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Poly-sgRNA/siRNA ribonucleoprotein nanoparticles for targeted gene disruption

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ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 nuclease (Cas9) can be used for the specific disruption of a target gene to permanently suppress the expression of the protein encoded by the target gene. Efficient delivery of the system to an intracellular target site should be achieved to utilize the tremendous potential of the genome-editing tool in biomedical applications such as the knock-out of disease-related genes and the correction of defect genes. Here, we devise polymeric CRISPR/Cas9 system based on poly-ribonucleoprotein (RNP) nanoparticles consisting of polymeric sgRNA, siRNA, and Cas9 endonuclease in order to improve the delivery efficiency. When delivered by cationic lipids, the RNP nanoparticles built with chimeric poly-sgRNA/siRNA sequences generate multiple sgRNA-Cas9 RNP complexes upon the Dicer-mediated digestion of the siRNA parts, leading to more efficient disruption of the target gene in cells and animal models, compared with the monomeric sgRNA-Cas9 RNP complex.

1. Introduction

Since its discovery, clustered regularly interspaced short palindromic repeats (CRISPR) -associated protein-9 nuclease (Cas9) has attracted tremendous interest as a very convenient tool for gene modification and has fueled numerous research publications [1–4]. The Cas9 protein derived from *Streptococcus pyogenes* bacteria recognizes a double-stranded target DNA gene *via* the protospacer adjacent motif (PAM) sequence in the target DNA and the 20-nucleotide guiding sequence in single-stranded guide RNA (sgRNA) complexed in the protein and cleaves the DNA. After the DNA breaks by Cas9, insertions and deletions (indels) are produced *via* non-homologous end joining (NHEJ), leading to a disruption of the gene [5,6]. This RNA-guided genome editing system can permanently knock out the target gene and knock in a new gene, providing an efficient strategy for the targeted gene therapy. Owing to its simple and unprecedentedly easy protocol for modification of genes, CRISPR/Cas9 technology has also demonstrated its potential in screening the functional genome and creating disease animal models [7,8].

However, NHEJ-based gene therapy may have potential risk due to mutated genes with unexpected pattern of indels. The off-targeted gene disruption by Cas9 is also potential risk that should be tackled away for the gene editing method to be fully applied to the therapy [9]. Another challenge to employing the CRISPR/Cas9 system for biomedical applications is that efficient delivery of the system into the target cells is required. Most studies of the CRISPR/Cas9 system rely on viral vectors or electroporation to perform the transfection of plasmids encoding Cas9 and sgRNA. On the other hand, safety concerns have been raised due to the integration of vector DNA into the undesired site of a gene causing genetic malfunctions [10,11] and damage of cells by electroporation [12]. As an alternative to the transfection of target cells with plasmids for Cas9 and sgRNA, several efforts have recently been made to deliver the Cas9-sgRNA ribonucleoprotein (RNP) complex directly to the intracellular regions using non-viral vehicles such as cationic lipids [13,14] and cell-penetrating peptides [15]. Nevertheless, non-viral vehicles are less efficient than viral systems and need to be improved for practical uses of CRISPR/Cas9.

In an effort to improve the delivery of the CRISPR/Cas9 system and its gene disruption efficiency, we here attempt to prepare polymeric sgRNA by using rolling circle transcription (RCT), which is a processive RNA polymerization technique that enables the synthesis of long RNA

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strands with repetitive sequences [16]. According to the previous research, RCT has been particularly useful for making polymeric RNA genes that form much stronger nano-complexes with cationic non-viral gene delivery carriers compared to the monomeric RNAs [17]. Polymeric RNA-based formulation led by the increased charge interactions has been beneficial in enhanced intracellular delivery of functional RNA molecules such as siRNA [17–19] and mRNA [20]. Here, we report a novel approach to deliver the CRISPR/Cas9 system efficiently with RCT, which has not been explored so far. The sgRNA sequence can similarly be polymerized by RCT, and the nanoparticles constructed by multiple sgRNA sequences and Cas9 *in situ* during RCT can be delivered more efficiently into the cells than a monomeric RNP using a cationic non-viral gene delivery system. When designing the template sequence for RCT, a Dicer substrate sequence, such as siRNA is incorporated following the sgRNA sequence so that Dicer, the endogenous double-strand RNA specific ribonuclease, would cleave the polymeric RCT product (poly-RNP) to produce monomeric RNP (mono-RNP) after intracellular delivery. In addition, this polymeric CRISPR/Cas9 system based on the poly-RNP provides higher serum stability, which is critical when the system is utilized for *in vivo* applications. Therefore, the poly-CRISPR/Cas9 system with enhanced delivery efficiency and intracellular activation by Dicer leads to improved knock-out rates of the target gene in both *in vitro* and *in vivo* models, compared to mono-CRISPR/Cas9 based on mono-RNP.

