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Polymer-Based Transfection Agents Used in CRISPR-CAS9 System

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Abstract: Genome editing is a method used to make desired changes in the target gene. Today, various methods are used for genome-editing studies; among them, one of the most widely used methods is the clustered, regularly interspaced short palindromic repeats (CRISPR). CRISPR-associated (Cas) genes and their corresponding CRISPR sequences constitute CRISPR-Cas systems. Due to its simplicity, it is likely that the CRISPR–Cas system could be used effectively in ex vivo gene therapy studies in humans. If this happens, the importance of CRISPR carrier systems will gradually increase. Viral and non-viral systems are used as delivery modalities in genome-editing studies. It has been proven that nanoparticles are the most promising tools for gene therapy due to their adjustable size, surface, shape, and biological behaviours. The polymeric carrier system has become the main non-viral substitute for gene delivery due to its reduced immunogenicity and pathogenicity. In this review, information about current studies related to polymeric carriers used in non-viral CRISPR delivery systems is presented.

CRISPR-CAS9 Sisteminde Kullanılan Polimer Bazlı Transfeksiyon Ajanları

Anahtar Kelimeler
CRISPR, Viral olmayan Transfeksiyon ajanları, Polimerik Trasnfeksiyon Ajanları,

Öz: Genom düzenleme, hedef gende istenilen değişiklikleri yapmak için kullanılan bir yöntemdir. Günümüzde genom düzenleme çalışmaları için çeşitli yöntemler kullanılmaktadır; bunlar arasında en yaygın kullanılan yöntemlerden biri kümelenmiş, düzenli aralıklarla yerleştirilmiş kısa palindromik tekrarlardır (CRISPR). CRISPR ile ilişkili (Cas) genleri ve bunlara karşılık gelen CRISPR dizileri, CRISPR-Cas sistemlerini oluşturur. Basitliği nedeniyle, CRISPR-Cas sisteminin insanlarda *ex vivo* gen terapisi çalışmalarında etkili bir şekilde kullanılmaya başlanmıştır ve CRISPR taşıyıcı sistemlerin önemi giderek artmaktadır. Genom düzenleme çalışmalarında dağıtım yöntemleri olarak viral ve viral olmayan sistemler kullanılmaktadır. Nanopartiküllerin ayarlanabilir boyutları, yüzeyleri, şekilleri ve biyolojik davranışları nedeniyle gen terapisi için en umut verici araçlar olduğu kanıtlanmıştır. Polimerik taşıyıcı sistem, azaltılmış immünojenisitesi ve patojenitesi nedeniyle gen aktarımı için viral olmayan ana ikame haline gelmiştir. Bu derlemede, viral olmayan CRISPR dağıtım sistemlerinde kullanılan polimerik taşıyıcılarla ilgili güncel çalışmalar hakkında bilgiler sunulmaktadır.

1. INTRODUCTION

The field of genome modification includes techniques used to obtain centered modifications inside an organism's DNA. Until today, the rapid transition of gene editing into clinical practice has occurred thanks to the emergence of programmable nucleases that allow scientists to perform gene editing in numerous cell types. Today, existing editing approaches include zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and clustered, regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated

nuclease 9 (Cas9). ZFN is a targetable DNA fragmentation protein that can be used to cleave DNA sequences at any site. On the other hand, TALENs can cause a double-strand break in the target nucleotide sequence, which leads to genome modification by triggering the DNA damage response pathway [1]. From 2002 to 2011, respectively, ZFNs and TALENs have been mainly used in genome editing in animal and human cells and plants; however, during this time, they have also exhibited some disadvantages that prevent their usage in certain cases. For example, ZFNs show limited specificity and can cause off-target mutations [2]. In addition, vector design for ZFNs and TALENs takes time and effort to perform [3]. Table 1 presents a comparison of the TALEN, ZFN, and CRISPR-Cas9 genome editing techniques.

Table 1. Comparison of the TALEN, ZFN, and CRISPR–Cas9 genome editing techniques [4]

	CRISPR/Cas9	**ZFN**	**TALEN**
Molecular elements	Single-stranded gRNA+Cas9 endonuclease	ZFN protein+Fok1 endonuclease	RVD of the TALE protein sequenced repeat region+Fok1 endonuclease
Recognition site and specificity	• Usually 20 bp directory sequence + PAM sequence • Tolerate spatial/consecutive multiple mismatches	• Each ZFN protein recognizes 3 bp DNA • Tolerate a small number of positional mismatch	• Each TALE protein unit recognizes a single base pair of DNA. • Tolerate a small number of positional mismatch
Challenges and restrictions	• A PAM sequence must come before the targeted area. • Utilizing oligo synthesis and standard cloning procedures	• Difficult to target regions with poor G nucleotide content • Requires significant protein engineering	• 5′ targeted base should be one T nucleotide for each TALEN monomer • Difficult and complex cloning methods
Advantages	Targeting multiple genes	Small protein size and suitable for viral vector	High recognition specificity and no need to link between repeats
Transfection difficulties	The widely used SpCas9 is medium in size and may cause packaging issues for some viral vectors, but smaller orthologs are available	Since ZFN expression elements are small, they are simple to use in a variety of viral vectors.	Due to the large size of functional components, it is difficult.

