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Scaffold-based delivery of CRISPR/Cas9 ribonucleoproteins for genome editing

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Abstract

The simple and versatile CRISPR/Cas9 system is a promising strategy for genome editing in mammalian cells. Generally, the genome editing components, namely Cas9 protein and single-guide RNA (sgRNA), are delivered in the format of plasmids, mRNA or ribonucleoprotein (RNP) complexes. In particular, non-viral approaches are desirable as they overcome the safety concerns posed by viral vectors. To control cell fate for tissue regeneration, scaffold-based delivery of genome editing components will offer a route for local delivery and provide possible synergistic effects with other factors such as topographical cues that are co-delivered by the same scaffold. In this chapter, we detail a simple method of surface modification to functionalize electrospun nanofibers with CRISPR/Cas9 RNP complexes. The mussel-inspired bio-adhesive coating will be used as it is a simple and effective method to immobilize biomolecules on the surface. Nanofibers will provide a biomimicking microenvironment and topographical cues to seeded cells. For evaluation, a model cell line with single copies of enhanced green fluorescent protein (U2OS.EGFP) will be used to validate the efficiency of gene disruption.

Keywords

Gene delivery; Cas9 protein; ribonucleoprotein; tissue engineering; electrospinning.

Running head

Scaffold-based delivery of CRISPR/Cas9

1. Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is an efficient and simple-to-implement genome editing technique ***(1,2)***. In CRISPR/Cas9 based genome editing, two main components are needed: Cas9 protein, which is an RNA-guided DNA endonuclease, and single-guide RNA (sgRNA), which binds to target sequence complementarily ***(1-3)***. The Cas9-sgRNA complex binds to a target sequence and induces a double-stranded break (DSB) at a specific target site. When a template is included, the DSB may be repaired by Homolog-Directed Repair (HDR) by inserting the template along the break site. Without this template, the DSB can be repaired by Non-Homologous End Joining (NHEJ) pathways that lead to deletion and insertion at the site. Hence, the gene or targeted locus will be disrupted.

There are a few methods to deliver Cas9 and sgRNA effectively. Generally, Cas9 can be delivered in the format of plasmid, mRNA or protein ***(4,5)***. Plasmid and mRNA will be transcribed and translated into Cas9 protein in the cell cytoplasm. Subsequently, the translated Cas9 proteins will be complexed with sgRNA. Comparatively, Cas9-sgRNA ribonucleoprotein (RNP) complexes are more efficient in transfection and have the least off-target effects ***(6)***. Nonetheless, these Cas9-sgRNA RNP complexes still require efficient delivery vehicles into the cells to achieve genomic editing. Here, non-viral approaches are desirable as they overcome the safety concerns posed by viral vectors ***(6,7)***.

Scaffolds are frequently used in tissue engineering and regenerative medicine approaches to support tissue regrowth and deliver topographical cues that can mimic the native tissue microenvironment. These scaffolds can also be modified to locally deliver cells and therapeutics, such as proteins, small molecules and nucleic acids to the injured tissues. Therefore, the incorporation of CRISPR/Cas9 systems with scaffolds offers a route to modulate cell behavior on the scaffold. Furthermore, such scaffold-mediated CRISPR/Cas9 systems can provide possible synergistic effects from other factors that are co-delivered by the same scaffold.

Immobilizing proteins or nucleic acids on scaffolds have been intensively reported ***(8,9)***. One method of immobilizing these biomolecules involves surface modification of the scaffold. In this chapter, a simple method of surface modification to functionalize electrospun nanofibers with CRISPR/Cas9 complexes will be described. Nanofibers will provide a biomimicking microenvironment and topographical cues to seeded cells. Here, the mussel-inspired bio-adhesive coating will be used as it is a simple and effective method to immobilize biomolecules on surfaces. Such an approach has been demonstrated to be effective in delivering nucleic acids in the form of siRNAs ***(10-12)*** and miRNAs ***(13-16)***, as well as Cas9-sgRNA RNP complexes ***(17)***. As a proof of concept, a model cell line with single copies of enhanced green fluorescent protein (U2OS.EGFP) will be used to validate the efficiency of genome editing.

