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Surface- and Hydrogel-Mediated Delivery of Nucleic Acid Nanoparticles

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Surface- and Hydrogel-Mediated Delivery of Nucleic Acid Nanoparticles

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Abstract

Gene expression within a cell population can be directly altered through gene delivery approaches. Traditionally for nonviral delivery, plasmids or siRNA molecules, encoding or targeting the gene of interest, are packaged within nanoparticles. These nanoparticles are then delivered to the media surrounding cells seeded onto tissue culture plastic; this technique is termed bolus delivery. Although bolus delivery is widely utilized to screen for efficient delivery vehicles and to study gene function in vitro, this delivery strategy may not result in efficient gene transfer for all cell types or may not identify those delivery vehicles that will be efficient in vivo. Furthermore, bolus delivery cannot be used in applications where patterning of gene expression is needed. In this chapter, we describe methods that incorporate material surfaces (i.e., surface-mediated delivery) or hydrogel scaffolds (i.e., hydrogel-mediated delivery) to efficiently deliver genes. This chapter includes protocols for surface-mediated DNA delivery focusing on the simplest and most effective methods, which include nonspecific immobilization of DNA complexes (both polymer and lipid vectors) onto serum-coated cell culture polystyrene and self-assembled monolayers of alkanethiols on gold. Also, protocols for the encapsulation of DNA/cationic polymer nanoparticles into hydrogel scaffolds are described, including methods for the encapsulation of low amounts of DNA (<0.2 μg/μL) and high amounts of DNA (>0.2 μg/μL) since incorporation of high amounts of DNA poses significant challenges due to aggregation.

Keywords: Gene delivery, Hydrogel, Surface-mediated, Transfection, Nonviral

1. Introduction

Gene expression within a cell population can be directly altered through gene delivery approaches, which have tremendous potential for therapeutic applications, such as gene therapy to correct genetic deficiencies or tissue engineering scaffolds, where gene delivery can present chemical factors to guide tissue

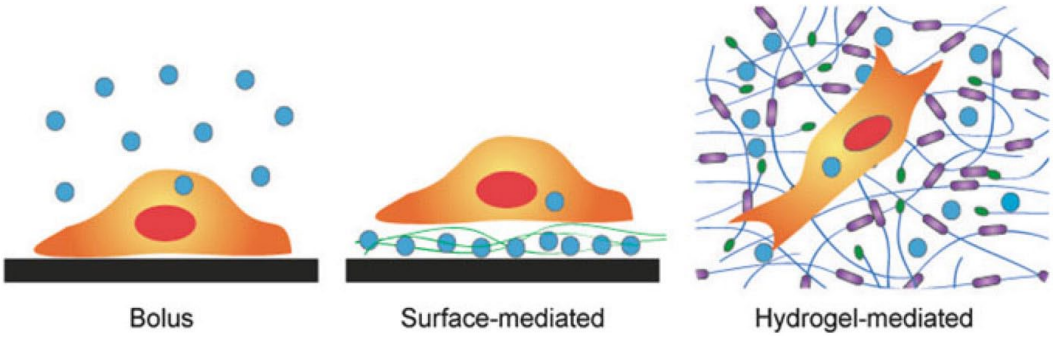

Figure 1. Bolus, surface- and hydrogel-mediated gene delivery.

formation in regeneration matrices for treatment of organ loss and failure. Furthermore, gene delivery is often critical to research applications, such as functional genomics, cell culture studies, and biotechnological assays. Gene delivery can be performed with both viral and nonviral vectors, the latter of which are the focus of the techniques described in this chapter. Traditionally for nonviral delivery, plasmids or siRNA molecules, encoding or targeting the gene of interest, are packaged within nanoparticles. These nanoparticles are then delivered to the media surrounding cells seeded onto tissue culture plastic; this technique is termed bolus delivery (Figure 1). Although bolus delivery is widely utilized to screen for efficient delivery vehicles and to study gene function in vitro, this delivery strategy may not result in efficient gene transfer for all cell types or may not identify those delivery vehicles that will be efficient in vivo. Furthermore, bolus delivery cannot be used in applications where patterning of gene expression is needed. In this chapter, we describe methods that incorporate material surfaces (i.e., surface-mediated delivery) or hydrogel scaffolds (i.e., hydrogel-mediated delivery) to efficiently deliver genes (Figure 1).

Surface-mediated delivery, also termed solid-phase delivery or reverse transfection, refers to the delivery of nucleic acid nanoparticles from a surface or biomaterial that supports cell adhesion. DNA nanoparticles are immobilized to the surface or biomaterial and cells are plated directly on top of the immobilized nanoparticles (Figure 1), which enhances the extent of transgene expression, along with increasing cell viability (1). This method of delivery was first described in 2002 using a specific avidin–biotin bond to tether nanoparticles to surfaces (2, 3) and was later extended to nonspecific absorption of the nanoparticles to a variety of biomaterial surfaces (1, 4–10). Nonspecific adsorption of nanoparticles is accomplished through noncovalent mechanisms (1, 4–14), including hydrophobic,

electrostatic, and van der Waals interactions. Nonspecific binding depends upon the molecular composition of the vector (e.g., lipid versus polymer) and the relative quantity of each (e.g., nitrogen-to-phosphate ratio or N/P), as well as properties of the surface (e.g., hydrophilicity, charge, presence of serum proteins).

