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Current Advances Toward the Encapsulation of Cas9

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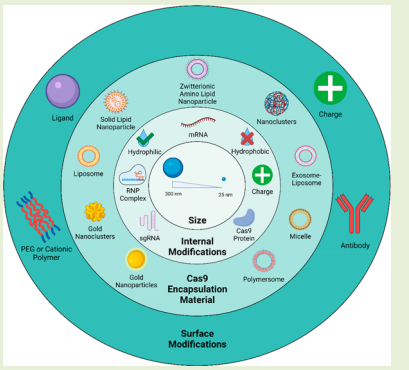
ABSTRACT: Genetic diseases present formidable hurdles in maintaining a good quality of life for those suffering from these ailments. Often, patients look to inadequate treatments to manage symptoms, which can result in harmful effects on the body. Through genetic engineering, scientists utilize the clustered regularly short palindromic repeat (CRISPR)-associated protein, known as Cas9, to treat the root of the problem. The Cas9 protein is often codelivered with guide RNAs or in ribonucleoprotein complexes (RNP) to ensure targeted delivery of the genetic tool as well as to limit off-target effects. This paper provides an overview of the current advances made toward the encapsulation and delivery of Cas9 to desired locations in the body through encapsulating nanoparticles. Several factors must be considered when employing the Cas9 system to allow gene editing to occur. Material selection is crucial to protect the payload of the delivery vector. Current literature indicates that lipid- and polymer-based nanoparticles show the most potential as delivery vessels for Cas9. Lipid nanoparticles greatly outpace polymer-based nanoparticles in the clinic, despite the benefits that polymers may introduce. When developing translatable systems, there are factors that have not yet been considered that are relevant to Cas9 delivery that are highlighted in this Viewpoint. The proper functioning of Cas9 is dependent on maintaining a proper internal environment; however, there are gaps in the literature regarding these optimal conditions. Interactions between charges of the Cas9 protein, codelivered molecules, and delivery vehicles could impact the effectiveness of the gene editing taking place. While the internal charges of nanoparticles and their effects on Cas9 are presently undetermined, nanoparticles currently offer the ideal delivery method for the Cas9 protein due to their adequate size, modifiable external charge, and ability to be modified. Overall, a cationic lipid-/polymer-based nanoparticle system was found to have the most prospects in Cas9 delivery thus far. By understanding the successes of other systems, translatable, polymer-based delivery vehicles may be developed.



1. INTRODUCTION

Despite the vast bioinformatic knowledge now available on disease pathophysiology and genetic causes with the success of the Human Genome project, patients with genetic diseases are still suffering from a lack of treatment options. Patients often turn to subpar approaches that mainly treat symptoms and can harm the patient's body with off-target side effects. Instead of treating symptoms, therapeutic approaches should aim to correct the genetic defects. Unfortunately, the number of novel gene-editing tools developed in the past decade aimed to correct genetic defects has greatly outpaced the ability to effectively deliver them into diseased cells.

Consequently, researchers look to the Cas9 protein, which is a clustered regularly interspaced short palindromic repeat (CRISPR)-associated endonuclease utilized to alter genomic information in the human body to engineer therapeutic responses.[1,2] It is important to note that Cas9 is not the only beneficial CRISPR system for gene editing, with other types of CRISPR-associated proteins being reviewed extensively elsewhere.[3,4] However, Cas9 has the most simplistic development, optimization, and application for human genome editing. CRISPR-Cas9 can participate in nonhomologous end joining (NHEJ) that results in frameshift mutations and potential gene knockouts.[5] NHEJ requires single-guide RNA (sgRNA) developed as a fusion of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA),[6] to introduce double-stranded breaks.[3] With the addition of a template or donor DNA to Cas9 and gRNA, the Cas9 system is capable of homology-directed repair (HDR), which could lead to gene correction.[7] Through this genome-editing technology, diseases and cancers caused by genetic irregularities have the potential to be irradicated.[8] However, the function of Cas9 is dependent on the ability to effectively deliver the protein *in vivo*, which is challenging. Cas9 is a 160 kDa protein, isolated from the CRISPR system in the *Streptococcus pyogenes* bacterium, making Cas9 xenogeneic.[9,10] Thus, Cas9 elicits an immune response in human application.[9,10] Its large size can limit *in*

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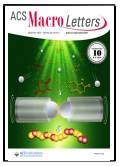

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vivo transport and prohibit cellular internalization.[10] Simultaneously, different areas of the body have various physiologies *in vivo*[6] which are capable, in varying capacities, of degrading Cas9.

While the body maintains a relative blood pH level of 7.4, other body fluids have different pHs.[10−12] The blood relies on hemoglobin and albumin to maintain a strict pH. However, interstitial fluids depend on weak buffers, such as phosphate and bicarbonate, so their pH is more variable than that of blood.[11,12] Furthermore, strenuous exercise leads to the production of lactic acid which in turn causes body fluid acidosis.[11,12] If the Cas9 protein interacted with fluids or tissues other than the blood, it could cause degradation. To function in the human body, the Cas9 protein must overcome these biological challenges and more, including the blood and cellular barriers, variation in pH throughout the body, and the reticuloendothelial system (RES) which removes foreign material. An alternative approach to modifying Cas9 to endure conditions *in vivo*, which could alter performance, is to encapsulate the protein in targeted nanoparticles (NPs) to ensure efficient delivery to the target site. Self-assembled NPs have already demonstrated their potential to encapsulate, protect, and deliver high molecular weight proteins.[13−15] Because of this, self-assembled NPs are a promising starting point for the delivery of Cas9. Acknowledging the presence of many recent review papers on CRISPR-Cas9 use *in vivo*,[5,16−20] in this Viewpoint we focus on analyzing the large parameter space available for nonviral delivery of Cas9 as a gene-editing technology. We highlight areas where the field of encapsulating nanoparticles needs to focus to increase the likelihood of clinical translation. To deliver the Cas9 protein in NPs, one must consider material, internal environment, charge−charge interactions, and physical constraints. This Viewpoint aims to critically analyze the current literature and provide considerations for the design of systems to encapsulate Cas9.

2. TYPES OF CAS9 FOR DELIVERY

Researchers are also investigating multiple types of Cas9 delivery mechanisms, including Cas9 ribonucleoprotein complexes (RNPs), which consist of Cas9 complexed with sgRNA, Cas9, and sgRNA independently or plasmid-based Cas9 to be used as a part of the CRISPR-Cas9 system to edit DNA *in vivo*[21] (Figure 1). Without donor DNA, all types of Cas9 can participate in NHEJ. However, HDR is possible via Cas9 with the addition of donor DNA, which has additional encapsulation challenges due to the large size and highly charged nature of DNA.

