A Review: possible optimization of Cas9-sgRNA nuclease delivery via ingested lipid-nanoparticles bioencapsulated within plant cell-based enfolding

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Abstract

The possibility of gene editing to correct disorders is one of the most impactful therapeutic agents, currently. CRISPR Cas9-sgRNA nucleases can be used to cleave and to delete harmful or pathogenic DNA sequences, which cause genetic disorders. Cas9 nuclease with palindromic repeats can cut and delete a single point mutation or multiple DNA target site sequences. The Cas9, attached to a sgRNA or a guiding RNA, finds and then cleaves the target DNA sequence. The Cas9-sgRNA method of cleavage has corrected DNA mutations that cause cataracts in the eyes, cystic fibrosis, and chronic granulomatous disease. However, there are issues for producing a less strenuous delivery of Cas9-sgRA to target DNA sequences. Delivering Cas-9 nucleases are negatively affected by off-target DNA sites, sgRNA design, off-target cleavage, Cas9 activation, and the method of delivery. This review focuses on oral and ingested delivery methods to effectively guide the transport of Cas9-sgRNA nucleases in vivo. A review of Cas9 delivery will present possible alternatives for nuclease delivery within optimized lipid-nanoparticles, plant, algae, and bacterial-based orally ingested edibles. This review will attempt to provide evidence in support of enhancing the Cas9 delivery through therapeutic bioencapsulated ingestion. In this review, it is suggested that the ingestion of encapsulated edibles carrying the nuclease can more directly target cells within the gastrointestinal tract for blood or lymph circulation.
Introduction

The sgRNA of the CRISPR CAS-9 system is attached to Cas9 for a specific genomic area that is complementary to 20 bases of the sgRNA, consisting of a 5’-NGG-3’ protospacer-adjacent motif or a PAM (18). Cas9 breaks the double stranded DNA structure at the specific gene loci and then repairs it through nonhomologous end-joining or homology-directed repair, HDR. Dominguez et al. (2016) define a CRISPR–Cas as a prokaryotic RNA a part of the adaptive immune system, protecting its cells from foreign DNA sequences. Dominguez et al. (2016) described the structure of CRISPR-Cas as a large protein with multiple domains but with only two active sites for nuclease activity. After researchers studied the Cas9 of S. thermophilus it was found to block the entrance of viruses (5). The CRISPR Cas-9 system has corrected gene mutations that cause diseases as cataracts and cystic fibrosis, however, there has not been a direct injection into mammalian organs to genetically edit gene mutations. Genome editing was initially applied to Drosophila melanogaster, and rapidly extends to a broad range of organisms (16).

Benefiting from the simplicity and the adaptability of CRISPR/Cas9, Cas9 gene editing can reveal the biological function of genes needed for correcting the gene mutations of multiple disease types. Genome editing can modify DNA sequences and treat genetic disorders through CRISPR. CRISPR-Cas9 consists of palindromic repeats that can change a single or multiple genos (14). CRISPR corrected mutations in NOX2. NOX2 is responsible for an immunodeficiency disorder called chronic granulomatous disease (6). The modified stem cells were transplanted into mice, and the NOX2 protein was functional for 5 months. Therefore, CRISPR/Cas9 is a proven and effective method for modifying gene mutations. Additionally, the delivery of the Cas9 nuclease into a target cell may require adjustments.

Further studies are necessary to explore the characteristics and improve the performance of CRISPR/Cas9, especially its specificity for off-target mutations and the delivery methods of the CRISPR/Cas9. For example, Zhang et al. (2014) discussed an issue of off-target mutations versus ZFNs and TALENs, which CRISPR/Cas9 offered a higher propensity for developing off-target mutations. In addition, the human cell has a large amount of DNA material, which frequently consists similar homologous DNA target sequences. As a result, identifying more CRISPR prokaryotic taxonyms for sequencing total bacterial genomes, can improve the choice of target sites and enhance sgRNA design.

