CRISPR/Cas9-Mediated Cancer Gene Therapy via Liposome-Templated Hydrogel Nanoparticles, *Adv. Funct. Mater.*, 2017, **27**(46), 1703036.
- 108 Y.-W. Lee, R. Mout, D. C. Luther, Y. Liu, L. Castellanos-García, A. S. Burnside, *et al.*, In Vivo Editing of Macrophages through Systemic Delivery of CRISPR-Cas9-Ribonucleoprotein-Nanoparticle Nanoassemblies, *Adv. Ther.*, 2019, **2**(10), 1900041.
- 109 H. Park, J. Oh, G. Shim, B. Cho, Y. Chang, S. Kim, *et al.*, In vivo neuronal gene editing via CRISPR-Cas9 amphiphilic nanocomplexes alleviates deficits in mouse models of Alzheimer's disease, *Nat. Neurosci.*, 2019, **22**(4), 524–528.
- 110 E. Y. Cho, J. Y. Ryu, H. A. R. Lee, S. H. Hong, H. S. Park, K. S. Hong, *et al.*, Lecithin nano-liposomal particle as a CRISPR/Cas9 complex delivery system for treating type 2 diabetes, *J. Nanobiotechnol.*, 2019, **17**(1), 19.
- 111 J. Y. Ryu, E. J. Won, H. A. R. Lee, J. H. Kim, E. Hui, H. P. Kim, *et al.*, Ultrasound-activated particles as CRISPR/Cas9 delivery system for androgenic alopecia therapy, *Biomaterials*, 2020, **232**, 119736.
- 112 S. Deng, X. Li, S. Liu, J. Chen, M. Li, S. Y. Chew, *et al.*, Codelivery of CRISPR-Cas9 and chlorin e6 for spatially controlled tumor-specific gene editing with synergistic drug effects, *Sci. Adv.*, 2020, **6**(29), eabb4005.
- 113 T. Wei, Q. Cheng, Y. L. Min, E. N. Olson and D. J. Siegwart, Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing, *Nat. Commun.*, 2020, **11**(1), 3232.
- 114 Q. Liu, C. Wang, Y. Zheng, Y. Zhao, Y. Wang, J. Hao, *et al.*, Virus-like nanoparticle as a co-delivery system to enhance efficacy of CRISPR/Cas9-based cancer immunotherapy, *Biomaterials*, 2020, **258**, 120275.
- 115 W. Sun, W. Ji, J. M. Hall, Q. Hu, C. Wang, C. L. Beisel, *et al.*, Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing, *Angew. Chem., Int. Ed.*, 2015, **54**(41), 12029–12033.
- 116 Y. Wang, P. K. Shahi, R. Xie, H. Zhang, A. A. Abdeen, N. Yodsanit, *et al.*, A pH-responsive silica-metal-organic framework hybrid nanoparticle for the delivery of hydrophilic drugs, nucleic acids, and CRISPR-Cas9 genome-editing machineries, *J. Controlled Release*, 2020, **324**, 194–203.
- 117 S. Samimi, N. Maghsoudnia, R. B. Eftekhari and F. Dorkoosh, Chapter 3 - Lipid-Based Nanoparticles for Drug Delivery Systems, in *Characterization and Biology of Nanomaterials for Drug Delivery*, ed. S.S Mohapatra, S. Ranjan, N. Dasgupta, R.K Mishra and S. Thomas, Elsevier, 2019, pp. 47–76.
- 118 P. R. Cullis and M. J. Hope, Lipid Nanoparticle Systems for Enabling Gene Therapies, *Mol. Ther.*, 2017, **25**(7), 1467–1475.
- 119 X. Cheng and R. J. Lee, The role of helper lipids in lipid nanoparticles (LNPs) designed for oligonucleotide delivery, *Adv. Drug Delivery Rev.*, 2016, **99**, 129–137.
- 120 X. Ge, L. Chen, B. Zhao and W. Yuan, Rationale and Application of PEGylated Lipid-Based System for Advanced Target Delivery of siRNA, *Front. Pharmacol.*, 2020, **11**, 598175.
