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Non-Viral Gene Delivery Vectors

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Chapter 16

Gene Delivery Method Using Photo-Responsive Poly(β-Amino Ester) as Vectors

Nan Zheng, Yang Liu, and Jianjun Cheng

Abstract

Nonviral vectors show great potential in delivering nucleic acids (NA) into many mammalian cells to achieve efficient gene transfection. Among these, cationic polymer is one of the most widely used nonviral gene delivery vectors, forming the polymer/NA complexes for the intracellular transportation and release of the genetic materials into the target mammalian cells. Here we describe the poly(β-amino ester) (PBAE) with the photo-responsive domain built in the polymers, as a UV-light-responsive nonviral gene delivery vector to deliver and release plasmid DNA (pDNA) into HeLa cells and achieve enhanced transfection efficiency.

Key words Nonviral gene delivery, Poly (β-amino ester)s, Photo-responsive, Transfection efficiency

1 Introduction

Gene therapy has emerged as a promising approach in treating various genetic diseases [1]. Compared with viral vectors, nonviral vectors have received growing attention and have been developed as safer alternatives to the viral vectors due to their limited immunogenicity and oncogenicity effect [2, 3]. Cationic polymer is one of the major class of nonviral vectors, which is capable of condensing negatively charged nucleic acids (NA) to form stable complexes (polyplexes) for intracellular delivery [4–6]. Among all cationic polymers being developed and studied, poly(β-amino ester) (PBAE) has attracted particular interest because of its ease of synthesis and high efficiency of gene delivery capability [7–9].

PBAE typically degrades through the hydrolysis of the backbone ester linkages. To enable controlled degradation of PBAE, we recently developed photo-responsive PBAEs by incorporating nitrobenzyl esters into the PBAE backbone [10, 11]. The photo-responsive PBAEs were synthesized through the poly-addition of (2-nitro-1, 3-phenylene)bis(methylene) diacrylate and a bisfunctional amine. Upon external UV-triggering, the nitrobenzyl ester

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bonds can be almost instantaneously cleaved and the PBAEs were degraded, releasing the complexed NA [12].

Here we describe the method of using the photo-responsive PBAEs as nonviral gene delivery vectors to deliver plasmid DNA (pDNA) encoded with enhanced green fluorescence protein (EGFP) (pEGFP) into HeLa cells. Upon UV irradiation, the release of DNA was demonstrated and the enhanced gene transfection efficiency was observed.

2 Materials

2.1 pDNA and Delivery Vectors (PBAEs)

1. pDNA encoding enhanced green fluorescence protein (EGFP) (pEGFP) (Elim Biopharm, Hayward, CA, USA) (*see* **Note 1**).
2. PBAEs were synthesized via Michael addition reaction (*see* **Note 2**).

2.2 Reagents for In Vitro Experiments

1. YOYO-1 (Life Technologies, Carlsbad, CA, USA) (*see* **Note 3**).
2. Ethidium bromide (EtBr).
3. RIPA lysis buffer. 250 mL of 0.1 M Tris, 210 mL of 0.1 M hydrochloric acid (HCl), and 40 mL of dH_2O to get the Tris–HCl buffer, pH 7.4. Add 5 g of NP40, 0.5 g of sodium dodecyl sulfate (SDS), and 4.383 g of sodium chloride (NaCl) into the freshly prepared Tris–HCl buffer and stir overnight.
4. 25 mM sodium acetate buffer (CH_3COONa), pH 5.2.
5. Human cervix adenocarcinoma cells (HeLa) (American Type Culture Collection, Rockville, MD, USA).
6. Complete cell-culture medium: Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (*see* **Note 4**).
7. Opti-MEM.
8. 3-(4, 5-dimethylthiahiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT).
9. Bicinchoninic acid (BCA) assay.
10. Dimethyl sulfoxide (DMSO).
11. 20 mg/mL of heparin in deionized water (dH_2O).
12. Polypropylene microcentrifuge tubes.
13. Vortex.
14. Spectrophotometer-spectrofluorimeter (e.g., SpectraMax® M2 Multi-detection reader).
15. Flow cytometer.