Due to the reasons mentioned above, the use of CRISPR–Cas9 in genome editing has been widely preferred since 2013. The CRISPR–Cas9 method is an RNA-guided endonuclease that can specifically target DNA sequences through nucleotide base pairing. This system is highly specific, versatile, and simple. It has become a valuable tool in biological research for both gene function and gene editing because of these properties [5]. With the reporting of the first CRISP structure by Ishino in 1987, it has reached the present day as a mechanism used by many bacteria and archaea to protect them from virus invasion [6, 7]. In 2002, after observing the existence of several similar structures in different archaea and bacteria, Jansen proposed the acronym for CRISPR [8].

Finally, the approach was first applied to mammalian cells in 2013 and since then, the CRISPR–Cas system has occupied an important place in genome-editing research [9]. CRISPR–Cas structures are categorized from type I to type VI [10]. The type I system is characterized by the formation of Cas3, which is a protein with both DNase and helicase activities that can be used to degrade targets. The type II CRISPR–Cas system uses Cas1, Cas2, Cas9, and a fourth protein (either Csn2 or Cas4) [11]. The type III-A CRISPR–Cas system is an adaptive immune system guided by prokaryotic RNA, which uses Csm (a protein–RNA complex that can perform transcription-dependent immunity against foreign DNA) [12].

The type II CRISPR–Cas system is derived from *Streptococcus pyogenes* and consists of three parts: CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA), and a Cas9 protein [13]. Cas9 is an enzyme that uses CRISPR sequences to recognize and separate specific DNA strands; thus, the Cas9 enzyme and CRISPR sequencing can be used together to edit genes in organisms and form the basis of CRISPR–Cas9 technology [14]. The regulation process has a huge range of applications, including the improvement of biotechnology products, basic organic studies, and disease treatment [15].

In general, Cas proteins obtained from bacteria have two nuclease domains and work together with single-guide RNA (sgRNA) to create a double-strand break in the target DNA sequence. sgRNA is the component that directs the Cas protein to the target DNA since it contains crRNA and tracrRNA and has a short sequence complementary to the target DNA sequence [9]. Another critical component is the protospacer adjacent motif (PAM), which is located in the target DNA sequence and guides the determination of the cut side of the Cas protein. This system has made it possible to perform applications such as mutation deletion, gene suppression and activation in the target region [14].

By applying this method in the field of genetic engineering, the structure of DNA can be changed precisely in areas where the genome is cut. Moreover, to recognize and repair damaged DNA, this method can be used in the field of genetic engineering by making use of the mechanism of cells. The successful application of the CRISPR–Cas9 system is based on the correction of the cut ends formed by the double-strand break in the DNA as a

result of the cutting created in the desired region of the genome/gene by non-homologous end-joining or a homologous recombination repair mechanism. By modifying the components of the system, the desired and designed genome/gene change can be realized. Also, through linking the engineered sgRNA sequence to the target DNA region by base pairing, the Cas protein recognizes the cleavage site and cuts both strands of the chain from three nucleotides near the PAM by way of nuclease activity, and subsequently, endogenous DNA repair is initiated. After this step, if the knockout of the target gene is aimed, the double-strand break is repaired by the non-homologous repair mechanism that is prone to error. Insertion mutations that occur during this time cause the loss of gene products.

1.1. CRISPR–Cas9 Carrier Systems

There are several obstacles to the practical application of the CRISPR–Cas9 Carrier Systems. These obstacles can be summarised as follows:

1. The stability of the CRISPR–Cas9 system against serum nucleases is very low.
2. The CRISPR–Cas9 system can stimulate the innate immune system.
3. This system may interact nonspecifically with non-target cells and serum proteins.
4. The CRISPR–Cas9 system can be easily cleaned by the kidney system.
5. It is difficult to enter target tissues through blood vessels
6. Degradation products of carriers can be toxic
7. Requires different approaches for different cell line [16-21].