2. Materials:

2.1. Preparation of electrospun fiber scaffolds with bio-adhesives

1. Polycaprolactone (PCL, M_w: 80 kDa and 40 kDa)
2. 2,2,2-trifluoroethanol (TFE)
3. 18 mm diameter glass coverslips
4. Carbon tape
5. 22G needle
6. 1mL syringe
7. Electrospinning setup: Syringe pump, rotating wheel with speed control, wires, power source
8. Silicone glue
9. 70% ethanol
10. Distilled water (sterilized by 0.02 μm syringe filter)
11. 3,4-dihydroxy-l-phenylalanine (L-DOPA)
12. Buffer for polymerization of DOPA: 10 mM Bicine, 250 mM NaCl, HCl (to adjust pH), pH 8.5
13. Laminin mouse protein
14. 1 × PBS

2.2. Preparation of complexes

1. sgRNA targeting EGFP:

   GAAATTAATACGACTCACTATAGGTGAACCGCATCGAGCTGAAGTTTTAGAGCTA GAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACC GAGTCGGTGCTTTTTT (see **Note 1**)
2. Cas9 protein: EnGen Cas9 NLS protein (NEB) (see **Note 2**)
3. Transfection reagent: Lipofectamine CRISPRMAX Cas9 Transfection Reagent (Invitrogen) (see **Note 3**)
4. Opti-MEM

2.3. Cell seeding/ culture

1. U2OS.EGFP cells
2. Dulbecco's Modified Eagle's Medium (DMEM) with glutaMAX
3. Fetal bovine serum (FBS)
4. Penicillin-Streptomycin (10,000 U/mL, PS)
5. Culture medium for U2OS.EGFP cells: DMEM with glutaMAX containing 10% FBS and 1% PS.
6. Trypsin-EDTA (0.05%)

2.4. Evaluation

1. 4 % Paraformaldehyde (PFA)
2. Triton-X 100
3. Rhodamine Phalloidin
4. DAPI
5. 1 × PBS
6. Proteinase K working stock: 0.6 U/mL in TE buffer
7. DNA purification kit

8. Genomic cleavage assay kit
9. Gel electrophoresis setup
10. Agarose
11. Tris/Acetate/EDTA (TAE) buffer or other appropriate buffers for agarose electrophoresis (separation of DNA)
12. Ethidium bromide or other appropriate dyes for staining and visualization of DNA (such as SYBR Green etc)

3. Methods:

3.1. Electrospun fiber scaffold preparation

1. Prepare electrospun fiber scaffolds by electrospinning ***(14, 17)***. (see **Note 4**)
2. Melt 1 g of PCL (40 kDa) at 60 °C for 30 minutes to form a PCL block in a mold (Aluminium foil may be used to make this mold with a dimension of 20 mm by 20 mm by 10 mm). After this PCL block is formed, trim the block down to 10 mm by 5 mm by 10 mm before using a cryostat to section this block into small strips of PCL films of 20 μm thickness.
3. Dissolve PCL (80 kDa) in TFE (14 % W/V) overnight.
4. Vortex the solution until homogenous.
5. Before electrospinning, adhere the small strips of PCL films (40 kDa) with 20 μm thickness onto glass coverslips (diameter ⌀ 18 mm) to create a 10 mm × 10 mm area. Place these PCL films onto the coverslips to form a square configuration before melting them on a hotplate, at 50 °C for 15 seconds. Remove coverslips from hotplate once these films turn transparent.
6. Fix 8 coverslips on a rotating collector (ϕ = 15cm) with carbon tapes.
7. Electrospin the PCL fibers onto the coverslips that are fixed on a rotating collector (2400 rpm). Apply a flow rate of 1.0 ml/h and voltages of +8 kV and -4 kV to the needle tip and rotating collector respectively. Fix the spinneret-to-collector distance at 21 cm. Allow electrospinning to occur for 6 min 30 sec (100 μl of 14 % W/V PCL solution electrospun) before removing the coverslips off the rotating collector.