3 Methods

3.1 Preparation of PBAE/DNA Complexes

1. Dissolve the PBAEs in DMSO to a final concentration of 100 mg/mL in a glass vial.
2. Dilute the polymers using 25 mM CH_3COONa buffer, pH 5.2, to the final concentration of 1 mg/mL in a 1.5 mL polypropylene microcentrifuge tube.
3. Determine the initial DNA concentration by measuring the absorbance (optical density, OD) at $\lambda = 260$ using the following calculation:

   $$\text{DNA concentration} = 50\ \mu g / mL \times OD_{260} \times \text{dilution factor}$$
   $$(\text{dilute the sample to give OD readings between 0.1 and 1.0}).$$

4. Dilute pEGFP in dH_2O to the final concentration of 0.2 mg/mL in a 1.5 mL polypropylene microcentrifuge tube.
5. Add 10 μL of 0.2 mg/mL polymer solution (*see* **Note 5**) to 2 μL of 0.2 mg/mL pEGFP solution (*see* **Note 6**) in a 1.5 mL polypropylene microcentrifuge tube, then vortex for 30 s and incubate for 20 min at room temperature (RT) to allow the formation of PBAE/DNA polyplexes with the polymer/DNA weight ratio of 5. Instead, add 20 μL of 0.2 mg/mL polymer solution to 2 μL of 0.2 mg/mL pEGFP solution to allow the formation of PBAE/DNA polyplexes with the polymer/DNA weight ratio of 10 (*see* **Note 7**).

3.2 UV-Triggered Polyplex Dissociation and DNA Release

1. Add 999 μL of dH_2O to 1 μL of 10 mg/mL EtBr to get a final 10 μg/mL EtBr solution.
2. Add 1 μL of 1 mg/mL pEGFP solution to 10 μL of 10 μg/mL EtBr solution in a 1.5 mL polypropylene microcentrifuge tube, vortex, and incubate the mixture for 1 h at RT to get 11 μL of EtBr-stained DNA solution.
3. Mix 50 μL of 0.2 mg/mL polymer solution with 11 μL of EtBr-stained DNA solution, vortex the mixture and incubate for 20 min at RT to allow the formation of polyplexes (polymer/DNA weight ratio of 10) (*see* **Note 8**). Add 39 μL of dH_2O to make the final volume of 100 μL.
4. UV-irradiate polyplex at $\lambda = 365$ nm, 20 mW/cm^2, for 5 min (*see* **Note 9**).
5. Add 0.5, 1, 2.5, 5, 25, and 100 μL of 20 mg/mL heparin to polyplexes solutions to make the final heparin concentrations of 0.1, 0.2, 0.5, 1, 2, 5, and 10 mg/mL, respectively.
6. Incubate the mixtures for 1 h at 37 °C.
7. Read the fluorescence intensity on a spectrofluorimeter at $\lambda_{ex} = 510$ nm and $\lambda_{em} = 590$ nm.

8. Calculate the DNA condensation efficiency (%) according to the following equation:

$$\text{DNA condensation efficiency}\,(\%) = \left(1 - \frac{F - F_{\text{EtBr}}}{F_0 - F_{\text{EtBr}}}\right) \times 100$$

Where F_{EtBr}, F, and F_0 denote the fluorescence intensity of pure EtBr solution, DNA/EtBr solution with polymer, and DNA/EtBr solution without any polymer, respectively (Fig. 1).

3.3 Intracellular Delivery of DNA

1. Seed HeLa cells onto 24-well plates at 2.5×10^4 cells/cm^2 in 0.5 mL/well of complete cell-culture medium.
2. When cells reach confluency, replace the complete cell-culture medium from each well with 0.2 mL/well of Opti-MEM.
3. Label DNA using YOYO-1 dye by mixing 2 μL of 1 mg/mL DNA solution, 3 μL of 5 mM/mL YOYO-1, and 5 μL of dH_2O to obtain a final concentration of 0.2 mg/mL YOYO-1-DNA. Determine the DNA concentration as described in Subheading 3.1, **step 3** (*see* **Note 10**).
4. Incubate the mixture for 20 min at RT in the dark.
5. Add 25 μL of 0.2 mg/mL polymers to 2.5 μL of 0.2 mg/mL YOYO-1-DNA solution in a polypropylene microcentrifuge tube (*see* **Note 11**).
6. Vortex the mixture, and incubate for 20 min at RT to allow the formation of polyplexes with the polymer/DNA weight ratio of 10 (*see* Subheading 3.1, **step 5**).