In surface-mediated delivery, DNA is concentrated at the delivery site and targeted to cells adhered to the substrate (1–3, 15). The major advantages of surface-mediated DNA delivery are the following: (1) decreased nucleic acid nanoparticle aggregation and degradation, since nanoparticles can be produced in the most favorable buffer for their formation and then immobilized to the surface, which preserves the size observed in solution and avoids exposure of the particles in solution to the cell culture media, which often induces aggregation and decomplexation; (2) cells cultured on the substrate are exposed to elevated DNA concentrations within the local microenvironment (but typically overall concentration of DNA is minimized when compared to bolus techniques), which enhances transfection and minimizes cytotoxicity (1–10, 16) ; (3) elimination of mass transport limitations for the nanoparticles to reach the cell; and (4) immobilization of DNA complexes to substrates allows for the ability to pattern transfection (9, 17), allowing for spatial control of gene delivery, which is critical for biotechnological and tissue engineering applications. Below, the reader will find protocols for surface-mediated DNA delivery focusing on the simplest and most effective methods, which include nonspecific immobilization of DNA complexes (both polymer and lipid vectors) onto serum-coated cell culture polystyrene and self-assembled monolayers (SAMs) of alkanethiols on gold.

Hydrogel-mediated delivery has been studied for over a decade primarily through the encapsulation of naked DNA during hydrogel formation. Naked DNA has been successfully incorporated inside hydrogels composed of collagen (18), pluronic-hyaluronic acid (19), dimethacrylated poly(lactic acid)-b-poly(ethylene glycol)-b-poly(lactic acid) triblock copolymers (20), alginate (21), oligo(polyethylene glycol) fumarate (22), and engineered silk elastin (23). Although naked DNA has shown gene expression and ability to guide regeneration in vivo (18, 24), limitations with low gene transfer efficiency and rapid diffusion of the DNA from the hydrogel scaffold motivated the use of DNA nanoparticles instead of naked DNA. DNA condensed either with cationic peptides, lipids, or polymers has been introduced into fibrin hydrogels (25–29), enzymatically degradable PEG hydrogels (30), and PEG-hyaluronic acid hydrogels (26). The delivery of genes from hydrogel scaffolds is becoming an attractive route to introduce foreign genes to cells for several reasons. First, in the biotechnology area, where new delivery agents are being investigated for in vivo gene transfer, the delivery of genes inside a matrix may be a better model for in vivo gene transfer. For example, hydrogels can be used to recreate disease models in vitro, including multiple cell types and complex extracellular matrices (e.g., cancer and organoid models). Second, in tissue engineering and regenerative medicine applications, it is often desired to transfect cells either infiltrating the scaffold

(endogenous) or that are transplanted. In the case of endogenous cell transfection, the hydrogel scaffold serves as a depot that contains genes or siRNA that can transfect cells as the cells infiltrate the scaffold or the matrix is degraded and the genes or siRNA are released. In the case of transplanted cell transfection, the matrix and cells are encapsulated together. The goal here is to transfect the transplanted cells at a later time to induce their differentiation or the differentiation of nearby cells. Below, the reader will find protocols for the encapsulation of DNA/cationic polymer nanoparticles into hydrogel scaffolds. We divide the encapsulation into low amounts of DNA (<0.2 μg/μL) and high amounts of DNA (>0.2 μg/μL) since incorporation of high amounts of DNA poses significant challenges due to aggregation.

2. Materials

For surface-mediated delivery, the following reagents are necessary.

2.1. Dulbecco's Phosphate-Buffered Saline (PBS): 1× Solution containing 2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, and 8.1 mM Na_2HPO_4 with no calcium or magnesium, prepared in MilliQ water, pH 7.4. Stored at room temperature. Sterile filtered.

2.2. 10% Fetal Bovine Serum (FBS): 10% FBS prepared in 1× PBS (prepared as described above). Prepare sterilely and aliquots may be stored at 4°C for a short term (<2 weeks) or −20°C for longer time periods.

2.3. TBS Buffer: 100 mM tris(hydroxymethyl)aminomethane–HCl, pH 7.4, 150 mM NaCl.

2.4. Opti-MEM® I Reduced Serum Media (1×) (Invitrogen™ by Life Technologies) (see Note 1).

2.5. Tissue Culture Polystyrene (TCPS): Delivery can be performed on any TCPS substrate (microscope slides, 6-, 12-, 24-, 48-, or 96-well plates or dishes, see Note 2), with the requirement that it is "tissue culture" polystyrene (indicating that it has been surface modified, typically through corona discharge or gas-plasma, to promote protein and cell adhesion (31, 32)). These reagents are needed for the preparation of SAMs:

2.6. Ethanol: 200 Proof, filtered through a 0.22 μm filter, degassed with N_2 gas for at least 15 min.

2.7. Argon Gas: It is recommended to store the alkanethiol solutions and gold substrates under argon gas. Do not store SAMs under argon as it can cause toxicity issues.

2.8. 11-Mercaptoundecanoic Acid (MUA): 2 mM solution in ethanol, described above.

2.9. Gold Substrates: Gold-coated glass slides, composed of a titanium adhesion layer and 100–500 Å of gold, can be purchased commercially or prepared using e-beam evaporation. Gold-coated slides should be cut into smaller pieces with a diamond-tipped glass cutter so that pieces fit into standard 48-well tissue culture plates (for this protocol, may be adjusted based on experimental setup).

The following reagents are used for DNA nanoparticle preparation:

2.10. Reporter Plasmid DNA: Transfection is typically assessed by either fluorescent reporter genes (e.g., GFP or DS-red) or luminescent reporter genes (e.g., Firefly or Renilla Luciferase), or both. Plasmids are available commercially with these reporter genes, typically driven by CMV promoters. These plasmids can be expanded using commercially available prep kits such as the endotoxin free Giga Prep kit from Qiagen (see Note 3) and their expression assayed using fluorescent microscopy or commercially available kits. DNA is typically stored in TE buffer (10 mM Tris–Cl, pH 7.4–8.0, 1 mM EDTA, pH 8.0) at −20°C. Prepare stock solution in TE of 1 μg/ μL for complexation protocol below.

2.11. Branched Poly(Ethylene Imine) (PEI) Stocks:

1. Prepare 10 mg/mL solution of PEI (Sigma, branched, 25 kDa) in 0.1 M $NaHCO_3$ (sodium bicarbonate) (pH 8.2). Branched PEI is a liquid.
2. Using 6,000–8,000 MWCO tubing, dialyze the PEI against MilliQ water for 24 h, changing water at least three times over the course of dialysis.
3. After dialysis, lyophilize the PEI solution.
4. When lyophilization is complete, weigh the lyophilized PEI and resuspend in TBS buffer at 1 mg/mL. Store stocks at −20°C.