To overcome these problems, new carrier systems have been introduced over time. Viral vectors (e.g., lentivirus, adeno-associated virus, and adenovirus) and physical strategies (e.g., microinjection, electroporation and osmocytosis-induced transduction, mechanical cell deformation, and hydrodynamic injection) are the most widely used delivery system strategies [22-25]. Viral vectors are widely used in CRISPR–Cas9 delivery, and viral carrier systems provide high efficiency in genome editing. However, viral carrier systems also show some basic limitations such as the risk of cancer development, the fact that it is a very long and difficult process to produce on a large scale and their propensity for immune-system stimulation [26, 27].

Physical carriers such as electroporation and microinjection have high degrees of transfection efficiency, but they are not suitable as methods to be adopted for *in vivo* purposes [28-30]. Instead, a hydrodynamic application strategy can be used for *in vivo* experiments; however, it causes various problems such as hepatomegaly, hypertension, and heart dysfunction [31, 32]. In recent years, many studies have been conducted on the development of nanoparticle systems to deliver the CRISPR–Cas9 system to target cells. By overcoming the aforementioned obstacles through the synthesis of advanced nanocarriers, the gene-delivery efficiency can be significantly increased [33-35]. Polymers exhibit significant potential due to their low carcinogenicity or immunogenicity, reduced restrictions with respect to load size, protection of cargo molecules, and creation of nanoformulations with the CRISPR–Cas9 system [36]. In addition, various modifications made on polymers can enhance their circulation time, the controlled release of cargo molecules and cell- or tissue-specific delivery capacities [37, 38]. This review focuses on polymeric nanocarrier systems used in the context of the CRISPR–Cas9 system.

1.2. CRISPR–Cas9 Polymeric Nanocarrier Systems

Polymeric nanoparticles are widely used for the transport of diverse types of nucleic acids [39]. Polymeric carriers can be synthesized from synthetic monomers [e.g., poly(lactic-co-glycolic) acid (PLGA) or polycaprolactone] or natural monomers (i.e., sugars such as chitosan) (Glass et al., 2018). Polymer can protect the cargo molecule from degradation and can increase specific release by functionalizing the cargo molecule with targeting moieties that will enable it to bind to surface receptors of the target cell [12]. However, there are few examples of polymeric carriers reported to date in the literature regarding the CRISPR–Cas9 delivery system. Nanoparticular systems can be used to transport all three forms of CRISPR–Cas9 components (i.e., Cas9 protein/sgRNA, Cas9 mRNA/sgRNA, and Cas9/sgRNA plasmid DNA) to desired cells.

In the literature, cationic polymers such as polyethyleneimine (PEI) have been studied for the transport of CRISPR–Cas9 systems. These polymers are highly positively charged, which can protect DNA from physical and chemical factors. In addition, it presents a high endosomal escape capability to liberate plasmid. So far, polymers such as PLGA, poly(amidoamine), PEI, and chitosan have been tested for delivery of the CRISPR–Cas9 plasmid to target cells, and they have demonstrated good genome-editing potential. They have high positive charges, can interact strongly with negatively charged nucleic acids, and can effectively escape from the endosome due to a strong proton-sponge effect; all of these ensure that it exhibits a high degree of gene-transfection efficiency [40]. In the research conducted by Ryu et al. [38], it was shown that commercial jet-PEI and 25-kDa branched PEI polymer-based DNA transfection reagent successfully delivered plasmid-encoding Cas9/sgRNA. However, the use of these nanocarriers has been restricted due to their high cytotoxicity [38]. Both the transfection and toxicity efficiency of PEI increase in parallel with its molecular weight. Therefore, low-molecular-weight PEI (LMWPEI) has been tested in CRISPR–Cas9 plasmid delivery since its low transfection efficiency is tolerable. Moreover, various modifications have been made to LMWPEI in order to improve its properties. Lino et al. [41] functionalized the LMWPEI polymer with polyethylene glycol (PEG) and cholesterol (CHOL) to obtain a lipopolymer. They showed notably that CHOL increased the permeability through the membrane and PEG increased the hemocompatibility and stability of the lipopolymer. This lipopolymer was then modified with specific aptamers for targeted delivery of