Plasmids are used to deliver Cas9, by integrating the protein into their structure.[9] They are cost-efficient and have higher stability than messenger RNA (mRNA) and sgRNA. However, the efficiency of gene editing is restricted by the vector's ability to reach the nucleus of the target cell, which means passing through the cell and nuclear membranes.[10,22,23] Plasmids can be constructed quite easily and can be modified, which makes them a good vector for Cas9.[24] One study found that utilizing plasmids to deliver payloads could lead to longer expression times *in vivo* because plasmid DNA is relatively stable.[22] However, increased expression time could also lead to harmful immune responses because plasmids can carry bacterial expression promoters which do not interact well with mammalian cells.[24] Another study found that a plasmid-based system is more stable than a combined system of just Cas9 mRNA and sgRNA, due to the poor stability of mRNA.[23]

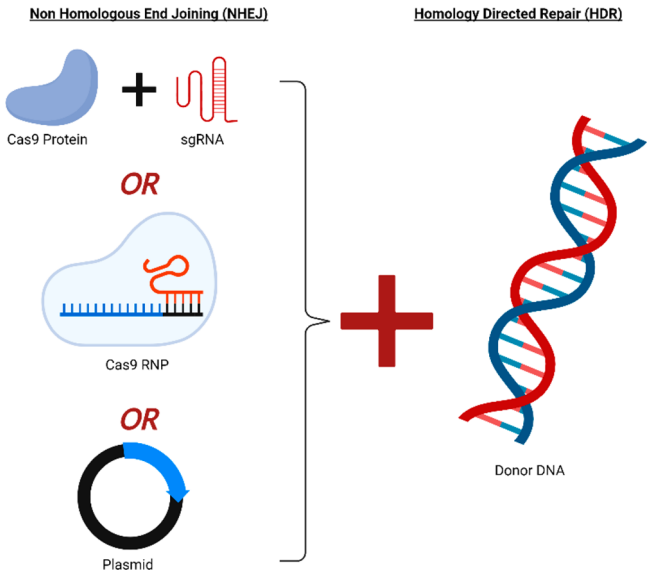

Figure 1. Types of Cas9 encapsulated in nanoparticles for delivery and their corresponding gene repair mechanisms. All types can lead to NHEJ after double-strand breaks, but the codelivery of donor DNA allows for HDR to occur. Figure made with Biorender.

One issue with using plasmids for *in vivo* studies is they begin gene editing slower than proteins and mRNA because the Cas9 plasmid DNA must be first transcribed into mRNA to begin the process, which may slow down treatment time.[22,24] Furthermore, delivery of plasmids with Cas9 DNA tends to experience off-targeting more than other delivery methods that directly deliver the Cas9 protein.[22−24] Plasmid DNA has also been found to have a half-life of around 10 min when injected into mice, which likely does not leave enough time for the payload to reach the nuclei of target cells to begin editing.[24] Although plasmids have been explored as favorable vectors for CRISPR-Cas9 delivery, the possibility of a negative immune response and slow gene editing presents considerable challenges for use. The benefit of delivering Cas9 in an RNP form or using mRNA is that it only needs to be delivered to the cytoplasm, as this is where translation will occur, instead of DNA methods which requires nuclear delivery, which can be extremely challenging.[5] All types of Cas9 (Figure 1) have effectively been encapsulated and delivered using nanoparticles with various materials, discussed in detail below.

3. DESIGN OF CAS9-ENCAPSULATING NANOPARTICLES

3.1. Material Selection. A major makeup of the NP system is the material. When selecting effective materials for encapsulation and delivery of Cas9, it is important to analyze the large potential parameter space (Figure 2) with the end goal in mind: a delivery vehicle to encapsulate Cas9 and decrease any potential detrimental interactions between the material makeup of the NP system and the protein. Polymeric systems are currently being outpaced by lipid-based systems with respect to translation to the clinic, as observed with the availability of Doxil since the late 1990s and now the COVID-19 vaccine[25] (Figure 3). Despite this fact, polymers have a greater translation potential in the encapsulation and delivery of targeted gene-editing technology. Gene-editing tools, such as CRISPR-Cas9, need to be precisely delivered to the target site to minimize off-targeting effects. Lipid-based systems are

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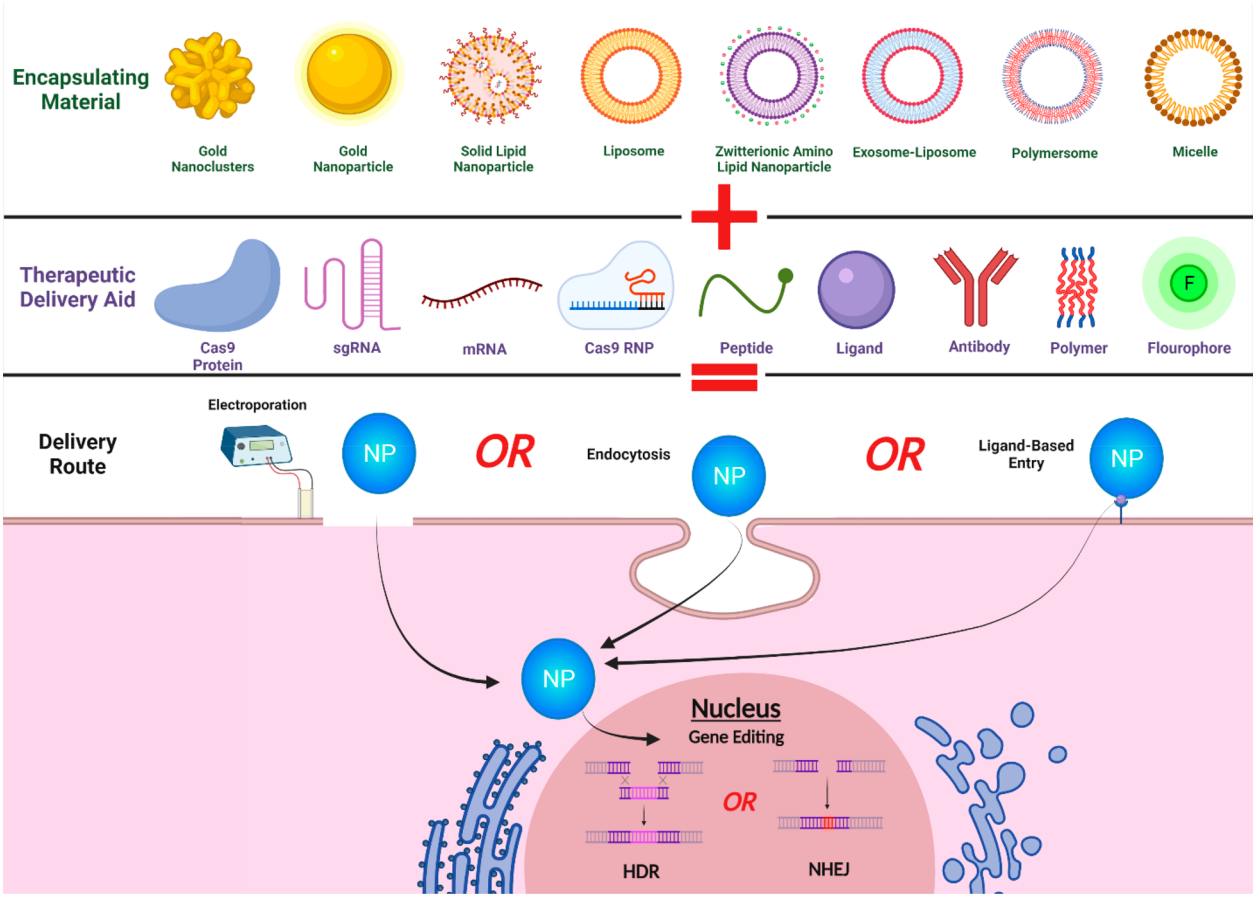