However, oral ingestion of Cas9 nuclease may be a possible effective route of delivery through the GI tract. Many immune cells are located near large aggregations of commensal bacteria in skin and in the GI tract. The microbiota provides a barrier to assist with the immune response. The host cells have a close interaction with microbiota, which provide a buffer system between microbes and epithelial cells. Called the “Mucosal firewall” it consists of epithelial cells, mucous, immunoglobin A, antimicrobial peptides, and immune cells (1). Jan-Peter Van Pijkeren at the University of Wisconsin-Madison and his team produced possible alternatives for antibiotics by combining CRISPR treatments with edible probiotics, inducing harmful bacteria to
self-destruct its DNA (13). In this review, alternatives for optimizing Cas9 nuclease delivery, through oral ingestion into the GIT, will be examined. The delivery issues for Cas9-sgRNA, alternative methods, and possible oral ingestion delivery of Cas9 will be discussed in this review.

**Survey Methodology**

Research Questions: What are the current challenges of the Cas-9 nuclease? How can the delivery of Cas9-sgRNA be improved? Can lipid-nanoparticles improve delivery of Cas9? Can oral ingestion of lipid-based nanoparticles with Cas9 become a more effective approach for its delivery? How may the GI tract become an advantageous route for Cas9 delivery?

The search terms included: CRISPR Cas9 description, Structure of the immune system within the gut, the structure of the Peyer’s patches, lipid-nanoparticles and Cas9 delivery, liposomes versus lysosomes, bioencapsulation and Cas9 current issues and challenges. Search Engines used were google scholar, google images, Science Direct, and ResearchGate. The total research articles viewed were approximately 20. The chosen research articles were a sum of 17 and four articles were eliminated.

The four articles were eliminated based on their title and abstract, which did not align with the search terms. The first article was eliminated because it reviewed the coagulation or blood clotting gene mutations corrected by CRISPR gene editing, the 2nd article’s title included the term Rheumatoid Arthritis, the 3rd article studied muscular dystrophy, the 4th article only discussed the history or the development of CRISPR nucleases.

1. Issues and Methods for CRISPR CAS9 Delivery

Some challenges for targeting genes include the sgRNA design, which guide the Cas9 to its target DNA site. Mice with the mutation in the Crygc gene have a point mutation or a 1 base pair deletion in the third exon of Crygc. The third exon of the Crygc mutation leads to a formation of a stop codon at the 76th amino acid. The change and insert of a stop codon cause the formation of an extended gamaC-crystallin structure, which produces the cataracts. The formation of cataracts occurs in homozygous and heterozygous mice as well in mice with the induced mutation. Researchers engineered five sgRNAs that matched loci within the range of the 1 base pair deletion because the 1 base pair point mutation forms a neo-protospacer adjacent motif or a neo-PAM that does not occur in the wildtype allele. The sgRNA-5 is concentrated upstream of the 1 base pair deletion of the normal DNA sequence.

Researchers transfected plasmids and the mammalian-codon-optimized Cas9 was expressed, consisting of the five sgRNAs injected into the wild-type embryonic stem cells. Their results from PCR amplification for the targets regions showed that sgRNA-2 and sgRNA-5 targeted the wild-type alleles successfully but with nonhomologous end-joining-mediated mutations of the Crygc. However, sgRNA-1 and sgRNA-3 had less amounts of NHEJ-mediation mutations. The embryonic stem cells with sgRNA-4 transfection showed no mutation present in
the ESC clones. All the sgRNAs cleaved at the Crygc mutant target sites in the heterozygous mutant ESC clones.

