CHAPTER

5

Cationic Polymers for Gene Delivery: Formation of Polycation–DNA Complexes and *in Vitro* Transfection

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I. INTRODUCTION

Although nonviral gene delivery systems were first introduced with cationic lipids, cationic polymers (or polycations) have gained increasing attention in recent years as a nonviral vector for gene therapy due to their nonimmunogenicity and low acute toxicity. In both systems, DNA is incorporated into a complex by electrostatic interactions between anionic phosphate groups of DNA and cationic groups of lipids or polycations under physiological conditions. A large number of polycations have been studied as nonviral vectors, such as poly-L-lysine (Wu and Wu 1987), poly(ethylenimine) (Behr *et al*., 1999; Ahn *et al*., 2002), poly(amidoamine) dendrimers (Haensler and Szoka, 1993), poly(2-dimethylaminoethyl methacrylate) (Cherng *et al*., 1996), and chitosan (Lee *et al*., 1998). Cationic polymers have flexibility in designing a carrier with well-defined structural and chemical properties on a large scale as well as the ability to introduce functional moieties (e.g., targeting moieties). In general, the ability to vary and control the physicochemical properties in cationic lipid–based system is relatively limited. This article describes methods that are used commonly in characterizing polycation-based gene delivery systems in *in vitro* transfection, focusing on complex formation between polycations and DNA.

II. MATERIALS AND INSTRUMENTATION

HEPES (Cat. No. H4034), bicinchoninic acid (BCA) protein assay kit (Cat. No. BCA-1), fetal bovine serum (FBS, Cat. No. F3885), 100× penicillin–streptomycin (Cat. No. P0781), trypan blue (Cat. No. T0076), methylthiazoletetrazolium (MTT, Cat. No. M5655), and poly(L-aspartic acid) (Cat. No. P5387) are from Sigma (St. Louis, MO). Polyethylenimine (PEI, Cat. No. 40,872-7), glycerol (Cat. No. 19,161-2), and dimethyl sulfoxide (DMSO, Cat. No. 27,043-1) from Aldrich (Milwaukee, WI). Dulbecco's modified eagle's medium (DMEM, Cat. No. 12100-046) and 10× trypsin–EDTA (Cat. No. 15400-054) are from Gibco-BRL (Carlsbad, CA). Agarose (Cat. No. 161-3101), ethidium bromide (EtBr Cat. No. 161-0433), TBE (Tris/boric acid/EDTA, Cat. No. 161-0733), bromphenol blue (Cat. No. 161-0404), and xylene cyanole FF (Cat. No. 161-0423) are from Bio-Rad (Hercules, CA). The plasmid maxi kit (Cat. No. 12162) is from QIAgen (Valencia, CA). pSV-β-galactosidase (Cat. No. E1081), β-galactosidase enzyme assay system (Cat. No. E2000), pGL3 control containing the SV40 promoter-driven luciferase reporter gene (Cat. No. E1741), and Luciferase assay system (Cat. No. E1500) are from Promega (Madison, WI).

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The spectrofluorometer (Spex FluoroMax-2) is from JY Horiba (Edison, NJ). The gel electrophoresis (Mini-PROTEAN 3 electrophoresis cell) system is from Bio-Rad. The microtiter plate reader (SOFTmax PRO) is from Molecular Device Corp. (Sunnyvale, CA). The luminometer (Lumat LB9507) is from Berthold Technologies (Oak Ridge, TN). The dynamic light scattering (90 plus) is from Brookhaven Instruments Corp. (Holtsville, NY).

III. PROCEDURES

A. Formation of Polycation/DNA Complexes (Polyplexes)

Solutions

1. *HEPES-buffered saline:* 15 m*M* HEPES, 150 m*M* NaCl, pH 7.4. To make 500 ml, add 1.787 g HEPES and 4.383 g NaCl to 480 ml water and adjust the pH to 7.4 with 1 *M* NaOH. Adjust the total volume to 500 ml with water. Sterilize the buffer by filtering through a filter with a 0.22-μm pore size.

2. *Plasmid stock solution:* Plasmid DNA (e.g., pSV-β-galactosidase or pGL3 control containing the SV40 promoter-driven luciferase reporter gene) is transformed into *Escherichia coli*. The transformed cells are grown in larger quantities (0.5–1.0 liter) of Luria and Bertan (LB) broth. The plasmid DNA is isolated using the plasmid maxi kit from QIAgen according to instructions of the manufacturer. The plasmid DNA is collected in HEPES-buffered saline and stored at 4°C. The purity is confirmed by 1% agarose gel electrophoresis, and DNA concentration can be measured by UV absorption at 260 nm.