Figure 2. Schematic of the large parameter space available when designing Cas9-encapsulating nanoparticles. Delivery of Cas9 requires special consideration of encapsulating material, with many options available and being explored preclinically, highlighted above. Attention needs to be paid to therapeutic payloads, where Cas9 protein and the multiple RNAs required can be delivered separately or together in an RNP. Simultaneously, targeting ligands, peptides, or antibodies are needed to reach the intended target. Additional polymer can be incorporated to increase the stability and circulation time, and fluorophores can be incorporated for nanoparticle tracking. Finally, material selection and therapeutic delivery aids can dictate the delivery route into the cell, where electroporation can be used in culture and endocytosis, and ligand-based entry can be used in cell culture and *in vivo*. Finally, the nanoparticle needs to be able to enter the nucleus, where gene editing can occur. Figure made with Biorender.

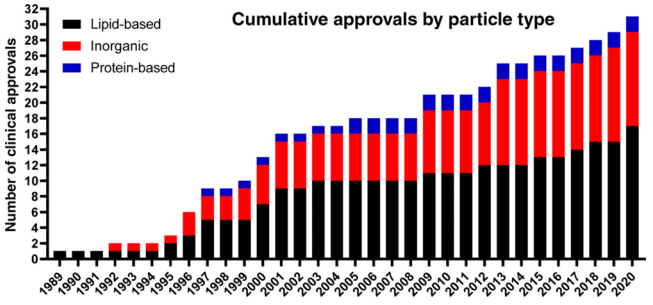

Figure 3. Nanoparticle approvals by material type. Republished with permission from Bioengineering & Translational Medicine, First published: 08 August 2021, DOI: 10.1002/btm2.10246.[25] CC by 4.0.

notoriously leakier than polymer-based systems,[26] which could be detrimental to the ultimate health of the patient if payloads are released at unintended sites. Polymer-based systems could benefit from understanding the successful nanoparticle delivery vehicles created for Cas9 thus far. Integrating this knowledge with stimuli-responsive, stable polymer-based nanoparticles could build toward translatable nanotechnology. This section will examine the prominent nonviral vectors to transport Cas9, highlighting the benefits and drawbacks of each, also summarized in Table 1.

Gold Nanoparticles (AuNPs). Although gold nanoparticles (AuNPs) do not encapsulate Cas9 for delivery, they have been the most used transport vector thus far and warrant comparative discussion to encapsulation techniques. AuNPs are inorganic, modifiable, biocompatible, and versatile.[27,28] Researchers can dictate the size of the NPs, which drives the ultimate fate of the AuNP, with small NPs being easily taken up by the kidneys and larger NPs being processed by macrophages and the liver.[29−32] AuNPs also can be easily conjugated with molecules that allow for easy click addition of targeting peptides to direct delivery and fluorescent molecules to track delivery.[31,33] Furthermore, AuNPs can be modified with targeting ligands to cross the blood−brain barrier (BBB), which makes delivering the Cas9 protein to brain tissue possible, as shown in Table 1.[31,34] However, naked AuNPs can be endocytosed into cells, so additional modifications are not required.[35] Another significant benefit is that AuNPs can have proteins and nucleic acids bound to the surface, which allows for more diverse loads.[34,36] Glass et al. note that AuNPs can deliver small drug molecules like DNA and RNA, as well as large molecules, like proteins.[37] Pissuwan et al. found that AuNPs may be capable of binding photoactivated ligands to their surface, thus allowing for photoactivated drug release.[33] Additionally, Ding et al. describe AuNPs as tunable vectors for

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Table 1. Materials Used as Delivery Vehicles for Cas9 Thus Far[a]

material type		general properties	benefits	drawbacks	citations
gold		inorganic; modifiable; biocompatible; versatile; visible in imaging applications	•Click-addition to direct delivery	•Binding of free Cas9 can be unstable	27−41
			•Track delivery through fluorescence	•Toxicity of size and ligands can cause cell death in vivo	
			•Can be endocytosed into cells	•Expensive therapeutic vehicle	
			•Can possibly bind photoactivated ligands to surface	•Tissue clearance dependent on size	
			•More diverse loads		
			•Can maintain multifunctional surfaces		
			•Versatile chemistry for binding of Cas9 RNP		
			•AuNPs using Donor DNA and DNA-Thiol led to increased binding stability		
			•AuNPs can form AuNCs, which encapsulate Cas9 due to charge−charge interactions		
lipid	solid lipids	easy production; limited immune response; tunable design; large carrying capacity	•Easy to manufacture	•Solid nature can lead to gelation or capillary blockage	37, 42−45, 101
			•Modifications allow for larger payloads	•low encapsulation of hydrophilic drugs	
			•Fundamental properties well-known	•crystallization during storage	
	liposomes	biocompatible, somewhat tunable structure	•Bilayer allows hydrophilic and/or hydrophobic drug encapsulation	•Injecting PEGylated liposomes many times can lead to accelerated blood clearance reducing the effects of treatment over time	9, 52−54, 66
			•Positively charged liposomes can cross cell membrane	•None of the Food and Drug Administration (FDA) approved liposomal drugs have demonstrated higher patient survival as compared to free drugs	
			•Positively charged liposomes with polyethylene glycol (PEG) can deliver CRISPR/Cas9 in vivo	•Weak stability can result in lower circulation time in vivo	
			•16 liposomal drugs approved for human use	•leakiness and loss of therapeutic payloads	
	zwitterionic amino lipids	organic tunable charge	•Co-deliver long RNAs with Cas9 mRNA (mRNA) and sgRNA	•Inability to optimally co- deliver mRNA and sgRNA, due to nucleic acids' kinetic differences	27, 44, 56, 57
			•Bound to PEG have properties that allow to evade immune responses in vivo, increasing possible circulation time	•High charge can affect uptake in cells	
			•Safely deliver payloads *in vivo*		
	exosomes/liposomes	size due to hybridization; high stability	•Endocytosed by mesenchymal stem cells	•Liposomes are much larger than exosomes and reduce trans-membrane delivery as they cannot cross as many barriers as their counterpart	56−59
			•Exosomal markers	•Synthetically created liposomes have different compositions than exosomes, which has possibility of producing cytotoxic and immunogenic responses	
			•Cross crucial barriers		
plasmid		cost-efficient higher stability than mRNA and sgRNA	•Constructed easily and can be modified to fit parameters	•Efficiency of gene-editing restricted by vector's ability to reach the nucleus of target cell	9, 10, 22−24
			•Could lead to longer expression times in vivo as plasmid DNA is relatively stable	•Increased expression time could lead to harmful immune responses, since plasmids can carry bacterial expression promoters which do not interact well with mammalian cells	
			•Plasmid-based system is more stable than a combined system of Cas9 mRNA and sgRNA, due to poor stability of mRNA	•Begin gene-editing slower than proteins and mRNA since Cas9 plasmid DNA must be first transcribed which may slow treatment time	
				•More off-targeting	
				•Plasmid DNA found to have a half-life of ∼10 min when injected into mice, which may not leave enough time for payload to reach nuclei of target cells	
polymer	polymersomes	amphiphilic self-assembled	•Hydrophobic and hydrophilic regions for binding	•Biocompatibility of the material must be considered	14, 26, 60, 71−73, 102