Challenges with the delivery of Cas9 occurred when sgRNA-2 and sgRNA-5 detected and changed the wildtype and the mutant genes for the mCrygc embryonic stem cells or ESCs. The sgRNA-1, sgRNA-3, and sgRNA-4 only targeted the gene mutation of mCrygc. The sgRNA-4 was selected due to its inducing deletions, insertions, and it closely targeted the mutant allele. To correct the mutation that causes cataracts, a mouse model was used. Zygotes were injected with Cas9 mRNA and sgRNA-4 and were the offspring of females that procreated with homozygous males, expressing the phenotypic cataracts. Approximately 91 percent of the zygotes matured into embryos and 22 mice were born (15). Of all the 22 mice none of the wildtype alleles were affected or changed, showing the accuracy of sgRNA-4 to only target the mutant alleles. Other challenges may occur with direct injection or through unguided Cas9. The Cas9 mRNA, sgRNA-4, and Oligo-1 were injected into the cytoplasm to test rather adding a single-stranded DNA oligo called Oligo-1 can make HDR-mediated precise genome more efficient. The co-injection was projected into the cytoplasm of heterozygous cataract mutation-bearing zygotes. The results included: the DNA sequencing 14 of the 29 mice displayed gene changes in the mutant allele (15). Nine of the fourteen mice did not form cataracts.

A disease caused by the genetic disorder called tyrosinemia type 1, is caused by a mutation in the fumarylacetoacetate hydrolase (FAH) enzyme. Fah is the final enzyme in the tyrosine catabolic pathway and process. The HTI mutation occurs at exon 8 with a point mutation of G to A, causing FAH. The mutation causes the exon 8 to be skipped, leading to the FAH protein to be denatured and lacking a functional structure. The lesser the amount of FAH as an enzyme, more toxins are aggregated, causing liver disease. To genetically edit the Fah gene three sgRNAs: FAH1, FAH2, and FAH3, researchers cloned each into the plasmid called pX330. The vector pX330 expresses sgRNA and Cas9 complementary to the target gene mutation. Mice were then injected with unguided Cas9 or with ssDNA oligos with pX330, dispatching Cas9 and one of the sgRNAs of FAH1 to FAH3. The unguided Cas9 injected mice needed to be euthanized due to their 20 percent loss of body weight (17). The FAH-2 injected mice after 30 days exhibited less liver damage and disease. After the histological analysis of the liver of the mice, applying serum biomarkers as aspartate aminotransferase, AST, alanine aminotransferase (ALT), and bilirubin to the liver, the Fah gene increased with less liver damage.

More methods for delivering the Cas9 nuclease include viral vectors, non-viral vectors, electroporation, and microinjections. The adeno-associated virus, AVV, can deliver Cas9. Using a viral vector, delivers Cas9 to a variety of cell types and cell lines. However, delivery through AVV presents issues within clinical testing. The transfection and integration of AVV-Cas9 delivery disrupts adjacent and significant genes. Other issues include the size of Cas9 proteins and the limited space within the viral vector. The smaller space within the AVV limits the packaging of Cas9 proteins. The AVV can only carry 4.7kb of DNA (7). Viral vehicles are limited in that they cannot surpass cellular obstacles and need chemical additions to efficient transport.
The non-viral delivery approach presents immense sufficiency to avoid insertion errors and is not limited by the small capacity of viruses. Non-viral delivery can regulate the dosage, endurance, and the specificity of the transport of Cas9. Routes of non-viral delivery include electroporation and microinjection. Through administering electroporation an electric current is applied into the cell membrane, creating pores in the cell. The pores are physically opened for inserting the Cas9. However, electroporation may degrade the cell membrane and has not been approved for clinical trials or for human patients. The increased release of electrical currents can disrupt electrical signals in the nerves and muscles. Hydromicroinjection uses water to create pores for injecting large biological molecules. Applying microinjection has only been tested in laboratory animals and can inject biomolecules into the cytoplasm of a cell. The main issue for microinjection is each cell of the target tissue will need an injection, which may be time consuming, it has successfully modified mutations in unicellular embryos. Next, the immense potential of Cas9 delivery through the structure and function of the immune system in addition to the Peyer’s patches within the GIT, will be discussed.