3. *Polycation stock solution, 5 mg/ml in HEPES-buffered saline:* Dissolve 50 mg of a cationic polymer in 10 ml HEPES-buffered saline. Sterilize the polymer solution through a filter with a 0.22-μm pore size.

Steps

1. DNA: Dilute the plasmid DNA stock solution to a final concentration of 20 μg/ml in HEPES-buffered saline.

TABLE I Amount of Polyethylenimine (PEI) for Forming Polyplexes with Various N : P ratios[a]

N : P ratio	0.2	0.5	1.0	1.5	2.0	3.0	4.0	5.0	6.0
PEI (μl)[b]	0.10	0.26	0.52	0.77	1.03	1.55	2.06	2.58	3.10

[a] Twenty micrograms of DNA is used.
[b] PEI stock solution at 5 mg/ml in HEPES-buffered saline.

2. Cationic polymers: Make a series of dilutions in HEPES-buffered saline (see Table I). The N : P ratio is defined as the molar ratio of amino groups in polycations to phosphate groups in DNA.
3. Add polycation solutions to plasmid DNA solutions at different N : P ratios and vortex gently.
4. Incubate at room temperature for 30 min to allow complex formation.
5. Store at 4°C.

B. Analysis of Polycation/DNA Complexes

1. Ethidium Bromide Displacement Assay

The degree of DNA condensation by polycations can be determined by an EtBr displacement assay using a fluorometer (Wadhwa *et al.*, 1995; Choi *et al.*, 1998). Ethidium bromide intercalates between stacked base pairs of double-stranded DNA to give a significant increase of fluorescence intensity. Addition of a polycation causes a large drop in fluorescence intensity due to displacement of ethidium bromide molecules from DNA, which indicates the condensation of DNA to form complex particles.

Solutions

1. *Ethidium bromide at 10 mg/ml*: The stock solution should be stored in a bottle wrapped in aluminum foil at 4°C and in the dark.

2. *Plasmid DNA stock solution*: *(See solutions in Section III,A)*

3. *Stock solutions of polycations*: *(See solutions in Section III,A)*

Steps

1. The spectrofluorometer is operated with an excitation wavelength (λ_{ex}) of 510 nm and an emission wavelength (λ_{em}) of 590 nm. Use slit widths set at 10 nm and an integration time of 3 s. Perform all experiments in triplicate.
2. Dilute the DNA stock solution to a final concentration of 10 μg/ml, including 0.4 μg/ml EtBr in a test curvette (total volume of 2 ml).
3. Incubate the DNA solution for 15 min to ensure interactions between DNA and EtBr.
4. Measure the fluorescence and calibrate to 100%.
5. Measure the background fluorescence with EtBr alone and set to 0%.
6. Add aliquots of the polycation stock solution sequentially to the DNA solution at various N : P ratios, mix gently, and measure the fluorescence after each addition.
7. Plot the graph the relative fluorescence (%) *vs* N : P ratio.

8. Determine or compare the abilities of different polycations to condense DNA.

2. *Agarose Gel Retardation Assay*

The complex formation between polycations and DNA can be observed by a decrease of mobility of DNA in agarose gel electrophoresis (Ahn *et al.*, 2002).

Solutions

1. *Ethidium bromide solution*: *See solutions in Section III,B*
2. *Loading buffer solution:* 0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanole FF, 5% glycerol
3. *Electrophoresis buffer solution*: The ionic strength and pH of the buffer can play major roles in complex stability. Standard electrophoresis buffers such as TBE (90 m*M* Tris–borate, 2 m*M* EDTA, pH 8.3) and TAE (40 m*M* Tris–acetate, 1 m*M* EDTA, pH 7.9) commonly prove satisfactory, but others such as TE (10 m*M* Tris, 1 m*M* EDTA) have been used.
4. *Dissociation buffer solution:* Dissolve 100 mg poly(L-aspartic acid) in 5 ml double distilled water.

Steps

1. Prepare polycation/DNA complexes at various N : P ratios in HEPES-buffered saline and incubate at room temperature for 30 min to allow the complexes to form properly.
2. Add aliquots of the polyplexes (20 µl) in Eppendorf tubes containing 2 µl gel-loading buffer and mix gently.
3. Load the complexes onto wells of the 0.8% agarose gel containing ethidium bromide (1 µg/ml). Also, apply the controls for free DNA and a free polycation to the gel.
4. Perform electrophoresis in 0.5× TBE buffer at 100 V until the bromphenol blue has migrated 5–7 cm through the gel.
5. Visualize and photograph the electrophoresed gel on an UV illuminator to show the location of DNA and complexes.
6. Incubate the gels in the dissociation buffer for 30 min to disturb polycation/DNA complexes and rephotograph the gel to show the presence of DNA dissociated from the polymer.