8. Secure the electrospun PCL fibers onto the coverslips with silicone glue. Using a fine paintbrush, apply a small amount of silicone glue along the parameter of the square configuration formed by small strips of PCL films (refer to step 5). Allow silicone glue to dry overnight at room temperature. Prior to surface modification, store scaffolds in 12-well plates for no more than a week at room temperature.
9. Sterilize the electrospun fibers by UV irradiation (30 min) followed by incubation in 70% ethanol for 15 min.
10. Wash scaffolds with sterilized DI water 3 times to rinse away the ethanol.

3.2. Creating adhesive surfaces with pDOPA

1. To create the pDOPA adhesive surfaces for Cas9:sgRNA protein-RNA lipofectamine complexes to adhere onto, coat the electrospun scaffolds with mussel-inspired pDOPA.
2. Dissolve L-DOPA in a buffer that consisted of 10 mM Bicine and 250 mM NaCl (pH 8.5), at a concentration of 0.5 mg/mL.
3. Wait 5 minutes for L-DOPA to be fully dissolved.
4. Incubate each electrospun scaffold in 1 mL of the DOPA solution on a shaker (80 rpm) for 2 h at room temperature.
5. After pDOPA coating, wash the electrospun fibers with sterilized DI water to remove unbound L-DOPA monomers.
6. Coat laminin onto the scaffolds at 10 μg per scaffold (1 cm^2) for 2 h at 37 °C. Dilute laminin in sterile 1× PBS. (see **Note 5**)

3.3. Formation of Cas9:sgRNA-lipofectamine complexes on the scaffold

1. For Cas9:sgRNA complex formation, mix sgRNA and Cas9 protein in Opti-MEM at a molar ratio of 1:1 according to the manufacturer's protocol. Here, lipofectamine CRISPRMAX is used.
2. For a 1 cm^2 scaffold, dilute 7.5 pmol of sgRNA and Cas9 protein each in 25 μL of Opti-MEM.

3. Thoroughly mix the diluted sgRNA and Cas9 with 2.5 µL of Cas9 Plus reagent (provided with Lipofectamine CRISPRMAX).
4. Concurrently, dilute 1.5 µL of Lipofectamine CRISPRMAX into another microtube with 25 µL of Opti-MEM and incubate for 1 min at room temperature.
5. Add the mixture with sgRNA, Cas9 protein and Cas9 Plus reagent (Cas9-sgRNA complexes) to the diluted Lipofectamine CRISPRMAX and incubate for 15 min at room temperature to form Cas9:sgRNA-lipofectamine complexes.
6. Transfer the mixture onto the scaffold (pDOPA coated fibers). Incubate for 1 h at 37°C.
7. Remove the supernatant and wash once with OptiMEM.
8. Seed cells at an appropriate density. For U2OS.EGFP, seed at 10,000 cells per cm^2 in 100 µL. Allow the cells to attach at 37°C before submerging the whole scaffold with an additional 900 µL of culture medium.

3.4. Evaluation (EGFP expression)

1. After 3 days of culture, fix the cells on scaffolds with 4 % PFA for 10 min at room temperature.
2. As cells tend to align and elongate along the aligned electrospun fibers, it may be difficult to detect the outline of cells. Therefore, additional staining for cellular cytoskeleton may be needed to count the number of cells for quantifying the changes in EGFP expression (see **Note 6**).
3. Add 0.1% Triton-X diluted in PBS to the scaffolds and incubate for 15 minutes.
4. Remove the supernatant and stain the cells with Rhodamine Phalloidin (1:500 in PBS) and DAPI (1:1000 in PBS) for 1 h at room temperature.
5. Wash the scaffolds 3 times with PBS.
6. Take images using an epifluorescence or confocal microscope.
7. Successful gene editing will result in knockout of EGFP. The knockout efficiency of EGFP can be quantified as the percentage of EGFP-positive cells per DAPI.
8. Additional evaluation is needed to validate the genome-editing efficiency. (see **Note 7**)