2.12. Linear PEI Stocks: Linear PEI (Polysciences, 25 kDa) comes as a very basic polymer with Cl^- as a counterion. To remove excess Cl^- ions, which would be present at neutral pH, the PEI is precipitated and dialyzed.

1. Dissolve 100 mg of PEI in 10 mL of DI water at room temperature. While stirring the solution bring the pH down to 7.4 using 1 N HCl to help dissolve it.
2. Precipitate the PEI by raising the pH to 12 with 1 N NaOH. Keep stirring the solution. The solution will turn white and blurry, but still stay in suspension. The PEI will be neutral since it will be deprotonated and it will become hydrophobic and insoluble in water.

3. Using 6,000–8,000 MWCO tubing, dialyze the precipitated PEI against DI water, pH 12 for 1 day to remove the chlorine ion (change water 2× every 2–3 h using 3–4 L DI water, pH 12 each time) and for 2–3 days against DI water (pH 7) to re-protonate.

4. The PEI will become partially soluble in water again. After dialysis lyophilize the PEI solution.

5. Redissolve the entire 100 mg in 10 mL water or, alternatively, weigh out the desired amount of powder and dissolve it in water for a 10 mg/mL solution. About 10 µL of HCl per 3 mg of PEI will be required to help it dissolve. After the PEI is dissolved adjust the pH to 7.4.

6. Aliquot 1 mg/tube and lyophilize the PEI solution a second time. Store the lyophilized stocks at −20°C and resuspend in water or the desired buffer/saline solution. A typical final concentration is 1 µg/µL. Adjust final pH to 7.4.

2.13. Lipofectamine™ 2000 (Invitrogen™ by Life Technologies) is used (see Note 4).

Materials for hydrogel-mediated delivery are the following:

2.14. Triethanol amine (TEOA) Buffer: 0.3 M TEOA (stored under argon). Pipette 19.2 mL of ultrapure water into a beaker, add 0.8 mL of TEOA, and mix. Set pH to 8.6–8.7 with 37% HCl (the initial pH is 9–10 and requires 20–40 droplets). Sterile filter the final buffer. Use immediately or alternatively the buffer can be placed into aliquots and stored at −20°C under argon until use.

2.15. PEG-VS:

1. Dissolve four-arm PEG-OH in tetrahydrofuran (THF) under inert atmosphere and heat to reflux in a Soxhlet apparatus filled with molecular sieves for 3–4 h.

2. Allow the solution to cool to the touch and add sodium hydride (NaH; please note that NaH is a highly flammable material when in contact with water), at fivefold molar excess over OH groups, followed by the addition of divinyl sulfone, at 50-fold molar excess over OH groups.

3. Allow the reaction to continue at room temperature (RT) under argon atmosphere with constant stirring for 3 days.

4. Neutralize the reaction solution with acetic acid, filter it, concentrate by roto-evaporation, and precipitate it in ice-cold diethyl ether.

5. Precipitation should be repeated three times to remove unreacted divinyl sulfone.

6. Dry the final product under vacuum and store under argon at −20°C.

7. The degree of functionalization is confirmed with 1H NMR (in $CDCl_{3)}$: 3.6 ppm (PEG backbone), 6.1 ppm (d, 1H, $CH_{2)}$, 6.4 ppm (d, 1H, $CH_{2)}$, and 6.8 ppm (dd, 1H, $–SO_2CH$).

2.16. HA-AC:

1. Dissolve 2 g hyaluronic acid (HA) (60 kDa) in 400 mL DI water.

2. Add 36.3 g adipic acid dihydrazide (ADH, 21.8 molar excess over carboxylic acid groups), dissolve completely, and adjust the pH to 4.75 with 1 N HCl.

3. Add 4 g 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, 3.95 molar excess over carboxylic acid groups) as a catalyst.

4. Maintain the pH at 4.75 until it becomes stable (~2 h) and let it react overnight.

5. Purify HA–ADH through dialysis (6,000–8,000 MWCO) against DI water for 3 days, changing the water at least 2× per day.

6. Collect the purified HA–ADH and lyophilize it until completely dry (~2 days). Dry HA–ADH can be stored at −20°C until the next step.

Using these reaction conditions, ~40% of the carboxyl groups are modified with ADH based on the trinitrobenzene sulfonic acid (TNBSA, Pierce) assay.

7. Make reaction buffer: 10 mM HEPES with 150 mM NaCl and 10 mM EDTA at pH 7.2.

8. Dissolve HA–ADH in reaction buffer for a final concentration of ~5.5 mg/mL. Mix using a stir bar and plate for about 2 h to completely dissolve.

9. Weigh out 1 g *N*-acryloxysuccinimide (NHS-Ac, ~3–4 molar excess over amine groups) and dissolve in 10 mL DMSO. Keep on ice.

10. React HA–ADH with NHS-Ac by adding 1 mL NHS-Ac in DMSO every 15–20 min and let it react overnight.

11. Purify HA-AC through dialysis (6,000–8,000 MWCO) against DI water for 3 days, changing the water at least 2× per day.

12. Collect the purified HA-Ac and lyophilize it until completely dry (~2 days). Dry HA-Ac can be stored at −20°C until used.

The degree of functionalization should be confirmed with 1H NMR (in D2O).