2. Materials and methods

2.1. Materials

MagListo™ 5M Genomic DNA Extraction Kit and all nucleic acids were purchased from Bioneer (Korea). T7 Endonuclease, Bbs1 endonuclease and HiScribe™ T7 High Yield RNA Synthesis Kit were obtained from New England Biolabs (USA). Power *Pfu* polymerase (ver 2.0) was purchased from NanoHelix (Korea). Lysozyme, imidazole, protease inhibitor cocktail (PIC), phenylmethylsulfonyl fluoride (PMSF), absolute ethanol and isoamyl alcohol were purchased from Sigma-Aldrich (USA). Rosetta™ 2(DE3) Competent Cells were supplied by Novagen (Germany). Unless stated otherwise, all buffers were obtained from Biosesang (Korea). SYBR® Gold Nucleic Acid Gel Stain, SuperSignal™ West Pico Chemiluminescent Substrate and HisPurTM Ni-NTA Resin were purchased from Thermo Fisher Scientific (USA). The Amicon Ultra-15 Centrifugal Filter Unit Ultra-4 (MWCO 100 kDa) was obtained from Merck Millipore (USA). Bacto™ Yeast Extract and BactoTM Tryptone were acquired from BD Biosciences (USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) were purchased from Gibco (USA). The well plates were purchased from SPL Lifesciences (Korea). The radioimmunoprecipitation assay (RIPA) lysis buffer was purchased from LPS solution (Korea). All antibodies were obtained from Abcam (UK). Dulbecco's PBS (DPBS) was purchased from Welgene (Korea). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Biobasic (Canada). NaCl was supplied from Daejung (Korea). Quantikine Elisa kit Mouse and IL-6 Quantikine Elisa kit Mouse TNF-α (USA) were purchased from R&D Systems (USA), Lipopolyhsaccharides form *Escherichia coli* 055:B5 were obtained from Sigma-Aldrich (USA). The Stemfect RNA transfection kit was purchased from Stemgent (USA).

2.2. Statistical analysis

One-way analysis of variance (ANOVA) with Turkey's multiple comparison test was used to establish the statistical significances. The *P*-values for the comparisons are inserted in every corresponding figure (***$P < 0.005$; **$P < 0.01$; *$P < 0.05$).

2.3. Expression and purification of NLS-Cas9

pET-NLS-Cas9-6xHis was purchased from Addgene (plasmid #62934, USA) and purified as described elsewhere [13]. Briefly, pET-NLS-Cas9-6xHis was transformed into Rosetta™ 2(DE3)pLysS competent cells (Novagen, USA). The resulting single colony was grown in Luria-Bertani (LB) media, and the overnight culture (5 mL) was inoculated into LB (1 L) in the presence of ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL) at 37 °C. The Cas9 protein was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside at 18 °C for 16–18 h. The pellets were harvested, resuspended in buffer A (50 mM Tris(hydroxymethyl)aminomethane hydrochloride) (Tris-HCl, pH 8.0), 1 M NaCl, 20% glycerol, 20 mM imidazole and 2 mM Tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich, USA), and lysed by sonication. After centrifugation at 8000*g* for 40 min at 4 °C, Cas9 was separated by Ni-NTA affinity chromatography. The eluted Cas9 was loaded onto a Hiprep SP HP 16/10 column (GE Health-care Life Sciences, USA) and purified using a linear gradient of NaCl from 0.1 M to 1 M in buffer B (50 mM Tris-HCl, pH 8.0, 20% glycerol, and 2 mM TCEP). The final purity and concentration of Cas9 was determined by a SDS PAGE gel and Bradford protein assay (Bio-Rad, USA) using bovine serum albumin as the protein standard, respectively.

2.4. Circularization of linear DNA

The DNA oligonucleotides were purchased from Integrated DNA Technologies (USA). To synthesize the circular DNA for the RCT reactions, four types of linear ssDNA (1 μM) (Table S1) were hybridized with equimolar amounts of the primer for RNA (Table S1) by heating at 95 °C for 2 min and cooling slowly to 25 °C over 1 h using a PCR thermal cycler (Bio-Rad). To ligate the nick in the circularized DNA, the solution was incubated with T4 DNA ligase (3 U/μL, Promega) and ligase buffer (300 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM dithiothreitol (DTT) and 10 mM adenosine triphosphate (ATP), pH 7.8, Promega) on a gentle rotator at room temperature overnight.

2.5. Preparation of poly-RNP nanoparticles

The circular DNAs (final concentration of 0.03 μM) were mixed with T7 RNA polymerase (10 U/μL, New England Biolabs, USA), rNTP mix (8 mM, New England Biolabs, USA), reaction buffer (80 mM Tris-HCl, 12 mM $MgCl_2$, 2 mM DTT, 4 mM spermidine, New England Biolabs, USA) and Cas9 protein (73 ng/μL). The mixed solution was then incubated for 20 h at 37 °C for the enzymatic process. The poly-RNP nanoparticles were purified by Amicon Centrifugal Filter (MWCO 100 kDa).

2.6. Characterization of poly-RNP nanoparticles

Atomic force microscopy (AFM, Park NX10, Korea) was used to obtain high resolution digital images of the RNA-Cas9 ribonucleoprotein particles. The AFM sample was dropped and dried onto a silicon wafer. All the AFM images were recorded using a Non-Contact Cantilever (PPP-NCHR 5M, Nanosensors, Korea) in non-contact mode at room temperature. The images were analyzed using XEI software (Park Systems, Korea). An internal image of the nanoparticles was examined with a JEM-2100F TEM (JEOL, Japan) operated at 200 kV. A fluorescent microscope (Nikon, Eclipse Ti, Japan) and Nucleocounter (NC-3000, Chemometec, Denmark) was used to image the nanoparticles. To progress the fluorescent microscope and image cytometry analysis of the RNP nanoparticles, the Cas9 protein was labeled with TMR (tetramethylrhodamine) before the RCT reaction. After preparing the RNP nanoparticles, they were stained with SYBR Green. The solution of the particles was analyzed on NC-Slide A2 (Chemometec). The results were visualized using NucleoViews NC-3000 software (Chemometec). The flow cytometry result was re-plotted by FlowJo (USA). The zeta potential and size distribution of the nanoparticles were measured using a