3.5. Evaluation (genomic cleavage)

1. After 3 days of culture, wash the scaffolds once with PBS.
2. Add 200 μL of Proteinase K solution to each scaffold and incubate the scaffolds in Proteinase K solution for overnight at 37 °C to extract genomic DNA.
3. Transfer the solution from scaffolds to the microtubes (one scaffold per tube). The optimal number of scaffolds per microtube depends on the required number of cells stated on the kit. If more DNA is desired, pool solution from more scaffolds as one sample. Flush the fibers with the solution a few times to ensure genomic DNA are washed out from the scaffolds.
4. Purify the genomic DNA using a DNA purification kit or established DNA purification protocol. (see **Note 8**)
5. After the pure genomic DNA is obtained, run a genomic cleavage assay using a genomic cleavage detection kit or established protocol. During the PCR step to amplify the DNA, use primers that are flanking the EGFP cleavage site: GACGTAAACGGCCACAAGTT (forward) and GCGGATCTTGAAGTTCACCT (Reverse). (see **Note 9**)
6. Successful genomic cleavage will result in two additional bands with DNA fragments of smaller sizes (~100bp and 300bp) in addition to the PCR product of EGFP (~400bp). Quantify the cleavage efficiency based on the intensity of the bands on the gel using the ImageJ 'Gel Analyzer' plugin.

4. Notes

1. Successful genome editing depends heavily on a good sgRNA design. The sequence here has been validated in multiple publications that target EGFP. For other genes, sgRNA has to be designed. This can be done through various online sgRNA designing platforms. Once the sgRNA is designed or the sequence is known, it can be either synthesized in the lab using synthesis kits or ordered directly from companies that can synthesize the required sgRNA.

2. There are a variety of Cas9 proteins commercially available. We have only tested EnGen Cas9 NLS protein from New England Biolabs which works well in this approach with U2OS.EGFP cells.
3. There is a range of commercially available delivery vehicles that are primarily lipid and cationic polymer-based. We have tested a few and found that lipofectamine CRISPRMAX works with high efficiency in our approach with USOS.EGFP cells. Note that this also depends on the types of cells being transfected as this may work differently for primary cells or difficult-to-transfect cells.
4. Here we describe a method to fabricate electrospun fibers scaffold on the coverslips to simplify observation and imaging. A similar protocol may be applied to the other scaffold designs but optimization will be needed.
5. Laminin coating may be optional depending on the cell type seeded. This needs to be optimized. In our preliminary trial, we found that laminin enhanced cell proliferation of U2OS.EGFP cells on the scaffolds.
6. Confocal microscopy helps in clearly identifying GFP^{+} and GFP^{-} cells with just GFP and DAPI signals on the fiber scaffolds. In this case, cytoskeleton staining may not be needed. Alternatively, other staining or methods that can visualize cellular outline may be used. However, we have tried using WGA to label cellular membranes, but it did not provide clear cellular outlines on the fiber scaffolds. Note that the fluorophore chosen shouldn't be in a similar range of wavelength that the GFP has.
7. For gene editing evaluation, genomic disruption assay using U2OS.EGFP is a fast and efficient method. However, for proper evaluation, genomic cleavage detection (GCD) assay is also needed to validate the results. Sequencing can also be performed to check for the indels. If a different scaffold design or when different reagents are used, loading efficiency and release profile may be checked through fluorescence tagged Cas9 or sgRNA. Alternatively, RNA assay (Ribogreen Assay) can be performed as a proxy for quantifying the sgRNA amount.

8. The topic of genomic DNA purification is beyond the scope of this chapter. Therefore, we only cover the methods until extracting genomic DNA from the scaffold efficiently. Established protocol or commercial DNA purification kits can be used to purify genomic DNA. We have tried Zymo DNA Clean & Concentrator and it worked well.
9. The topic of genomic cleavage assay is beyond the scope of this chapter. Established protocol or commercial genomic cleavage detection kits can be used to detect genomic cleavage. We have tried GeneArt Genomic Cleavage Detection Kit (Thermofisher) and it worked well. The primers here were designed for EGFP and sgRNA presented in this protocol. For other genes of interest, the primers shall be designed to flank the cleavage site that the Cas9:sgRNA complexes bind to.

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