2.17. Peptide Crosslinker Aliquots: Michael addition chemistry can be used to cross-link PEG-VS or HA-AC into a hydrogel. Any di-thiol-containing molecule would work; however, to make the hydrogel degradable by cells protease degradable peptides are generally used. The peptide Ac-GCRDG-PQGIWGQDRCG-NH_2 (synthesized through solid-phase peptide synthesis or commercially purchased) has been used by several groups (for reviews see Refs. 33, 34). Aliquots of the peptide are made to prevent its exposure to oxygen and disulfide bond formation. The peptide crosslinker powder is dissolved in 0.1% TFA, aliquoted into microcentrifuge tubes, and lyophilized. Because TFA is a volatile salt the resulting lyophilized powder contains only the peptide. The amount to be aliquoted depends on the number of cross-links desired or *r* ratio, which is the molar ratio of –SH groups to –VS or –AC groups. For example, for 100% modified PEG-VS hydrogels with 6% PEG an *r* ratio close to 1 is used, while for a 40% modified HA-AC with 3% gel an *r* ratio close to 0.3 is used. Typically enough material is aliquoted to be used in a single use. No freeze–thaw cycles are recommended.

2.18. RGD Peptide Aliquots: Michael addition is used to modify PEG-VS or HA-AC with cell adhesion peptides such as RGD. Similar to the peptide crosslinker aliquots, RGD peptide aliquots are made to prevent disulfide bond formation. RGD peptide powder (Ac-GCGYGRGDSPG-NH_2, synthesized through solid-phase peptide synthesis or commercially purchased) is dissolved in 0.1% TFA, aliquoted into microcentrifuge tubes, and lyophilized. The amount to be aliquoted depends on the amount of RGD desired inside the hydrogel material (typically 30–500 μM final concentration for HA hydrogels and 30–200 μM for PEG-VS gels). Typically enough material is aliquoted to be used in a single use. No freeze–thaw cycles are recommended.

2.19. Reporter Plasmid DNA: To assess transfection from cells seeded inside hydrogel scaffolds, secreted proteins are recommended. The two most commonly used are secreted alkaline phosphatase mammalian expression vector (pSEAP, Genlantis, San Diego, CA) and Gaussia luciferase (pGLUC, New England Biolabs, Ipswich, MA). Both plasmids can be expanded using commercially available prep kits such as the endotoxin free Giga Prep kit from Qiagen and their expression assayed using commercially available kits.

2.20. Sigmacote-Coated Slides:

1. Prepare Sigmacote® -coated glass slides by immersion of slides in Sigmacote® solution using a 50 mL conical tube for 1 min and air drying for 3 min. Repeat this 3× for each slide (see Note 5).

2. After the third coating, place slides into oven above 100°C for 2 h to overnight to allow for complete drying.

3. Wash the slides with ethanol to sterilize before use. You should notice that the water slides off the slide easily.

2.21. Agarose: Dissolve 670 mg low-melting-point agarose in 1 L MQ H_2O, allow 1–2 h to mix using a stir bar and stir plate, and filter solution using a 0.22 µm filter. Store stock solution at RT.

2.22. Sucrose: Dissolve 3.5 g sucrose in 10 mL Millipore water and filter solution using a 0.22 µm filter. Store stock solution at 4°C.

3. Methods

3.1. Surface-Mediated DNA Delivery

For substrate-mediated gene delivery, the properties of the surface are critical to both immobilization strategies and transfection efficiencies, as are the properties of the DNA complexes. Surface-mediated delivery has been reported on a variety of surfaces, including SAMs (9, 10), and standard tissue culture polystyrene (PS) plastic (1, 4–6, 35). As the highest transfection levels were reported on carboxylic acid-terminated SAMs (9) and serum-coated PS (1), methods for these two surfaces are reported in this chapter. Methods are included for both polymer- and lipid–DNA complexes (Figure 2).

3.1.1. For Surface Preparation of Serum-Coated Polystyrene Proceed as Follows (See Also Notes 6–8)

1. Add 100 µL of 10% FBS per cm^2 (e.g., add 100 µL per well of a 48-well plate).
2. Let plate incubate at room temperature for 2 h (see Note 9).
3. After incubation, remove 10% FBS solution from wells and wash with 1× PBS (see Note 10).

3.1.2. For Surface Preparation of COO^--Terminated SAMs Proceed as Follows (*See Note 11)*

1. Prepare 2 mM solution of MUA in filtered, degassed ethanol (see Note 12).
2. Wash gold substrates with copious amounts of acetone followed by ethanol. Dry gold substrates under a stream of N_2 gas.
3. Place gold surfaces into 2 mM MUA solution and allow monolayer formation to proceed for 45 min (see Note 13).

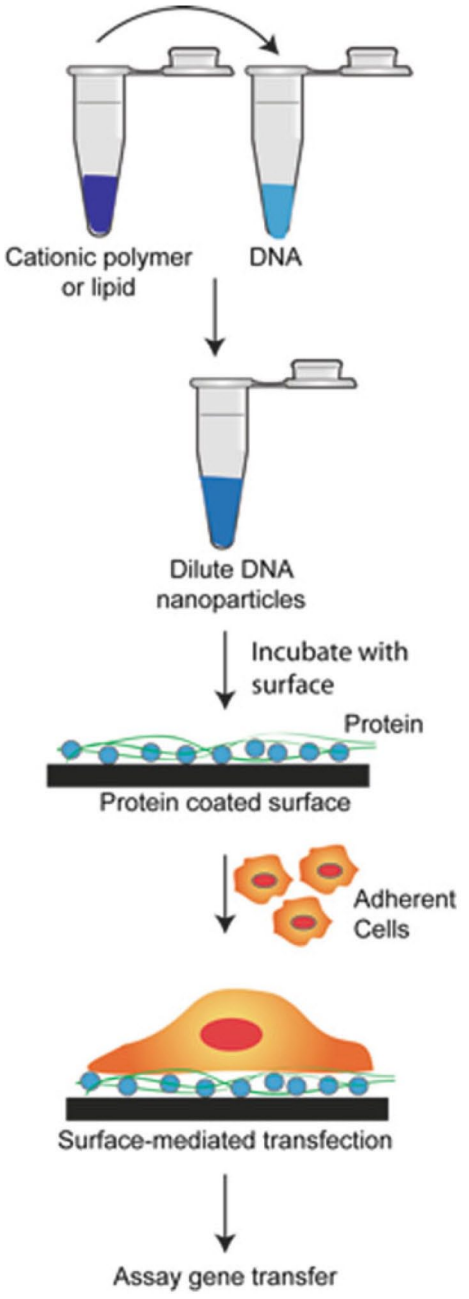

Figure 2. Visual protocol for surface-mediated gene transfer.