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Table 1. continued

material type	general properties	benefits	drawbacks	citations
		•Can be adjusted to desired size with hydrophobic block molecular weight	•Been in use for less than a decade	
		•Thickness of bilayer allows for higher physical and chemical stability		
		•Meant to mimic structure and function of organelles		
		•High stability and low fluidity		
		•Long circulation time		
		•Ability for controlled release		
		•Tunability		
		•Can be synthesized with an outer shell which helps avoid RES and protects payloads		
micelles	versatile biocompatible	•Hydrophobic core and hydrophilic shell allow encapsulation of hydrophobic materials and outer-shell binding	•Not as tunable	74, 75, 77, 94, 95, 103
		•Hydrophilic shell reduces nonspecific uptake of NPs by the RES, which can increase circulation time	•Synthesis material determines diffusion and editing efficiencies	
		•Small enough to avoid filtration in kidneys	•sgRNA loaded alone was less stable than coencapsulated sgRNA with Cas9	
		•Versatility	•One study mentioned not significant E7 inhibition when NgAgo present	
		•Can contain stimuli-sensitive agents that allows controlled drug release		
		•Exterior shell allows for location targeting and fluorescence labeling		
		•Positive relationship between mRNA and its codelivered sgRNA		

[a]This table presents a summary of properties of each type of drug delivery system discussed in the above text. It also provides benefits and drawbacks for each system.

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nucleic acids because they can maintain multifunctional surfaces which allow for multiple payloads, such as targeting agents and drugs to bind simultaneously.[36]

Despite their potential benefits, the modification of the AuNPs can cause the binding of Cas9 to be unstable.[32] Lee et al. investigated the binding tendencies of Cas9 RNPs to unmodified AuNPs and found that the adsorption was possible but temporary as it did not survive several rounds of washing.[35] On the other hand, the group notes that binding Cas9 RNP to AuNPs, using DNA−thiol to complementarily attach donor DNA, led to increased binding stability. This allowed them to delete targeted genes in Ai9 mice in which successful deletion of a gene leads to expression of red fluorescent tdTomato protein after intramuscular injection.[35] From this, one can deduce that different modifications made to the AuNPs can have varying results in terms of Cas9 binding stability.

Likewise, AuNPs can also cause cell toxicity, depending on their size and attached ligands. Sizes ranging from 5 to 10 nm tend to accumulate in organs and are difficult to be metabolized by the liver and kidney.[32,38] When looking at 5 and 10 nm PEGylated AuNPs, there was a decrease in white blood cells, indicating an inflammatory response.[32,38] Although AuNPs are thought to be nontoxic vectors for drug delivery, one group found that 10 and 60 nm AuNPs demonstrated higher cell toxicity than those of sizes 5 and 30 nm.[38] Other studies note the importance of the ligands bound to the AuNPs.[32,39] Specifically, the toxicity of cationic ligands *in vitro* could lead to cell death *in vivo* if bound to AuNPs.[32] Due to the lack of concrete toxicity classifications of different-sized and ligand-bound AuNPs, their effect on cell death cannot be concluded. Although AuNPs have been the most extensively explored delivery vehicle for Cas9 RNPs thus far, the surface binding of Cas9 RNP is not ideal and could lead to a loss of expensive therapeutic payload. Because of this, delivery tools that have the potential to encapsulate and protect Cas9 have begun to be explored.

A novel system made up of AuNPs and gold nanoclusters (AuNCs) has been used to encapsulate and deliver Cas9. Ju et al. created a AuNC delivery vehicle with diameters of ∼104 nm made up of gold and Cas9 that cluster together in a pH 7.4 environment, mimicking physiologic pH. In an acidic environment of pH 4.5, AuNCs begin to fall apart due to partial protonation of surface carboxylic groups, leading to release of the Cas9. Upon incubation with AuNC−Cas9 (ratio 20:1) and sgRNA designed to knockout E6 oncogene, 13.81 and 19.3% of HeLa cervical cancer cells were induced with early and late-stage apoptosis, respectively. Left unclear is information on precisely how sgRNA was codelivered with AuNC−Cas9, leaving open the question of potential systemic codelivery, where free sgRNA would not have a sufficient half-life for independent therapeutic delivery.[40] Another study looks at codelivery of gRNA with Cas9 in plasmid form via AuNCs, which facilitates nuclear delivery. Protamine-capped AuNCs (d = 1.56 nm) exhibited a positive charge, which allowed for complexation with negatively charged DNA and effective delivery into U2OS human osteosarcoma cells. When using U2OS.GFP cells and delivering Cas9 and $gRNA_{EGFP}$, an indel rate of 29% was observed, indicating effective gene editing.[41] Together, these papers show the potential of AuNCs to combine the beneficial properties of AuNPs with the protection provided by encapsulation. Major concerns are still associated with the use of gold in NP formulations, including their potential toxicity and concerns regarding clearance from the target tissue.

Lipid Nanoparticles. Lipid NPs (LNPs) are popularly used vectors for drug delivery because they have many desirable properties, such as easy production, little to no immune response, tunable design, and large carrying capacity.[42] Lipid NPs are made of components ranging from fatty acids and phospholipids to solid lipids, and they are easy to manufacture.[43,44] Furthermore, the size of LNPs can be modified to allow for larger payloads, such as Cas9.[43] Another attractive factor to consider is that lipid-based drug delivery systems have been well studied, so their fundamental properties are thoroughly known.[42] Additionally, many studies exploring lipid NPs delivering Cas9 RNP have been published, as highlighted in Table 1, demonstrating potential applicability as drug delivery vehicles after further studies in large animal models and clinical trials.[37]

In a study performed by Mullard et al., CRISPR-Cas9 was encapsulated with an RNA construct inside of a lipid nanoparticle, known as NTLA-2001. These NPs were introduced to patients with ATTR amyloidosis, which causes an abundance of the transthyretin protein (TTR) to build up in specific tissues including the heart, nerves, and digestive system. CRISPR-Cas9 with RNA construct was able to introduce frameshift mutations into TTR in liver cells *in vitro* to inhibit production of the mutated protein.[45] In the phase 1 clinical trial, data reported by Gillmore et al., six patients were treated with a single infusion of either 0.1 mg or 0.3 mg per kg of NTLA-2001, and by day 28, their TTR serum levels had fallen by 52% on the low dose and by 87% on the higher dose with minimal side effects. This treatment study is currently looking into mouse and monkey models and recruiting patients for a higher dose to further study the long-term effects and safety for human patients.[46] Although this trial utilizes one type of lipid NP, there are several other kinds that have shown promise. Also targeting TTR as an amyloidosis treatment, Finn et al. developed a biodegradable lipid that they integrated into a PEG-supported lipid nanoparticle called LNP-INT01. Co-encapsulated Cas9 mRNA and sgRNA designed for the mouse (TTR) gene in LNP-INT01 were injected through the tail vein of female CD-1 mice. A single injection led to robust gene editing effects, with >97% knockdown of serum TTR levels that remained stable for 52 weeks. This likely resulted due to the ∼72% DNA editing observed across the liver, indicating the trafficking of LNP-INT01 to the liver while maintaining Cas9 mRNA and sgRNA activity.[47] These initial results indicate that CRISPR-Cas9 can edit cells to treat genetic disorders when placed in lipid NPs.