2. The Immune System, Peyer’s Patch, and Drug Delivery within the Gastrointestinal Tract

Mucous in the intestines provide a barrier between microbiota and epithelial tissue. The goblet cells produce the mucous. The epithelial cells form antimicrobial peptides to reduce a physical interaction with commensal microflora. The antimicrobial peptides act as enzymes to degrade bacterial cell walls and its inner membranes. The peptides help to create and maintain a physical barrier between the host intestines and the microbiota. The immune response in the intestines includes FOXP3 for the regulatory Treg cells, which maintain balance in the mucous (1). The degradation of Treg cells causes effector responses in the gut. Tissue co-factors as Vitamin A, MUC2, a mucous glycoprotein formed from intestinal goblet cells differentiate dendritic cells for regulation. Treg cells in the colon also have a high affinity for antigens processed by commensal bacteria.

The immunoglobulin type A is derived from commensal bacteria and produced by dendritic cells. The microflora interacts with the epithelial cells, T cells, and B cells in the Peyer’s Patch. The microbiota-immune relationship yields IgA, which bind specific antigens only processed by commensal bacteria. Microflora bacteria bound to dendritic cells move through the lymph node but cannot cross the IgA barrier. Then, the B cells carrying IgA traverse to the lamina propria of the intestines to release the IgA antibody. The transfer of IgA from the B cell monitors the host to microflora-bacterial gene expression. The host-commensal bacteria immunity blocks bacteria from attaching to the epithelial lining of the GI tract.

The general mechanism involves a drug compacted within a liquid and solid lipid. The drug travels through the small intestines, and then binds to M cells that line the lumen of the intestines. The drug transports through the M cells to be engulfed and phagocytized by NCLs. The drug is carried by dendritic cells to the Peyer’s patch and released into the lymph nodes for lymphatic circulation. In the lymph nodes the dendritic cells release the drugs to enter blood circulation. Therefore, the route of drug ingestion and delivery through the intestines may be a high-yielding method for a direct interaction between the immune cells, the circulatory system,
and Cas-9-sgRNA nucleases. However, Cas9 nucleases will require packaging into lipid-based nanoparticles to successfully cross the lipid bilayer of the M cells.

3. Lipid nanoparticles and the possible Bioencapsulated Liposome Ingestion of CAS9-sgRNA

Lipid nanoparticles have much potential for improving CRISPR-CAS9 delivery. Because DNA is negatively charged, DNA can be encased within cationic or positively charged lipid nanoparticles. The lipid encased CRISPR Cas9 nucleases can cross the lipid cell membrane as a result of its positive charge. The liposome reduces an immune response, rendering it a safer delivery method. The Cas9 and sgRNA are intensely anionic, therefore, can be packed within lipid nanoparticles. However, there are issues present with encapsulation within lipid-nanoparticles.

The issues include: 1) the Cas 9 nuclease liposomes, entering the cell membrane, are immediately engulfed and degraded by lysosomes and 2) delivering the liposome rarely reaches the cell nucleus. Crossing the nucleus presents an arduous obstacle with a lower delivery efficiency. Lipofectamine can be a possible method and solution. It is positively charged, can bind DNA targets, diffuse through the cell membrane, and avoid endocytosis. The Cas9 and sgRNA plasmid DNA was transfected into human pluripotent stem cells. The Cas9 and sgRNA have successfully been delivered through Lipofectamine to correct Facial anomalies syndrome (ICF) syndrome with a high rate of transfection efficacy at 63% (11). Also, other methods to optimize lipid nanoparticle delivery will be reviewed.

The pH-sensitive liposome can avoid lysosome degradation. The pH-sensitive unit can be added to the liposome through blending pH-sensitive lipids with polymers. The liposome will remain stable in a neutral pH and intact until it reaches a lower pH environment, which is more acidic, pH of 5, in the cytoplasm (10). After entering the cell through an endosome, a controlled and gradual release of the contents inside the pH-sensitive liposome occurs, bypassing the lysosome degradation. Tumors are highly acidic with a pH of 5; however, Monteiro et al., (2018) currently crafted a pH-sensitive liposome consisting of paclitaxel or PTX. The pH-sensitive-PTX liposomes can extend its reach to tumors, and then release the encapsulated drugs as it contacts the acidic tissue masses. Hassett et al. (2019) enhanced lipid nanoparticles with ionizable lipids, called MC3, for delivering a nucleic acid-based vaccine for influenza. Their vaccine trials with 100 micrograms of the ionizable lipid-based mRNA vaccine yielded a 100% seroconversion or a total detection of the antibodies produced.