3. *Measurements of Particle Size*

Solutions

1. *Plasmid DNA stock solution in double-distilled water*: *See solutions in Section III,A*
2. *Stock solutions of polycations in double-distilled water*: *See solutions in Section III,A*

Steps

1. Turn on dynamic light-scattering equipped with a He–Ne laser at a scattering angle of 90°.
2. Set parameters for software. Set viscosity to 0.890 centipoise (cP), refractive index (RI) medium to 1.333 in water. However, if complexes are in 150 m*M* NaCl, set viscosity to 1.145 cP and RI medium to 1.340; if in HEPES, set viscosity to 1.546 cP and RI medium to 1.363. Set the temperature to 25°C for all the solutions.
3. Prepare 500-µl complexes at 20 µg/ml in test tubes and filter through a 0.45-µm filter. Cap the sample and allow it to equilibrate for 30 min before initiating measurements.
4. Calculate the particle size and size distribution using nonnegative least squares (NNLS) algorithms. When the difference between the measured and calculated baselines is less than 0.2%, accept the correlation function. If not, the concentration of complexes can be controlled to give a reasonable signal.
5. Measure the mean particle size, polydispersity factor using the Stokes–Einstein equation, and the cumulant method.

C. *In Vitro* Transfection

This procedure describes the *in vitro* transfection of 293T cells using cationic polymers, such as PEI and PLL, as a vector. The protocol can be used for all adherent cell types with slight modifications. For suspension type cells, cells need to be spun down before changing media.

Solutions

1. *HEPES-buffered saline*: *See solutions in Section III,A*
2. *Phosphate-buffered saline (PBS):* 137 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.47 m*M* KH_2PO_4, pH 7.4. To make 1 liter of 10× PBS, dissolve 80.06 g NaCl, 2.01 g KCl, 11.50 g of Na_2HPO_4, and 2.00 g KH_2PO_4 in 800 ml water. Adjust the pH to 7.4 with 1 *N* HCl. Adjust the total volume to 1 liter with water. To make 1 liter of 1× PBS, mix 100 ml 10× PBS (described earlier) with 900 ml of water. Sterilize the buffer through a filter with a 0.22-µm pore size.
3. *Tissue culture medium:* Tissue culture medium may vary, depending on the requirements of the cell line. Typically, Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin–streptomycin, and 2 m*M* L-glutamine are used to maintain cell lines.
4. *Plasmid stock solution: See solutions in Section III,A*
5. *Stock solutions of polycations: See solutions in Section III,A*

6. *0.05% trypsin–0.02% EDTA solution (1×):* Add 10 ml of 10× trypsin–EDTA to 90 ml of PBS and store at 4°C

7. *0.5% trypan blue solution:* Dissolve 0.5 g trypan blue in 100 ml PBS and filtrate through a filtration paper to remove possible crystals. Store at –20°C

Steps

1. Passage 293T cells 3–4 days before the transfection experiment.
2. Detach the cells with trypsin–EDTA solution and determine the cell number and cell viability using trypan blue. Mix 50 µl cell suspension and 50 µl of 0.5% trypan blue solution. Bring the mixture into a counting chamber and count the number of uncolored (vital) and blue (dead) cells in a number of squares using a microscope. When more than 10 cells are counted per square, dilute the cell suspension and count again.
3. Seed cells in a 6-well tissue culture plate at a density of ~2×10^5 cells/well in 2 ml completed DMEM and incubate overnight at 37°C in a humidified atmosphere under 5% CO_2.
4. Prepare polycations/DNA complexes at various N : P ratios in HEPES-buffered saline and incubate for 20 min at room temperature (see steps in Section III,A).
5. Remove culture media from the cells and replace with 2 ml of serum-free DMEM.
6. Introduce 400 µl polyplexes to each well and incubate for 4 h at 37°C in an incubator.
7. Aspirate transfection media, replace with 2 ml of completed DMEM, and culture the cells in an incubator for 48 h at 37°C.
8. Evaluate the transfection efficiency.

D. Determination of Transfection Efficiency

1. β-*Galactosidase Activity*

The β-galactosidase activity in transfected cell lysates can be determined using the substrate *O*-nitrophenyl-β-D-galactopyranoside (ONPG).