In a study performed by Yin et al., a specific Fah mutation in mice was able to be repaired using lipid nanoparticles to deliver Cas9 mRNA paired with sgRNA through the mechanism of HDR. A vector was designed using an adeno-associated virus (AAV) and an HDR template which contained a 1.7 kb sequence homologous to the Fah genomic region that was being targeted. Cas9 mRNA nanoparticles (nano.cas9) were delivered with the AAV-HDR to prove that the Fah mutation was corrected and showed Fah splicing was restored in more than 6% of hepatocytes in the adult mouse liver. Overall, this treatment was well-tolerated in the mouse models and fully rescued body weight loss and liver damage in tyrosinemia mice. It was also noted that the efficiency of gene editing depends on the dose of Cas9 mRNA as well as sgRNA and that

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further improvement of HDR efficacy may be seen by optimizing the HDR template design and inhibition of the NHEJ pathway.[48] This shows that LNPs are capable of encapsulating template DNA with Cas9, allowing for participation in gene correction. LNPs are also capable of being specifically targeted to disease sites.

Successful HDR has also been seen in a recent study performed by Farbiak et al. where Cas9 mRNA, single-guide RNA, and donor DNA were encapsulated in dendrimer-based lipid nanoparticles (dLNPs). The success of editing was quantified and found to edit >91% of all cells with 56% HDR efficiency *in vitro* and >20% HDR efficiency in xenograft tumors *in vivo*. In some genetic disorders HDR is a preferred approach, as NHEJ could get rid of previously existing activity in partially active proteins. Instead, double-strand breaks (DSBs) induced through HDR can be repaired when it is close to an endogenous DNA sequence which is typically known as donor DNA. Because of this, HDR has more requirements than NHEJ systems, leading to its limited advancement for future studies. However, Farbiak et al. showed that RNPs can find a target sequence within DNA where the sgRNA can bind using antiparallel complementarity and can then cleave the DNA, inducing a DSB. The ssDNA HDR donor template is then copied and added into the genomic DNA. This dLNP system was able to be optimized by using degradable, ionizable dendrimer-based lipids that can be positively charged at low pH so that RNAs bind during self-assembly. At neutral pH these lipids are uncharged to reduce toxicity but able to be positively charged again at the maturing endosome pH to facilitate endosomal release which has contributed to its success.[49]

The effect of small changes to lipid structure making up LNPs is highlighted in a novel selective organ targeting (SORT) approach, where the addition of permanently cationic lipids to 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)-based LNPs (concentrations of 5 to 100% of total lipids) targets delivery of mRNA or Cas9. SORT LNPs were designed to gene edit phosphatase and tensin homologue (PTEN), as a tumor suppressor, with encapsulated Cas9 mRNA and sgRNA for PTEN. SORT LNPS with 20% DOTAP led to 13.9% indels in the liver but not at all in the lung or spleen, while 50% DOTAP led to 15.1% indels in the lung exclusively.[50] Another study by this research group highlights the effectiveness of CRISPR-Cas9 delivery to targeted organs using multicomponent lipid nanoparticles (iPLNPs). iPLNPs are composed of multitailed ionizable phospholipids (iPhos), *N*-methyldioctadecylamine, cholesterol, and 1,2-dimyristoyl-*rac*-glycero-3-methoxy (PEG-2000) (25:30:30:1 mol/mol). Cas9 mRNA and sgRNA were codelivered using different lipid configurations of 9A1P9-5A2-SC8 and 9A1P9-DDAB iPLNPs in Ai9 mice to activate the tdTomato protein and investigate whether the iPLNPs could be used for SORT. 9A1P9-5A2-SC8 iPLNPs were found to gene edit in the liver tissue by confocal fluorescence microscopy, while 9A1P9-DDAB iPLNPs enabled gene editing in the lungs, demonstrating the system's ability to gene edit via SORT.[51] This SORT approach is promising toward more targeted delivery of Cas9, limiting off-targeting DNA editing effects that can be detrimental to human health over time.

One type of self-assembled lipid NP is the liposome. Liposomes are artificial vesicles formed in aqueous solution by amphiphilic lipids spontaneously creating a lipid bilayer.[9,52] They are popularly studied as delivery vectors for CRISPR-Cas9.[53] These NPs are composed of a bilayer to allow for hydrophilic and/or hydrophobic drug encapsulation.[9] Furthermore, positively charged bilayers could allow for the encapsulation of negatively charged nucleic acids, such as mRNA.[9] These positively charged liposomes can also cross the negatively charged cell membrane.[9] A potential drawback of using liposomes is their weak stability, which could result in lower circulation time *in vivo*.[9,52] However, this can be slightly mediated through the addition of polyethylene glycol (PEG), a polymer commonly added to increase circulation times of liposomes.[54,55] Zhen et al. note that positively charged liposomes with PEG can deliver CRISPR-Cas9 *in vivo*.[53] The location of delivery is not specified. However, injecting PEGylated liposomes multiple times can lead to accelerated blood clearance of the NP, which reduces the effects of the treatment over time.[53] Therefore, there need to be more studies conducted regarding PEGylated liposomes to avoid this phenomenon.

Zwitterionic amino lipids (ZALs) can be used to make organic-lipid-based NPs that can codeliver long RNAs with Cas9 mRNA and sgRNA.[27,44] ZALs are charged liposomes with zwitterionic sulfobetaine head groups.[27] These ZAL nanoparticles (ZNPs) bound to PEG have properties that allow them to evade harmful immune responses *in vivo*, thus increasing possible circulation time.[28,44] A study done by Miller et al. found that CRISPR-Cas9 gene editing was enabled by codelivering Cas9 mRNA and sgRNA *in vivo* using ZNPs.[27,56,57] The team observed the expression of tdTomato in the kidneys, lungs, and liver of their engineered mice due to the intravenous delivery of ZNPs, although a specific target was not mentioned.[27] A drawback of utilizing ZNPs is their inability to optimally codeliver mRNA and sgRNA, due to the nucleic acids' different rates of reactions which result in the RNAs being effective at varying time intervals.[27] The team notes that mRNA takes a longer time than sgRNA to induce change as it is longer and needs to be translated.[27] Consequently, Miller et al. concluded that effective gene editing would require the ZNPs to contain a ratio equal to or higher than 3:1 of mRNA:sgRNA.[27]