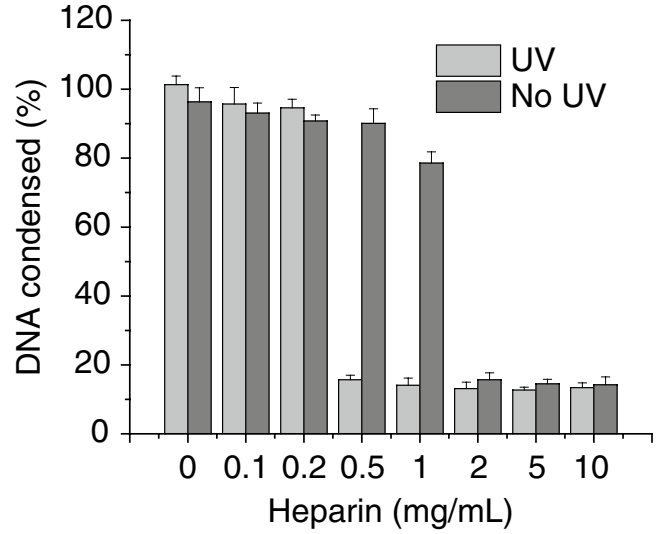

Fig. 1 DNA release from UV-irradiated and non-irradiated polymer/DNA polyplexes in the presence of heparin at various concentrations ($n = 3$)

7. Add 100 μL of polyplexes containing 0.5 μg of YOYO-1-DNA to each well seeded with cells (*see* **Note 12**).
8. After incubation for 4 h at 37 °C, wash the cells thrice with 1 mL of PBS (*see* **Note 13**).
9. Add 500 μL of RIPA lysis buffer to each well to lyse the cells and mix vigorously.
10. Rock the plate for 20 min at RT.
11. Monitor the YOYO-1-DNA content of 50 μL/well lysates in a 96-well plate by means of a spectrofluorimeter at $\lambda_{ex} = 485$ nm and $\lambda em = 530$ nm.
12. Prepare a set of 50 μL/well YOYO-1 DNA standards by diluting the 0.2 mg/mL YOYO-1 DNA into RIPA lysis buffer with 1, 0.5, 0.2, 0.1, 0.05, 0.02, and 0.01 μg/mL concentrations and read the fluorescence (*see* Subheading 3.3, **step 11**).
13. Prepare BCA working solution based on the BCA kit protocol (*see* **Note 14**), add 200 μL to each well containing 20 μL/well of cell lysates and the standards and then incubate for 30 min at 37 °C in a 96-well plate.
14. Read the absorbance at $\lambda = 562$ nm by means of a spectrophotometer.
15. Express the YOYO-1-DNA uptake level as ng of DNA/mg of cellular proteins (Fig. 2).

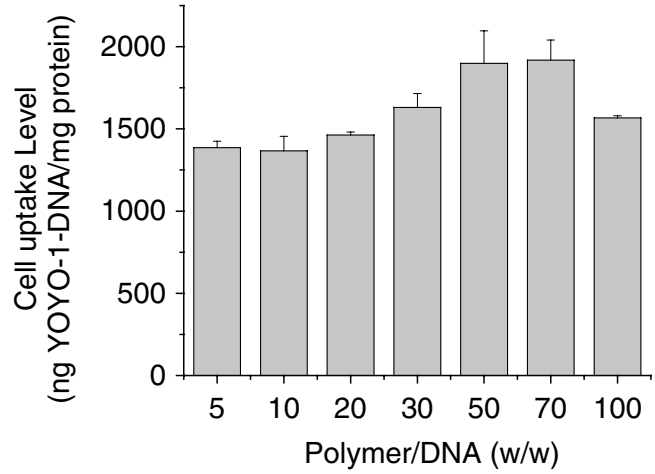

Fig. 2 Uptake level of polymer/DNA polyplexes with various weight ratios following incubation at 37 °C for 4 h ($n = 3$)