The size and dosage of oral ingested particles absorbed within the GIT can be a factor for its efficacy as well. For example, Brocks and Davies (2018) modeled the pharmacokinetics and the absorption of drugs or other particles across the enterocytes of the gut. They confirmed a competition between the lymphatic flow of drugs versus the blood flow, which is 500 times faster. The expected positive result for an oral ingested drug entering the lymphatic flow depends upon the drug size, a molecular weight less than 500g/mol, and the dosage. Larger drugs enter the blood flow instead of the lymphatic system to become cleared from the blood circulation by
the liver. A smaller dosage of a drug enters the lymphatic flow, delivering it more efficiently to
the lymph and nodes. However, the Cas9 nuclease will require efficient packaging to
successfully navigate through digestive enzymes of the stomach after ingestion.

The bioencapsulation of liposomes carrying Cas-9-sgRNA nucleases with plant, algae,
and bacterial-based oral delivery could become a prospective option. For example, consider the
most proven and studied vaccine delivery to induce an enormous adaptive and humoral immune
response include edible vaccines. Vaccines ingested signal an immune response through the gut-
associated lymphoid tissue or known as GALT. The GALT consists of the lymph nodes, the
Peyer’s Patch, and lymphoid tissues in the GI tract. The Peyer’s patch is 75% B cells and
includes 20% of T cells (3). When oral vaccines are ingested, the antigens are transferred
through the mucosal layer via the M cells into the Peyer’s patches and presented to the T-Cells.

However, oral vaccines need to surpass the mucosal tolerance to overcome and attract
more effector cells than regulatory cell types. Oral vaccines need to be revised to increase their
absorption by M cells. Three possible ways that enhanced oral vaccines are through plant, algae,
and bacteria-based bioencapsulation. Plant-based encapsulation allow for ample folding of
proteins as antigens and are more affordable. Plant-based delivered vaccines have been in use
since the 1990s. Also, because of plants thick and sturdy cell walls, the plant cells tightly
protected antigen proteins from degrading or denaturing. With the delivery of oral vaccine
components, the cross-contamination with animalistic disease-causing antigens was avoided.
Also, plant cell-based oral delivery prevents bacterial corruption.

Algae-Based oral vaccines, encapsulated in green microalgae called *Chlamydomonas reinardtii*, readily acquired and amplified the yield of antigens. The growth rate of algae is
extremely rapid. The FDA has identified and approved green algae as safe and it can be stored at
4 degrees Celsius for 20 months (3). The cell walls in algae increased the stability for the
bioencapsulation form, blocking antigen denaturing by the enzymes in the GI tract. Gram-
positive bacteria as Lactic acid bacteria do not cause disease and have been utilized for many
years, decades. The Lactic acid bacteria have been used to preserve food and used to compose
antibodies against HIV infection. Because the bacteria-base delivery allows for a more facile
acceptance in the GI tract among other commensal bacteria, the antigen compressed inside will
not degrade.

Bacteroidetes and Firmicutes are the only bacteria in the gut microbiome that use
enzymes called cellulosomes that bind to the cellulose of plant cell walls. Activating the
cellulosome, bound to cellulose, the glyosidic bonds are broken. The potency of bacterial
cellulosomes was proven through an in-situ hybridization study (9). In this study there was an
enormous measure of Bacteroidetes in the mucous layer and its substrates were predominantly
mucopolysaccharides. In the mucous layer, bacteria in the gut grasped and attached to a plant
cell with its pili consisting of cellulosomes. The plant cell was ruptured, releasing the Green
Fluorescent Protein. The GFP was not visible in the stomach, but in the upper GI tract near the
ileum and the villi absorbed in the epithelial cells. The release of visible GFP signaled a
successful oral delivery of a plant-based encased protein.
Conclusion