Researchers have also explored hybrid lipid NPs such as exosomes−liposomes. Exosomes are small vesicles secreted by cells and have a high enough stability to function in different body fluids.[57−59] Furthermore, they are small enough to avoid phagocytosis and can deliver nucleic acids, lipids, and proteins.[57,59] They can also cross the BBB and placental membrane because they contain the same membrane of the cells they were excreted from, which means they contain native proteins and do not require any extra modifications.[57,59] Additionally, exosomes can stay in circulation long enough to deliver target drugs and can cross through the vascular endothelium.[59] However, as a consequence of their small size, exosomes cannot encapsulate large molecules such as Cas9, motivating the creation of exosomal−liposomal hybrid lipid NPs.[56,58,59] Lin et al. report that the incubation of exosomes with liposomes for 12 h led to an effective combination based on analysis of size distributions of the hybrid NPs and Western blots, which showed traces of exosomal markers in the hybrid NPs.[59] These hybrid vectors were able to deliver CRISPR-Cas9 and also be endocytosed by mesenchymal stem cells.[59] However, utilizing hybrids has some drawbacks. For example, liposomes are much larger than exosomes, causing hybrids to have reduced trans-membrane delivery because they cannot cross as many barriers as independent exosomes.[58] Further-

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more, synthetically created liposomes have different compositions than exosomes, which have the possibility of producing cytotoxic and immunogenic responses.[59]

There has been much progress made in developed liposomal drugs that can be delivered *in vivo*. In their 2019 publication, Mukherjee et al. note that 16 liposomal drugs have been approved for human use.[9] However, none of the Food and Drug Administration (FDA)-approved liposomal drugs have demonstrated higher patient survival when compared to the free drugs.[9] Although LNPs have their own set of advantages, such as exosomes−liposomes being able to cross crucial barriers, they also have setbacks, such as liposomes having low circulation times, that need to be further studied to overcome. Due to this, vectors that can cross essential barriers and maintain high circulation times have been gaining interest as potential carriers for Cas9 delivery systems. It is also important to note that clinically successful lipid NPs typically involve the use of polymer PEG to increase circulation half-life and *in vivo* stability.

Polymer Nanoparticles. Here, we specifically discuss self-assembled polymeric nanoparticles that can be created through the self-assembly of amphiphilic copolymers and allow for encapsulation of hydrophobic and hydrophilic drugs. However, some polymeric NPs may be synthesized using hydrophobic polymers alone. Furthermore, these self-assembled polymer nanoparticles can aggregate into different structures, including polymersomes and micelles, which can serve various purposes depending on the design.[60] Various targeting ligands can be attached to the exterior using modular bioconjugation approaches, enabling targeted delivery.[52,57] These NPs are gaining popularity because they can effectively protect payloads and stabilize DNA.[57,61,62] Additionally, some of these self-assembled polymer NPs have the potential for sustained drug release and long circulation time due to their core−shell structure, which make them desirable vectors for drug delivery *in vivo*.[52] Polymeric NPs can also avoid triggering immune responses in the body when the surface is comprised of PEG, which is hydrophilic and has low toxicity.[28,39,43,44] Polymer NPs have some clear categorical benefits, and due to the wide variety of polymeric materials available, they can also be created to respond to specific disease conditions.

Synthetic polymers can be selected that respond to various relevant biologic stimuli that can be associated with pathophysiologic changes: pH-responsive, enzyme-responsive, temperature-responsive, and redox-responsive polymers fall into this category. Synthetic polymers can also be selected to respond to external stimuli that can be applied to a target area, including ultrasound and light.[60,62−66] Natural polymers such as chitosan, fibrin, and alginate have their own attractive qualities including being abundant, cheap to obtain, and easy to alter.[67−69] Chitosan is a derivative of chitin, which is one of the most abundant polysaccharides.[68] This polysaccharide is nontoxic, cheap to obtain, biocompatible, and biodegradable.[27,39] Fibrin is a monomer of fibrinogen, which plays a prominent role in coagulation in the body during wound healing and has been used for delivery of payloads *in vivo*.[45] It demonstrates slightly acidic properties under specific conditions for certain applications and can interact with positively charged proteins.[60] Alginate is a negatively charged polysaccharide that is biodegradable and biocompatible, making it applicable for *in vivo* deliveries.[46] Due to its preparation in moderate conditions, alginate NPs can be loaded with proteins that are sensitive to heat.[60] Furthermore, alginate has been shown to be able to protect proteins and peptides in the stomach's acidic conditions.[46]

Polymers can be used as a coating that encapsulates Cas9 in a deliverable, effective form, providing protection. In 2019, Chen et al. reported the development of nanocapsules (NC) with a diameter of 25 ± 6 nm made from a glutathione-cleavable cross-linked polymer that encapsulated Cas9 RNP. When injected in Ai14 mice using two local routes of administration, gene editing was observed. When NCs were bound to ATRA, a known ligand for a major protein that traffics to the retinal pigment epithelium and injected intraocularly, a significant increase in fluorescence expression was observed and quantified as an increase in fluorescent area to >4% of the tissue. Ai14 mice were also injected with NCs intramuscularly, which had a less dramatic gene editing effect, quantified by a >2% fluorescence expression in the injection site. Clearly, this paper demonstrates that local administration of encapsulated Cas9 can lead to gene editing, but the paper was lacking in quantification of *in vivo* gene edits.[70] Synthetic and natural polymers have been used to create self-assembled polymer NPs including polymersomes and micelles.

Polymersomes are NPs created through the self-assembly of block copolymer amphiphiles with a hydrophilic fraction between 25 and 40%, which allows them to have hydrophobic and hydrophilic regions for drug loading.[50−53] The structure of polymersomes consists of an aqueous interior and brush and a polymer bilayer that can be adjusted to a desired size through adjustment of the hydrophobic block molecular weight.[14,71] The polymers utilized in polymer synthesis can be synthetic or natural, as stated above; therefore, the biocompatibility of the material must be considered.[14,26] Although polymersomes have been in use for less than a decade, they are still a popular method for drug, protein, and nucleic acid delivery.[14,26,71−73] They demonstrate several useful properties for use *in vivo*, such as higher stability and lower fluidity than liposomes due to their bilayer membrane, long circulation time due to minimal nonspecific adhesion to cells, and the ability for controlled release due to their low permeability.[14,26,71,72] Another benefit of utilizing polymersomes is their tunability as a result of their synthesis using block copolymers; their structure, size, surface properties, and function can be adjusted to fit the desired parameters.[14,26,71,72] Polymersomes can also be synthesized with an outer shell which helps them avoid the RES and protects their payloads.[60] Despite the potential, there are currently no studies about polymersomes delivering Cas9 *in vivo*.