- 121 Z.-Y. He, Y.-G. Zhang, Y.-H. Yang, C.-C. Ma, P. Wang, W. Du, *et al.*, In vivo ovarian cancer gene therapy using CRISPR-Cas9, *Hum. Gene Ther.*, 2018, **29**(2), 223–233.
- 122 X.-Z. Yang, S. Dou, T.-M. Sun, C.-Q. Mao, H.-X. Wang and J. Wang, Systemic delivery of siRNA with cationic lipid assisted PEG-PLA nanoparticles for cancer therapy, *J. Controlled Release*, 2011, **156**(2), 203–211.



- 123 E. Samaridou, J. Heyes and P. Lutwyche, Lipid nanoparticles for nucleic acid delivery: Current perspectives, *Adv. Drug Delivery Rev.*, 2020, **154-155**, 37–63.
- 124 K. Garber, Alnylam launches era of RNAi drugs, *Nat. Biotechnol.*, 2018, **36**(9), 777–778.
- 125 S. Ramishetti, I. Hazan-Halevy, R. Palakuri, S. Chatterjee, S. Naidu Gonna, N. Dammes, *et al.*, A combinatorial library of lipid nanoparticles for RNA delivery to leukocytes, *Adv. Mater.*, 2020, **32**(12), 1906128.
- 126 Y. Dong, K. T. Love, J. R. Dorkin, S. Sirirungruang, Y. Zhang, D. Chen, *et al.*, Lipopeptide nanoparticles for potent and selective siRNA delivery in rodents and nonhuman primates, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**(11), 3955–3960.
- 127 L. Miao, J. Lin, Y. Huang, L. Li, D. Delcassian, Y. Ge, *et al.*, Synergistic lipid compositions for albumin receptor mediated delivery of mRNA to the liver, *Nat. Commun.*, 2020, **11**(1), 2424.
- 128 Y.-N. Zhang, W. Poon, A. J. Tavares, I. D. McGilvray and W. C. W. Chan, Nanoparticle–liver interactions: Cellular uptake and hepatobiliary elimination, *J. Controlled Release*, 2016, **240**, 332–348.
- 129 R. Rai, S. Alwani and I. Badea, Polymeric Nanoparticles in Gene Therapy: New Avenues of Design and Optimization for Delivery Applications, *Polymers*, 2019, **11**(4), 745.
- 130 G. Lin, H. Zhang and L. Huang, Smart Polymeric Nanoparticles for Cancer Gene Delivery, *Mol. Pharm.*, 2015, **12**(2), 314–321.
- 131 X. Zhang, C. Xu, S. Gao, P. Li, Y. Kong, T. Li, *et al.*, CRISPR/Cas9 Delivery Mediated with Hydroxyl-Rich Nanosystems for Gene Editing in Aorta, *Adv. Sci.*, 2019, **6**(12), 1900386.
- 132 S. Chesnoy and L. Huang, Structure and function of lipid-DNA complexes for gene delivery, *Annu. Rev. Biophys. Biomol. Struct.*, 2000, **29**, 27–47.
- 133 Y. Sun, H. Hu, N. Zhao, T. Xia, B. Yu, C. Shen, *et al.*, Multifunctional polycationic photosensitizer conjugates with rich hydroxyl groups for versatile water-soluble photodynamic therapy nanoplatforms, *Biomaterials*, 2017, **117**, 77–91.
- 134 J. Chen, C. Wu and D. Oupický, Bioreducible hyperbranched poly(amido amine)s for gene delivery, *Biomacromolecules*, 2009, **10**(10), 2921–2927.
- 135 Y. Liu, J. Du, J. S. Choi, K. J. Chen, S. Hou, M. Yan, *et al.*, A High-Throughput Platform for Formulating and Screening Multifunctional Nanoparticles Capable of Simultaneous Delivery of Genes and Transcription Factors, *Angew. Chem., Int. Ed.*, 2016, **55**(1), 169–173.
- 136 P. P. Tripathi, H. Arami, I. Banga, J. Gupta and S. Gandhi, Cell penetrating peptides in preclinical and clinical cancer diagnosis and therapy, *Oncotarget*, 2018, **9**(98), 37252–37267.