4. After monolayer formation, rinse SAMs with ethanol and dry surfaces under stream of N_2 gas.
5. Allow surfaces to equilibrate in PBS for 15 min to ensure deprotonation of terminal functional groups at physiological pH.

3.1.3. PEI–DNA Complexes Are Prepared with Branched PEI, 25 kDA with 3 m g of DNA in 300 m L Per Well at an N/P of 20 (See Notes 14 and 15)

1. Add 177 µL of TBS (see Note 16) to a microfuge tube labeled "DNA." Add 3 µL of plasmid DNA stock solution (1 µg/µL) and mix by pipetting up and down (see Note 17).
2. Add 111.8 µL of TBS to a microfuge tube labeled "PEI." Add 8.2 µL of PEI stock solution (1 mg/mL) and mix by pipetting up and down (see Note 18).
3. Add the contents of the PEI tube to the DNA solution (DNA tube) (see Note 19), vortex gently for 10 s, and let complexes form for 15 min at room temperature (see Note 20).

3.1.4. Lipofectamine 2000–DNA Complexes Are Generated with 1 m g of DNA in 300 m L Per Well, and a DNA:Lipofectamine 2000 Ratio of 1:2 (See Notes 14 and 15)

1. Add 147 µL of Opti-MEM media to a microfuge tube labeled "DNA." Add 3 µL of plasmid DNA stock solution (1 µg/µL) and mix by pipetting up and down.
2. Add 144 µL of Opti-MEM media to a microfuge tube labeled "LF2000." Add 6 µL of Lipofectamine 2000 stock solution and mix by pipetting up and down.
3. Add the contents of the LF2000 tube to the DNA solution (DNA tube) (see Note 21), pipette up and down, and let complexes form for 20 min at room temperature (see Note 20).

3.1.5. After Formation, Complexes are Immobilized

1. Once complex formation is complete (for either PEI–DNA complexes OR Lipofectamine 2000–DNA Complexes), add 300 µL of complexes to surfaces (for either FBS-coated PS or COO^--terminated SAMs) and let deposit for 2 h at room temperature.
2. After 2 h of complex deposition, remove unbound complexes by washing surfaces twice with TBS (for PEI–DNA complexes) or Opti-MEM (for Lipofectamine 2000–DNA complexes) (see Notes 22 and 23).

3.1.6. After Complex Immobilization to Surfaces, Cells Are Seeded onto the Complexes and the Transfection Efficiency Analyzed

1. Seed 300 µL of cells in each well (15,000 cells/well) (see Note 24).
2. Incubate cells at 37°C and 5% CO_2 for 24–48 h.
3. Assay for transfection using fluorescence microscopy or luminescence assay (depending on the reporter gene present on plasmid) after 24–48 h (see Notes 25–27).

3.2. Hydrogel-Mediated Gene Delivery: Direct Encapsulation (<0.2 m g DNA/ m L Hydrogel)

The encapsulation of DNA into hydrogel scaffolds will require different protocols depending on the quantity of DNA loaded. Low amounts of DNA, <0.2 µg/µL, may be loaded into hydrogel scaffolds without significant aggregation. The following protocol is for the synthesis of a 100 µL 3% HA or 100 µL 6% PEG hydrogel scaffold with 0.05 µg DNA/ µL of hydrogel. We will be incorporating DNA complexed with linear PEI at an N/P ratio of 7 (see Note 28).

3.2.1. HA-RGD/PEG-RGD Is Synthesized as Follows

1. Weight out and dissolve HA-AC (3 mg) or PEG-VS (6 mg) into 38 µL or 27 µL TEOA buffer. For the HA polymer, incubate for 20 min at 37°C to speed up the dissolution process. Ensure that polymer solution pH is 8.2–8.3 at this point.
2. Take an RGD peptide aliquot out from the −30°C freezer and place it on ice.
3. Use the dissolved HA-AC or PEG-VS to dissolve the RGD aliquot. Vortex thoroughly and allow to incubate for 30 min at 37°C.
4. Add 5 µL of 0.3 M TEOA to HA-RGD (see Note 29).
5. Keep the newly reacted HA-RGD or PEG-RGD on ice.

3.2.2. Thereafter, DNA Nanoparticles (Polyplexes) Are Formed at an N/P Ratio of 7

1. Place 5 µg of DNA into a sterile microcentrifuge tube and dilute to 10 µL in Millipore water.
2. Place 4.57 µg of PEI into a sterile microcentrifuge tube and dilute to 8 µL in Millipore water.
3. Add the PEI solution to the DNA solution, vortex gently for 15 s, and incubate for 10 min at room temperature. After the 10-min incubation place the DNA nanoparticles (polyplexes) on ice for at least 5 min.

3.2.3. The Hydrogel Is Synthesized as Follows

1. Take a crosslinker aliquot out of the freezer and place in −20°C cooler.
2. Subculture cells and dilute to 25,000 cells/ µL or 20,000 cells/ µL. The final desired cell concentration in hydrogel is 1,000–5,000 cells/ µL of gel. Place cells on ice.

The next steps must be done quickly to avoid premature gelation in the tube!

3. Bring the polyplex solution to 0.3 M TEOA by adding 2 µL of 3 M TEOA and immediately add the entire 20 µL polyplex solution to the HA-RGD or the PEG-RGD. Place on ice.
4. Add 20 µL or 31 µL of the 25,000 cells/µL or 20,000 cells/µL cell stock to the HA-RGD/DNA nanoparticle or PEG-RGD/DNA nanoparticle solution and mix thoroughly but gently. Place on ice (see Note 30).
5. Dissolve the crosslinker in 17 µL or 22 µL of ice-cold TEOA buffer and quickly add to the HA-RGD/Cell/polyplex suspension or the PEG-RGD/Cell/polyplex suspension. Mix thoroughly using a positive displacement pipette or a wide orifice tip (see Note 31). The final gel solution should have a pH of 8.0–8.1.
6. Quickly cast the desired gels either as one large 100 µL gel or several small gels into the gel caster (Figure 3). Place gel caster at 37°C for 30 min.