Abbasi et al. created polyplex micelles with PEG and poly(*N*′-(*N*-(2-aminoethyl)-2-aminoethyl) aspartamide), in which they were able to encapsulate Cas9 mRNA and sgRNA. The presence of PEG was important for proper diffusion in the brain tissues after intracranial injection; therefore the materials used for the synthesis of micelles must be considered for efficient gene editing to take place.[74] They found that loading the Cas9 mRNA and sgRNA together actually improved the stability of the sgRNA when compared to polyplex micelles loaded with sgRNA alone. The increased stability is attributed to the presence of two RNAs taking up more space than a single RNA.[74] Furthermore, the team injected the polymeric micelles in the frontal lobe of Ai9 mice and found that the polyplex micelles with Cas9 mRNA and sgRNA enabled the most gene editing as observed through the expression of the tdTomato protein in neurons, astrocytes, and microglia, which demonstrates the translatability of their

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micelle−Cas9 system.[74] The qualitative data were collected through microscopy and were quantified by calculating the area of pixels fluorescing red, indicating tdTomato expression.[74]

Tan et al. created three polycationic micelles composed of poly[ethylene oxide-*b*-2-(dimethylamino) ethyl methacrylate-*b*-*n*-butyl methacrylate] (PEO-*b*-PDMAEMA-*b*-PnBMA) with increasing PEO lengths from 5 to 10 kDa and a fourth sample with no PEO.[75] These micelles were complexed with the Cas9 protein and gRNA.[75] Cas9 and gRNA were successfully loaded into the micelle polyplex NPs, demonstrated by dynamic light scattering (DLS) and cryogenic electron microscopy.[75] They also noted that the micelles with PEO length of 10 kDa performed the best gene editing in an engineered HEK 293T TLR cell line (human embryonic kidney cells) when compared to the other micelles.[75] The addition of cationic charges to polymeric NPs is beneficial in aiding cellular penetration, as was observed by Luo et al. Cationic lipid-assisted PEG-*b*-poly(lactic-*co*-glycolic) (CLAN) nanoparticles formed via film rehydration effectively delivered Cas9 plasmids *in vitro* to macrophages and monocytes, observed via Western blot. CLAN encapsulated with Cas9 plasmid with an additional sgRNA sequence targeting Ntn1 was injected through the tail vein of C57BL/6 mice. This led to an indel frequency as high as ∼30% in monocytes and macrophages specifically, indicating the *in vivo* translatability of this polymer NP technology.[76]

Lao et al. developed micelles made from thermoresponsive Pluronic F127 and quaternary ammonium-terminated poly(propylene oxide) (F127/PPO-NMe_3) micelles to deliver Cas9 plasmids and a gene-editing enzyme *Natronobacterium gregoryi* Argonaute (NgAgo) to inhibit the human papillomavirus (HPV) E7 oncogene in HeLa cells. They note that the micelles effectively delivered the Cas9/NgAgo and hindered the HPV18-E7 oncogene *in vivo* and *in vitro*, thus slowing the progression of HPV-linked cancer.[77] Although the micelles show promise, the team mentions that there was not significant E7 inhibition, which they attributed to the presence of NgAgo.[77] The publications above have proven micelles to be capable of encapsulating/complexing and delivering Cas9 *in vitro* and in some cases *in vivo*. From their versatility to biocompatibility, micelles have great potential as Cas9 vectors.

Polymer NPs can also be used in HDR-type editing. Nguyen et al. have discovered ways in which HDR templates can be modified to improve their efficiency. For example, truncated Cas9 target sequences (tCTSs) were added to the ends of the HDR template which interacted with the RNPs to help transport the template to the nucleus. Also, stabilizing Cas9 RNPs into nanoparticles with polyglutamic acid not only enhances gene editing but also reduces toxicity and enables lyophilized storage without loss of activity. A combination of these two modifications was shown to improve gene-targeting efficiency even at reduced HDR template doses tested in various cell types. In the end, improvements to HDR efficiency were found to be dependent on the use of nuclear localization sequences in the Cas9 RNP, along with the use of gRNA and preincubation of the Cas9−NLS RNP with the tCTS-modified HDR template to prove enhanced genome targeting efficiency without requiring modification of the protein or gRNA itself.[78]

The papers discussed in this section demonstrate how the use of polymeric materials to encapsulate Cas9 provides the benefits of chemical versatility, decreased leakiness, loss of payload, and increased circulation time.[14] However, the self-assembled polymeric material field is plagued by a lack of high-quality scaleup production practices, limiting the number of particles that can be created for any given system.[79]

3.2. Internal Environment. Another crucial aspect to examine when encapsulating Cas9 is the internal environment within the NPs (Figure 2) because the integrity and functionality of the protein must be maintained throughout delivery. As shown in Figure 2, different molecules can be coencapsulated with Cas9; therefore, their interactions should be studied to avoid degradation. Ideally, the environment should not degrade Cas9 or any other protein/nucleic acid in the NP to ensure safe delivery. The pH of the buffer and solvents used for NP formation and encapsulation have a clear effect on Cas9 stability and behavior. Wei et al. examine the benefits of utilizing phosphate buffer saline (PBS) and ethanol when making NPs for Cas9 delivery.[21] Using dialysis against PBS which has a neutral pH, NPs are able to self-assemble without an extreme pH.[21] The study suggests that using neutral buffers instead of acidic buffers in the protocol for producing NPs will prevent the breakdown of the Cas9 protein.[21] Furthermore, Wei et al. found that Cas9 RNP loses its structure in acidic buffers, which increases the molecular size of the complex from 10 nm to around 150 nm and makes it difficult to encapsulate.[21]

Another major factor to consider with NP preparation for Cas9 delivery is storage temperature. A company that sells the Cas9 protein, Integrated DNA Technologies, notes that the molecule should be stored at −20 °C to maintain functionality.[80] They note that storing the protein at such a low temperature slows degradation and allows it to be functional for at least 2 years.[80] A few studies determined Cas9 proteins acquired from different bacteria would have different thermal limits.[81,82] The Cas9 protein from *Streptococcus pyogens* bacteria was characterized as not being functional at or under 42 °C.[81] On the other hand, researchers found that the Cas9 protein found in *Geobacillus thermodenitrificans* is functional between 20 and 70 °C.[81] Depending on the origin of the Cas9 protein and its modifications, the protein could likely be functional at different temperatures and environments. Currently, there is a literature gap regarding the optimal conditions for Cas9.

As a result of researchers already gathering data on the Cas9 protein functionality and forging ahead to clinical applications, they have not identified the fundamental conditions in which Cas9 can function. This could ultimately be detrimental when translating to clinical application, as storage and solvent conditions are of pivotal importance as seen with the COVID-19 vaccine.[53] There is limited information about the storage of the Cas9 protein and the buffers it can exist in. Consequently, one may look to general protein storage conditions to apply to Cas9. For short-term storage (2−4 weeks), proteins can be kept at 4 °C in a simple buffer solution.[83−85] For longer-term storage (1 year), proteins can be stored at −20 °C in 25−50% glycerol, ethylene glycol, simple phosphate, or Tris buffers.[83−85] All of these solutions have pH values near 7, which indicates they are neutral.[62,86−89] It is also known that proteins should not be exposed to hypertonic salt solutions for long periods of time, as plasmolysis could lead to damage.[90] Future studies on the environment needed to maintain Cas9 structure and function are of pivotal interest to the field when translating to clinical nanoparticle-based applications.

3.3. Charge−Charge Interactions. Like many proteins in the body, Cas9 and its encapsulating NPs interact with

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different molecules that possess various charges and can vary depending on the route of administration. The magnitude of the charges can impact the effectiveness of the Cas9 protein and a NP's ability to enter the cell membrane and nucleus to release their payload.[91−93] The Cas9 protein must remain functional to ensure gene editing takes place, so analyzing its surrounding charges is essential.[27,93]

Internal Interactions. Encapsulated Cas9 interacts with its delivery mates, such as sgRNA or mRNA and the NP. Therefore, to ensure the safe and effective delivery of the Cas9 protein, its charge−charge interactions with its codelivered molecules and encapsulating NPs must be examined to prevent the breakdown of the molecule or loss of functionality. Further research must be conducted to clarify Cas9 stability when in contact with different material charges, internal and external.