Solutions

1. *Phosphate-buffered saline: See solutions in Section III,C*
2. β-*Galactosidase enzyme assay system with reporter lysis buffer (Promega) including reporter lysis buffer (5×), assay 2× buffer,* β-*galactosidase, 1 M sodium carbonate*

Steps

1. Remove growth media from the cells and wash twice with 2 ml PBS.
2. Add 200 µl of a lysis buffer to cover the cells and rock the 6-well plate slowly several times to ensure complete coverage of the cells.
3. Incubate at room temperature for 15 min.
4. Transfer the cell lysate to a microcentrifuge tube.
5. Centrifuge at top speed in a microcentrifuge for 2 min and transfer the supernatant to a fresh tube.
6. Pipette 50 µl of the cell lysates into wells of a 96-well plate.
7. Meanwhile, make a series of dilutions for the standard curve of β-galactosidase in 1× lysis buffer between 0 and 5.0×10^{-3} units. Prepare 50 µl of each β-galactosidase standard per well.
8. Add 50 µl of assay 2× buffer to each well of the 96-well plate and mix by pipetting.
9. Incubate at 37°C until faint yellow color has developed or the highest standard shows an absorbance of 2 or more.
10. Stop the reaction by adding 150 µl of 1 *M* sodium carbonate.
11. Measure the absorbance of the samples at 420 nm in a microtiter plate reader and calculate the β-galactosidase amount of a sample by comparing with the linear standard curve.
12. The β-galactosidase amount in each sample is normalized to milligrams of protein. Protein concentrations in cell lysates can be measured using a BCA protein assay kit according to instructions of the manufacturer.

2. Luciferase Activity

Solutions

1. *Phosphate-buffered saline: See solutions in Section III,C*
2. *Luciferase assay system (Promega) including a lysis buffer and luciferase assay reagent*

Steps

1. Remove growth media from the cells and wash twice with 2 ml PBS.
2. Add 200 µl of a lysis buffer to cover the cells and rock the 6-well plate slowly several times to ensure complete coverage of the cells.
3. Incubate at room temperature for 15 min.
4. Transfer the cell lysate to a microcentrifuge tube.
5. Centrifuge at top speed in a microcentrifuge for 2 min and transfer the supernatant to a fresh tube.
6. Dispense 20 µl of the supernatant into a luminometer tube.
7. Set the read time to 10 s (the read time can be varied).

8. Initiate reading by injecting 100 µl of Luciferase assay reagent into the tube.
9. Luciferase activity in each sample is normalized to milligrams of protein. Protein concentrations in cell lysates can be measured using a BCA protein assay kit according to instructions of the manufacturer.

E. *In Vitro* Cytotoxicity Assay

Cytotoxicity of polycation/DNA complexes can be evaluated by the MTT assay. The assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form dark blue formazan crystals by active mitochondria dehydrogenases. This conversion only occurs in living cells.

Solutions

1. *MTT stock solution, 5 mg/ml in PBS*
2. *Tissue culture medium:* DMEM
3. *Polyplexes solution*

Steps

1. Seed cells in 24-well microplates at a density of ~4 $\times 10^4$ cells/well in 1 ml of completed DMEM and incubate overnight at 37°C in a humidified atmosphere under 5% CO_2.
2. Remove culture media from the cells and replace with 1 ml of serum-free DMEM.
3. Introduce 40 µl of polyplexes to each well and incubate for 4 h at 37°C in an incubator.
4. Aspirate transfection media, replace with 1 ml of completed DMEM, and culture cells in an incubator for 48 h at 37°C.
5. Remove old media and replace with new completed DMEM.
6. Add 50 µl of 5 mg/ml MTT solution to each well and incubate for 4 h at 37°C.
7. Remove MTT-containing media and add 750 µl of dime to each well and pipette up and down to dissolve formazan crystals formed by live cells.
8. Measure the absorbance at 570 nm using a microtiter plate reader
9. Calculate the absorbance percentage relative to that of untreated control cells.

IV. COMMENTS

Complex formation between polycations and DNA can be affected by several factors, such as the nature of the cation, the molecular weight of the polycation, the molecular architecture of the polycation, and the N : P ratio. Full complexation to form stable nanoparticles between 20 and 100 nm is necessary for effective transfection. Generally, shorter polycations need a higher N : P ratio to achieve full condensation.

V. PITFALLS

1. Large aggregates or precipitated materials can be observed in some cases. They lower the transfection efficiency and may cause toxic effects to cells. They may occur when the N : P ratio is too low or the pH of the transfection medium is too high. Generally, small cationic particles can be formed at the excess of positive groups of polycations compared with negative groups of DNA.

2. The size of polycation/DNA complexes may vary in different buffers mainly due to their different ionic strengths.

3. In some cases, complex particles may not be spherical. Particles with any extended structures may be ignored with light scattering, which leads to inaccurate results. Therefore, the morphology of complex particles must be checked using transmission electron microscopy or atomic force microscopy, as well as characterizing their size and distribution using light scattering.

4. Cell transfection can be performed in the absence or presence of serum. The effect of serum in the transfection medium will vary depending on the nature of polycations.

5. Cells of high passage number are transfected inefficiently.

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