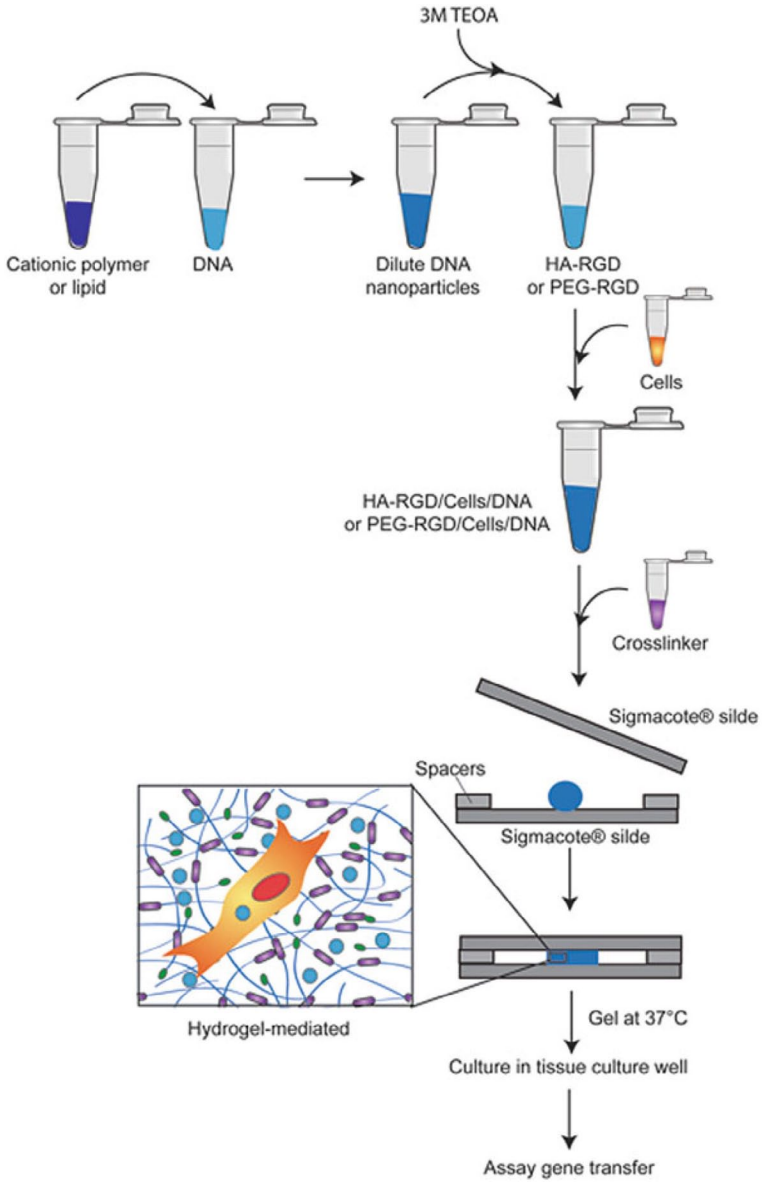

Figure 3. Visual protocol for hydrogel-mediated gene transfer for low concentrations of DNA.

7. Place the gel(s) into the desired tissue culture plate size. The large gel can be cut with a biopsy punch to result in several small gels. 96- or 48-well plates are recommended for culturing to avoid diluting out the reporter protein.
8. By collecting the media of the cells and performing the appropriate assay, Phospha-light SEAP reporter gene assay system or Biolux gaussia luciferase assay kit, transfection can be monitored. Gene transfer has been observed as early as 2 days and as late as 7 days depending on the cell type and how well the cells migrate through the material.

3.3. Hydrogel-Mediated Gene Delivery: Caged Nanoparticle Encapsulation

For high concentrations of DNA polyplexes direct encapsulation results in severe aggregation. The Segura laboratory has developed a process that can be used to encapsulate high concentrations, up to 5 μg/ μL, into hydrogel scaffolds (36). As an example, the following protocol is for the synthesis of 100 μL HA-AC/MMP hydrogels with 100 μg of DNA or 100 μL PEG-VS/MMP hydrogels with 100 μg of DNA. First, polyplex powder is prepared:

1. Dilute pDNA (100 μg) with 1,000 μL MilliPore water H_2O in a 15 mL conical tube.
2. In a separate 15 mL tube, add 100 μL (350 mg/mL) sucrose and 2,200 μL MilliPore water.
3. Add 91.3 μL of L-PEI to the diluted sucrose solution.
4. Add the dilute PEI/sucrose solution to the pDNA solution, vortex for 15 s, and incubate at room temperature for 15 min.
5. Add 1.5 mL of (.67 mg/mL) agarose to DNA/PEI solution, vortex for 10 s, and immediately dip into liquid nitrogen.
6. Allow 5 min to freeze completely and lyophilize.
7. Remove tubes from lyophilizer and place tubes at −20°C until ready for use.

3.3.1. HA-RGD/PEG-RGD Synthesis Is Carried Out Slightly Different as Described in Subheading 3.2

1. Weight out and dissolve HA-AC (3 mg) or PEG-VS (6 mg) into 38 μL or 27 μL TEOA buffer. For the HA polymer incubate for 20 min at 37°C to speed up the dissolution process.
2. Take an RGD peptide aliquot out from the −20°C freezer and place it on ice.
3. Use the dissolved HA-AC or PEG-VS to dissolve the RGD aliquot. Vortex thoroughly and allow to incubate for 30 min at 37°C.
4. Add 25 μL of 0.3 M TEOA to HA-RGD (see Note 29).
5. Keep the newly reacted HA-RGD or PEG-RGD on ice.