Since sgRNA or mRNA are frequently codelivered with Cas9, ensuring the RNA does not have adverse effects on the protein's function is crucial.[93] Although the Cas9 protein is positively charged, when complexed with negatively charged sgRNA to make Cas9:sgRNA RNPs, the overall structure has a negative charge.[93] Cationically charged particles have an easier time crossing the lipid membrane than anionically charged particles.[91,92] Consequently, the Cas9:sgRNA RNP has a low independent potential to cross the cellular membrane, making an encapsulation approach more promising for therapeutic efficacy.[93] Because Cas9 and sgRNA/mRNA have opposing charges, they complex together and can be delivered together.[27,93]

Along with its codelivered payloads, Cas9 also interacts with the internal part of NPs. Therefore, the charge interactions between Cas9 and NPs should also be considered. As previously mentioned, charge can affect the structure and properties of proteins, so it is necessary to ensure that the Cas9 does not degrade. Currently, there are no studies on the internal charge of NPs and their effect on Cas9, so there are no conclusions on how the charge interactions affect the Cas9 complex.

External Interactions. The surface charge of the chosen NP plays a crucial role in the delivery of Cas9 across the negatively charged cell membrane and the effects the structure will have on cytotoxicity.[21,52,74,75,94−96] As displayed in Figure 2, NPs have different routes of entry into the cell membrane, such as electroporation, endocytosis, and ligand-based entry. Charge can influence endocytosis of a NP in the cell membrane. Several studies found that cationic NPs are much more likely than anionic NPs to pass through the cell membrane, due to destabilizing the lipid bilayer.[91,92,97,98] Although cationic NPs show higher drug delivery efficacy and better gene transfer, they also demonstrate higher cytotoxicity levels than anionic NPs, as positively charged NPs tend to cause more damage to cellular structures such as mitochondria and lysosomes.[92] On the other hand, anionic NPs do not naturally cross the cell membrane but help delay membrane destabilization at certain conditions, allowing the membrane to survive high pH levels.[55,57] One study found that negatively charged NPs cause intracellular damage that usually leads to apoptosis or programmed cell death, which does not make them desirable for Cas9 delivery.[92]

The surface charge of the NPs encapsulating Cas9 plays a role in the internalization and release of the protein.[92] Miller et al. noted in their article that cationic lipid, zwitterionic phospholipid, and PEG can create efficient lipid NPs because the positively charged lipids promote the release of RNAs when pH levels lower.[27] The cationic liposome complex known as Lipofectamine is popularly used *in vitro* when delivering CRISPR-Cas9 complexes due to its ability to bind negatively charged molecules.[93,96] Furthermore, several studies show that the positive charge of cationic liposomes and DNA complexes improves delivery.[96] As mentioned in a previous section, there have been attempts to create exosome−liposome NPs for *in vivo* deposits of Cas9 which could be useful as exosomes contain various surface charges depending on the cells they originated from.[99] Consequently, these exosomes have specific roles in cell-signaling pathways, which could be exploited to cross charge-responsive barriers.[99]

While NPs can possess positive or negative surface charges, plasmids consistently have a negative surface charge, which creates a repulsion between the vector and the cell membrane.[10,96] For example, Zhang et al. note in their article that the negative charge of Cas9/sgRNA-fused plasmid DNA reduced its ability to become internalized by the negatively charged lipid membrane.[10] With further investigations, this system could be optimized for Cas9 delivery.

3.4. Volume Constraints. Along with the internal environment and charge, the effect of volume constraints of encapsulating Cas9 on structure and functionality must be studied. The functionality of a protein has a direct relationship to its structure, with structural changes due to stress, degradation, or space constraints contributing to the loss of a protein's function.[100] As Cas9 is a long protein, measuring at around 160 kDa, it requires a suitable environment to exist without an altered folded structure.[4,5] When Cas9 is complexed with sgRNA in a plasmid, its volume is 410 000 bp, which is a large load for NPs to carry.[10] This demonstrates that there is a certain size that NPs must be to properly encapsulate Cas9. Similarly, a Cas9 mRNA complex is around 4,500 nt, which makes delivering it difficult for synthetic carriers.[27] To ensure the effective delivery of Cas9 and its codelivered molecules, the size of NPs must be complementary to the size of their payload. Currently, there is a literature gap regarding the exact size NPs must be as to not compromise Cas9. As aforementioned, this is likely due to researchers prioritizing forging ahead with cutting-edge Cas9 research rather than formalizing optimal conditions for the protein. As the study of Cas9 continues, likely literature will emerge to examine the specific parameters that NPs need to fall within to effectively house Cas9, sgRNA, and mRNA.

4. CONCLUSION AND FUTURE PERSPECTIVES

NPs offer an ideal delivery method for the Cas9 protein, allowing this gene-editing protein to be protected while attempting targeted *in vivo* delivery and minimizing off-target effects. NPs such as AuNPs, lipids, polymers, and lipid−polymer hybrids all offer unique attributes that can be exploited for medical use in humans. Because of this, it is important to critically analyze the parameters required for each specific genetic disease when selecting the most appropriate material for application. While AuNPs and lipid-based NPs have led to clinically translated products, these systems are not able to be fine-tuned to the extent of polymer-based NPs. AuNPs are limited by their available sizes and high cost, making them suitable for certain applications like imaging but less suitable for the delivery of high molecular weight gene-editing protein Cas9. Lipid-based systems are limited in their chemical versatility, with NPs comprised of natural lipids, limiting their stimuli-responsive properties. Their leakiness can

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also be a major concern when delivering costly therapeutic payloads, like Cas9 and sgRNA which are expensive to design and to produce. As such, future research should aim to use more tunable and versatile polymer-based NPs, with polymeric materials already approved by the FDA for other biomaterials applications.

Currently, genetic diseases affect the quality of life of many patients, and the CRISPR-Cas9 NP system offers promising treatments. Once the material has been selected, other properties of NPs must be analyzed for efficient encapsulation and delivery of Cas9. The internal environment is a necessary component to ensure the integrity of Cas9 once it is encapsulated. Namely, the presence of solvent during encapsulation can lead to protein denaturation and limit the therapeutic benefits of Cas9. Furthermore, the charge−charge interactions that Cas9 has are important to consider because they affect internalization, cytotoxicity, drug delivery, and imaging efficacy.[53,55−57] Likewise, Cas9 must also exist in a NP that can contain its large size to prevent denaturing.[100] Overall, a cationic−lipid/polymer-based system was the one that several studies found to have the most potential for *in vivo* Cas9 delivery. In the future, as the delivery of Cas9 through NPs is further studied and effective methods are recorded, it is likely that the technology can be adjusted to fit each person and illness. Polymeric materials offer the opportunity to introduce stimuli-responsive, stable delivery vehicles for Cas9 that can be tuned to respond to specific disease pathologies and to more carefully reach the intended tissue.

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Notes

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