- 137 H. Lu, J. Wang, Y. Bai, J. W. Lang, S. Liu, Y. Lin and J. Cheng, Ionic polypeptides with unusual helical stability, *Nat. Commun.*, 2011, **2**(1), 1–9.
- 138 Y.-W. Won, P. P. Adhikary, K. S. Lim, H. J. Kim, J. K. Kim and Y.-H. Kim, Oligopeptide complex for targeted non-viral gene delivery to adipocytes, *Nat. Mater.*, 2014, **13**(12), 1157–1164.
- 139 E. Choi, J. Han, X. Tan, J. Oh, D. Lee, T. Rhim, *et al.*, Combined delivery of temozolomide and the thymidine kinase gene for treatment of glioblastoma, *J. Drug Targeting*, 2017, **25**(2), 156–162.
- 140 R. Bahadur K.C., B. Thapa and N. Bhattarai, Gold nanoparticle-based gene delivery: promises and challenges, *Nanotechnol. Rev.*, 2013, **3**, 269–280.
- 141 J. K. Patra, G. Das, L. F. Fraceto, E. V. R. Campos, M. D. P. Rodriguez-Torres, L. S. Acosta-Torres, *et al.*, Nano based drug delivery systems: recent developments and future prospects, *J. Nanobiotechnol.*, 2018, **16**(1), 71.
- 142 R. Vankayala, C. L. Kuo, K. Nuthalapati, C. S. Chiang and K. C. Hwang, Nucleus-targeting gold nanoclusters for simultaneous in vivo fluorescence imaging, gene delivery, and NIR-light activated photodynamic therapy, *Adv. Funct. Mater.*, 2015, **25**(37), 5934–5945.
- 143 C. D. Walkey, J. B. Olsen, H. Guo, A. Emili and W. C. W. Chan, Nanoparticle Size and Surface Chemistry Determine Serum Protein Adsorption and Macrophage Uptake, *J. Am. Chem. Soc.*, 2012, **134**(4), 2139–2147.
- 144 H. J. Kim, A. Ishii, K. Miyata, Y. Lee, S. Wu, M. Oba, *et al.*, Introduction of stearyl moieties into a biocompatible cationic polyaspartamide derivative, PAsp(DET), with endosomal escaping function for enhanced siRNA-mediated gene knockdown, *J. Controlled Release*, 2010, **145**(2), 141–148.
- 145 F. Pittella, M. Zhang, Y. Lee, H. J. Kim, T. Tockary, K. Osada, *et al.*, Enhanced endosomal escape of siRNA-incorporating hybrid nanoparticles from calcium phosphate and PEG-block charge-conversional polymer for efficient gene knockdown with negligible cytotoxicity, *Biomaterials*, 2011, **32**(11), 3106–3114.
- 146 H. Sakurai, K. Kawabata, F. Sakurai, S. Nakagawa and H. Mizuguchi, Innate immune response induced by gene delivery vectors, *Int. J. Pharm.*, 2008, **354**(1), 9–15.
- 147 M. Schlich, R. Palomba, G. Costabile, S. Mizrahy, M. Pannuzzo, D. Peer, *et al.*, Cytosolic delivery of nucleic acids: The case of ionizable lipid nanoparticles, *Bioeng. Transl. Med.*, 2021, e10213.
- 148 A. K. Varkouhi, M. Scholte, G. Storm and H. J. Haisma, Endosomal escape pathways for delivery of biologicals, *J. Controlled Release*, 2011, **151**(3), 220–228.
- 149 B. Ashok, N. A. Peppas and M. E. Wechsler, Lipid- and Polymer-Based Nanoparticle Systems for the Delivery of CRISPR/Cas9, *J. Drug Delivery Sci. Technol.*, 2021, **65**, 102728.
- 150 R. E. Taylor and M. Zahid, Cell Penetrating Peptides, Novel Vectors for Gene Therapy, *Pharmaceutics*, 2020, **12**(3), 225.
- 151 J. D. Gillmore, E. Gane, J. Taubel, J. Kao, M. Fontana, M. L. Maitland, *et al.*, CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis, *N. Engl. J. Med.*, 2021, **385**(6), 493–502.