3.3.2. Hydrogels Are Synthesized in the Following Way

1. Take a crosslinker aliquot out of the freezer and place in −20°C cooler.
2. Subculture cells and dilute to 25,000 cells/μL or 20,000 cells/μL. The final desired cell concentration in hydrogel is 1,000–5,000 cells/ μL of gel. Place cells on ice.

The next steps must be done quickly to avoid premature gelation!

3. Place polyplex powder in a 1.5 mL microcentrifuge tube and mix with HA-RGD/cell or PEG-RGD/cell suspension.
4. Add 20 μL or 31 μL of the 25,000 cells/μL or 20,000 cells/μL cell stock to the HA-RGD/DNA nanoparticle or PEG-RGD/DNA nanoparticle solution and mix thoroughly but gently. Place on ice.

5. Dissolve the crosslinker in 17 µL or 22 µL of ice-cold TEOA buffer and quickly add to the HA-RGD/Cell/polyplex suspension or the PEG-RGD/Cell/polyplex suspension. Mix thoroughly using a positive displacement pipette or a wide orifice tip (see Note 30). The final gel solution should have a pH of 8.0–8.1.
6. Quickly cast the desired gels either as one large 100 µL gel or several small gels into the gel caster (Figure 4). Place gel caster at 37°C for 30 min.

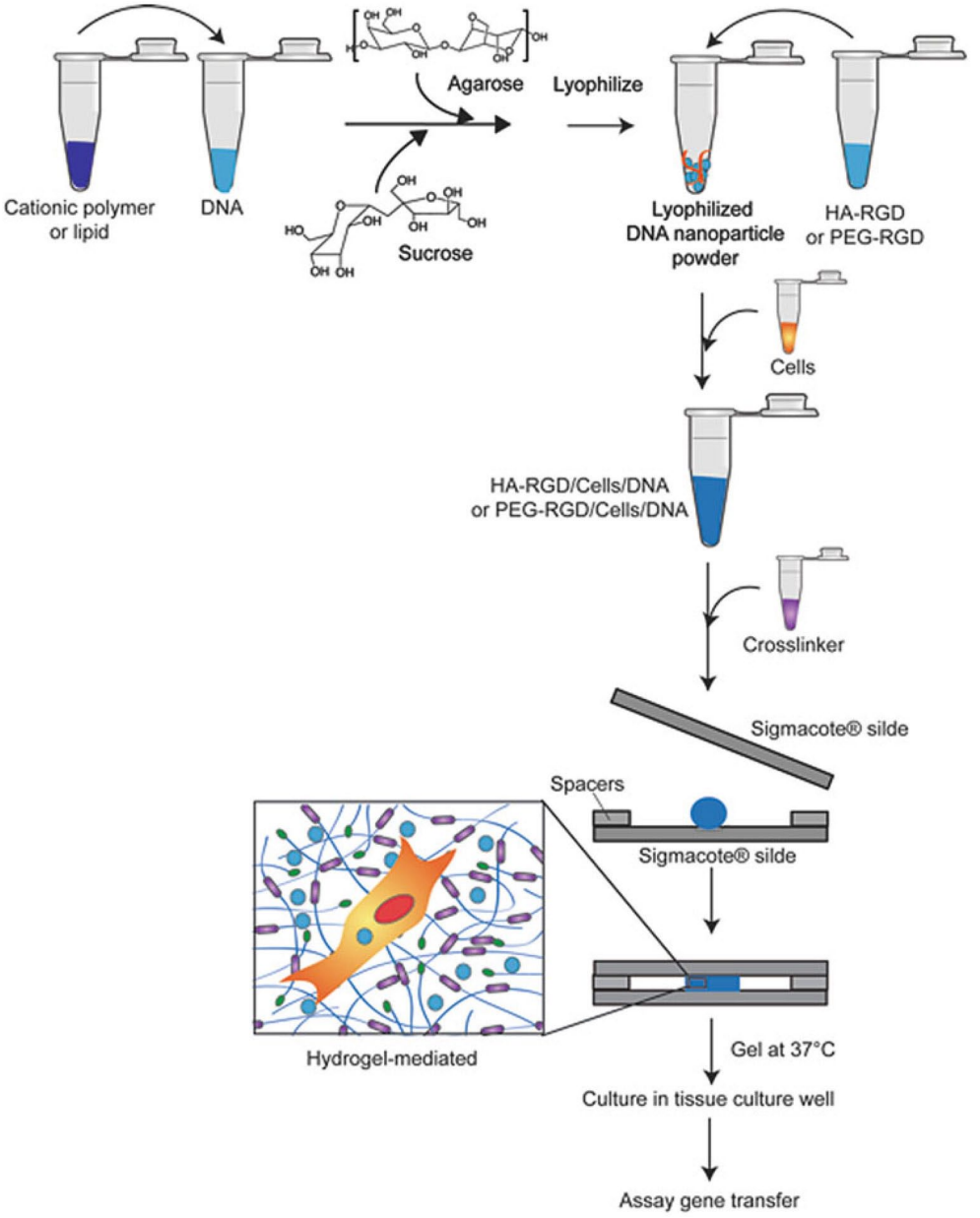

Figure 4. Visual protocol for hydrogel-mediated gene transfer for high concentrations of DNA.

7. Place the gel(s) into the desired tissue culture plate size. The large gel can be cut with a biopsy punch to result in several small gels. 96- or 48-well plates are recommended for culturing to avoid diluting out the reporter protein.
8. By collecting the media of the cells and performing the appropriate assay, Phospha-light SEAP reporter gene assay system or Biolux gaussia luciferase assay kit, transfection can be monitored. Gene transfer has been observed as early as 2 days and as late as 7 days depending on the cell type and how well the cells migrate through the material.