the CRISPR–Cas9 system [41]. Li et al. [34] developed a polymeric 'core-shell' complex with LMWPEI coated with RGD-R8-PEG-HA copolymer, which is a copolymer containing RGD-R8 peptide. In this arrangement, the peptide RGD-R8 had a significant effect on an integrin receptor that was overexpressed in tumours and tumour blood vessels and which gave the polymer tumour-targeting ability. In addition, Li et al. [42] showed that the anionic HA (hyaluronic acid) fragment reversed the cationic charge of the fluorinated LMWPEI and the PEG fragment made the complex more stable, thereby it reduced nonspecific interactions with the human body. In their research, Liu et al. [43] modified LMWPEI by fluorination. They revealed that the polymer subsequently became both hydrophilic and hydrophobic after fluorination, and nanopolymers with good resistance to the lipid bilayer were easily absorbed by cells, while escaping from endosomes also occurred [43]. Zhang et al. [44] assessed the in vitro transmission of the CRISPR–Cas9 system mediated by the cationic polymer PEI–cyclodextrin (PC) in HeLa cells. Their study showed that these nanocomplexes produced with Cas9/sgRNA plasmids were positively charged and could facilitate proper cell uptake and transfection. Ultimately, the efficient packaging and concentration of the plasmid encoding Cas9 and sgRNA in PC was realized. This study also emphasized that although it was similar to high-molecular-weight PEI in terms of structure, PC showed lower cytotoxicity, which allowed PC to function at high dose levels or repeated transfections [44]. Kang et al. [45], covalently linked conjugated branched PEI (bPEI) to the Cas9, then combined this grouping with sgRNA. This nanocomplex targeting the mecA gene was successfully transferred to *Staphylococcus aureus* despite the bacteria's thick cell wall. In addition, it was found that the efficiency of the polymer-conjugated Cas9 was relatively higher compared to the efficiency of genome editing of standard lipid-based formulations or natural Cas9 complexes [45].

PBAE has features such as low toxicity, high water solubility, good biocompatibility, pH sensitivity, and rapid drug release at acidic pH values [46]. A new nanostructure consisting of poly(β-amino ester) (PBAE) was designed by Zhu et al. [46] to transfer CRISPR/short-hairpin RNA (shRNA) to HPV16 transgenic mice. E6 and E7 human papillomavirus (HPV) are oncoproteins that play an essential role in the development of cervical cancer and drug resistance. Carboxylated branched poly(β-amino ester) nanoparticles produced by Rui et al.[45] ensured the efficient and rapid delivery of Cas9/sgRNA to the target cell cytoplasm and escaped from endosomes. This system demonstrated *in vitro* knockout and knockin efficiency results as 75% and 4%, respectively [47].

Xu et al. [48] showed a type of cationic CHOL-assisted PLGA nanoparticles (CLAN) consisting of PEG-PLGA and BHEM-CHOL (N,N-bis(2-hydroxyethyl)-N-methyl-N-(2-cholesteryloxycarbonyl aminoethyl) ammoniumbromide). While PEG was used to support the long-term circulation of the nanoparticles, cationic lipids were used to neutralize the negative charge of the polymer. These scholars also designed a CLAN library with different PEG densities and surface loads to produce the appropriate CLAN and then tested macrophage uptake *in vivo* to screen for the ideal option. Their results showed that nanoparticles with the highest surface loading and relatively low PEG density had the best uptake by macrophages [48]. PEGylation is widely used with the reticuloendothelial system to reduce opsonization and clearance. It can also be applied as a strategy for CRISPR–Cas9 delivery [49].

Chitosan is a positively charged, nontoxic and biodegradable polymer. Chitosan, as a widely used polymer for gene delivery systems, also exhibits potential for the delivery of the CRISPR/Cas9 system [43]. Zhang et al. [50] combined chitosan with mPEG for CRISPR–Cas9 delivery and showed that this nanocomplex protected DNA from nuclease nebulization and digestion.

2. CONCLUSION

Genome-editing studies are a field whose scope and importance are increasing with new and variable methods emerging. Especially given its simplicity and applicability, genome editing with CRISPR is likely to be a much more frequently used method in the future. The use of polymers is an increasingly common way by which the vectors used in genome editing are delivered. A wide range of nanocarriers has already been applied as non-viral delivery systems in human gene therapy. Advancements in materials science and nano-ecology allow the emergence of synthetic vectors with optimum physicochemical properties and tissue/cell targeting capabilities. Polymeric *non-viral* vectors have advantages such as the avoidance of potential toxicity and immunogenicity, good reproducibility, and adherence to good manufacturing practices.

New application strategies are being developed for the delivery of CRISPR–Cas9 components into the cell efficiently. Among these, cationic polymers are susceptible to modifications that allow targeting as well as basic properties such as low immunogenicity and carcinogenicity, and cargo protection. In conclusion, it can be said that the application of polymers in CRISPR–Cas9 delivery is an interesting area that warrants future research.

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