The review presented discussed possible methods for optimizing the delivery of Cas9 nucleases. Delivering Cas-9 nucleases are negatively affected by off-target DNA sites, sgRNA design, off-target cleavage, Cas9 activation, and the method of delivery. This review focused on delivery methods to effectively guide the transport of Cas9-sgRNA nucleases through the GI tract in vivo. Delivery through the digestive and GI tract may provide a valuable alternative method. The GI tract consist of 70% of the total immune system. The lining of the GIT contains a layer of microflora bacteria, a mucosal layer, and epithelial cells, and immune cells. The Peyer’s Patch is located within the GIT, containing immune B cells and T cells. The Peyer’s patches within the gut offers a fastidious transport of prescription drugs and other lipid nanoparticles. The ingestion of lipid-based nanoparticles travels through the epithelial lining of GIT via M cells, attach to dendritic cells transferring them to the Peyer’s patches where they are presented to B-Cells and T-Cells. The immune cells deliver the lipid nanoparticles to the lymph nodes or into blood circulation.

This review attempted to present alternatives for delivering CRISPR Cas9-sgRNA nucleases through the gastrointestinal tract. The process for a possible delivery through the ingestion of nanoparticles as lipid nanoparticles was described. Evidence for a more successful Cas9 delivery through the GIT was explained by revisiting the structure and function of the immune system within the GIT. For example, ingested nanoparticles as drugs can immediately travel through the epithelial lining of the lumen of the GIT and interact directly with immune cells in the Peyer’s patches. Lipid nanoparticles are a possible method that can immensely impact the delivery of Cas9 nucleases through the GIT to the Peyer’s patches to the lymphatic and circulatory systems. Because the Cas9 nucleases are negatively charged, they can be tightly packed into positively charged lipid nanoparticles.

Sheathing Cas9 nucleases into lipids can allow prompt diffusion through the lipid bilayer of cell membranes lining the lumen of the GIT. However, delivering the Cas9-liposomes are engulfed quickly by lysosomes, rarely entering the cell nucleus. Because liposomes lack viral subunits, it lessens an immune response, but liposomes could be re-designed to more precisely target cells and tissues. Plant, algae, and bacteria-based bioencapsulation can be a possible option for re-designing Cas9-liposomes. Plant cells have strong cell walls, which enclose antigens, averting its degradation. Using plant cells for encapsulation can prevent contamination with mammalian and bacterial material. Algae-base liposomes can protect the contents inside from GI tract enzymes. Algae-based encapsulation also have strong and durable cells walls to protect Cas9 nuclease cargo. Bacterial-based liposomes can have an even safer travel through the GIT because Lactic acid bacterial encapsulation will be accompanied with more commensals within the GIT microflora.

However, more emphasis is given to bioencapsulation with plant cells. Because the enzymes in the human stomach can not digest or degrade the cellulose in the plant cell walls, the Cas9-sgRNA contents within plant-based casing should not degrade. When the plant-based Cas9-sgRNA liposome receptacle enters the GI tract, commensal bacteria begin to metabolize the plant cell walls, and then the contents are released to transport across the GIT lumen via the
M cells. The contents permeate into the circulatory or the immune system. However, more research is needed to more effectively elucidate and apply methods that optimize delivery of CRISPR Cas9 nucleases. Specifically, continued improvement to optimize lipid nanoparticle delivery of Cas9 cargo components should be a future goal for further research. Further study needed could include confirming the efficacy of combined plant-based bioencapsulation with optimized lipid nanoparticles as an outer and mid covering of the Cas9 contents. There could be a possibility for oral digestion of a plant cell-based outer covering, to protect the pH-sensitive liposome, covering the inner Cas9 nuclease core from stomach enzymes. In addition, when designing a plant-based bioencapsulated lipid-nanoparticle for Cas9 delivery, its size, molecular weight, and dosage need to be considered to assure its absorption by the lymphatic system. Perhaps, applying pharmaceutical drug release and kinetics to enhance Cas9-sgRNA delivery can provide more methods for further research.

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References