4. Notes

1. Invitrogen recommends the use of Opti-MEM® I Reduced Serum Media for transfection with Lipofectamine 2000 (37). We have found that its use does enhance transfection, but for many cell lines other serum-free media can be used for formation of the lipoplexes.

2. Most of our protocols have been optimized with and utilize 48-well plate format. The least effective well for surface-mediated delivery is the 96-well plate, as those wells are often not perfectly flat and thus complexes deposit irregularly.

3. Any protocol for DNA preparation from bacterial cultures is sufficient, but "endotoxin-free" buffers are essential for transfection-grade DNA.

4. This chapter includes protocols for one lipid-based transfection reagent (Lipofectamine 2000) and one polymer-based transfection reagent (PEI). However, surface-mediated delivery has also been accomplished with jet-PEI (3, 16), Lipofectamine LTX (35), FugeneHD (Rocher) (unpublished results), and Effectene (Qiagen) (35), as well as viral vectors (15, 38–43). The encapsulation of lipoplexes and jetPEI/DNA polyplexes into hydrogel scaffolds has also been possible using a similar protocol (36).

5. After use, pour the Sigmacote® back into a dark glass bottle (not back into the clean stock solution). This Sigmacote can be reused 3–4×.

6. Methods are per cm^2, and can be scaled accordingly for different surface areas.

7. These methods can be modified to other surfaces including poly(lactide-co-glycolide) (PLGA) (7, 8, 11, 42) and collagen (11, 43).

8. This protocol can also be modified to use purified extracellular matrix/serum proteins, including fibronectin, collagen, and laminin (5, 7), with fibronectin typically resulting in the highest transfection levels.

9. Serum incubation can proceed for longer periods of time, even overnight at 4°C, without negatively affecting transfection levels.

10. Perform this step right before adding complexes, as described under "Subheading 3.1.6."

11. Incorporation of poly(ethylene glycol) groups into carboxylic acid-terminated SAMs may increase transfection (10). In this case, prepare SAMs by immersing gold substrates into a mixed alkanethiol solution containing both MUA as well as $HS(CH_2)_{11}(OCH_2CH_2)_6$ OH, in ratios identical to what is desired in the final SAM (typically 40% or less of the PEGylated thiol).

12. Prepare alkanethiol solution fresh for each experiment and use amber bottles or those covered with aluminum as thiols are light sensitive. Cover with argon if not used immediately.

13. Typically we use gold substrates of ~0.5 cm^2 cut from a gold-coated microscope slide, as described above. These small pieces of gold are best handled with three-prong forceps. We typically form SAMs in foil-lined glass scintillation vials that contain 7–8 mL of MUA solution.

14. Both DNA amount and N/P ratio (or DNA:lipid ratio) need to be optimized for each cell type; the conditions used in this chapter represent good starting optimization points for NIH/3T3s; also these amounts are for a single well, so scale accordingly for multiple wells. For Lipofectamine™ 2000, Invitrogen recommends a Lipofectamine™/DNA ratio (μL/μg) between 0.5 and 5 for optimum transfection (37). For branched PEI, N/Ps are recommended between 10 and 25.

15. Adsorption is typically 10–20% of total DNA added to surfaces (1, 9) ; thus use approximately 5–10 times as much DNA as would typically be used in bolus conditions.

16. Can also use 150 mM NaCl to form polyplexes.

17. 60% of total complex volume should be with DNA.

18. 40% of total complex volume should be with PEI.

19. This order of addition is critical to transfection success.

20. During this time prepare surfaces for complex immobilization; for FBS-coated PS, remove the FBS and rinse with PBS as described above or for SAMs, incubate with PBS to ensure deprotonation.

21. We have found that even for lipids this order of addition is critical to transfection success.

22. Longer adsorption times result in larger mass of DNA complexes immobilized, but can also be associated with loss of activity; thus adsorption time is another variable that can be manipulated.

23. Be sure that cell suspension is prepared and cells are ready to seed immediately after this rinsing step.

24. Cell density is for NIH/3T3s; this value will need to be optimized for each cell type.

25. Time after delivery to assay transfection may also need to be optimized.

26. For transfection studies on SAMs, move each SAM to a new well before proceeding with luminescence or protein assay to ensure that transfection is only measured in cells adhered to SAM and not on surrounding PS.

27. For transfection studies on SAMs, gold quenching of fluorescence may limit observation of transfection by fluorescent reporter genes. To reduce quenching, be sure to use upright microscope or fix cells and then place SAMs face down into well before imaging.

28. Encapsulation of polyplexes into fibrin, collagen, and UV cross-linked hydrogel scaffolds is also possible using the same protocols (36).

29. This 5 µL or 25 µL volume could be used to introduce other factors to the hydrogel such as growth factors. Further, this volume could be added to the cell volume to make the cell stock less concentrated.

30. The cell concentration in the gel and the cell concentration used in the stock solution should be optimized per cell type. For example, we have found that for MSCs 25,000 cells/µL is the highest concentration that can be used before severe cell death is observed post encapsulation. It is recommended to make different cell stock concentrations and make hydrogels without DNA/siRNA nanoparticles to determine the ideal cell concentration.

31. The amount of crosslinker added should be ~0.3 moles of thiol/mole of acrylate for the HA-AC hydrogel and 0.9 thiol/mole of vinyl sulfone for the PEG hydrogel. This is assuming a 40% modified HA molecule and a 100% modified PEG-VS polymer. This ratio can be varied to make hydrogels with different mechanical properties. It is recommended to use a spreadsheet program such as Microsoft Excel to calculate the ratio of thiols to acrylate or vinyl sulfone. Further the amount of crosslinker added will also be dependent on the activity of the crosslinker. Since the crosslinkers are bi-thiol molecules they are easily oxidized into disulfides. To determine the activity of the crosslinker, a thiol quantification assay such as the Ellman's assay should be performed. The moles of the crosslinker used to achieve a desired thiol/acrylate or vinyl sulfone ratio should be adjusted accordingly.

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