3.4 In Vitro Transfection

1. Seed HeLa cells in 24-well plates at 2.5×10^4 cells/cm^2 in 0.5 mL of complete culture medium and culture cells until they reach 70% confluence.
2. When cells reach confluence, replace the complete culture medium from each well with 0.2 mL/well fresh aliquots of Opti-MEM.
3. Add 125 μL of 0.2 mg/mL polymers to 2.5 μL of 0.2 mg/mL DNA solutions in a polypropylene microcentrifuge tube, vortex the mixture and incubate for 20 min at RT to allow the formation of polyplex with the polymer/DNA weight ratio of 10 (*see* **Note 11**).
4. Add 127.5 μL of polyplexes containing 0.5 μg of DNA/well to each well seeded with cells (*see* **Note 12**).
5. After incubation for 4 h at 37 °C, replace the culture medium from each well with 500 μL/well of complete cell-culture medium.
6. UV-irradiate the cells at $\lambda = 365$ nm, 20 mW/cm^2 for 0.5, 1, 2, 3, and 10 min (*see* **Note 9**).
7. Incubate the cells for further 44 h.
8. Evaluate the EGFP expression levels by flow cytometry and express the results as percentage of EGFP positive cells (Fig. 3).

3.5 Cytotoxicity

1. Seed HeLa cells at 3×10^4 cells/cm^2 on 96-well plates and cultured in 100 μL/ well of complete cell-culture medium.
2. After 24 h, remove the complete cell-culture medium from each well and add 100 μL/well of Opti-MEM.

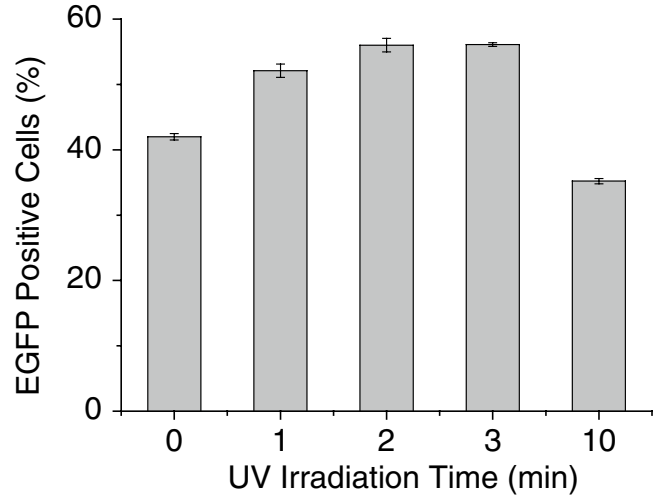

Fig. 3 Transfection efficiencies (TE) of polymer/DNA polyplexes at weight ratio of 50 in HeLa cells in response to UV irradiation ($\lambda = 365$ nm, 20 mW/cm^2) for various time

3. Add 25 μL of 0.2 mg/mL polymers to 0.5 μL of 0.2 mg/mL DNA solutions in a polypropylene microcentrifuge tube. Vortex the mixture and incubate for 20 min at RT to allow the formation of polyplex with the polymer/DNA weight ratio of 10.
4. Add 25.5 μL of polyplexes containing 0.1 μg of DNA/well to each well containing cells (*see* **Note 12**).
5. After incubation for 4 h at 37 °C, replace the old culture medium from each well with 500 μL/well of complete cell-culture medium.
6. UV-irradiate the cells at $\lambda = 365$ nm, 20 mW/cm^2 for 3, 5, or 10 min, and culture them for further 44 h (*see* **Note 9**).
7. Prepare 500 mg/mL of MTT solution by dissolving the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide into DMSO, then dilute it in PBS to the final concentration of 5 mg/mL.
8. Add 20 μL of MTT solution to each well and incubate for 4 h at 37 °C (*see* **Note 15**).
9. Read the absorbance at $\lambda = 570$ nm, with a reference $\lambda = 650$ nm using a microplate reader.
9. Calculate cell viability as percentage viability of control cells (%) according to the following equation:

$$\text{Cell viability}(\%) = \frac{A_p}{A_c} \times 100$$

Where A_p, and A_c denote the absorbance values of cells with the treatment of polyplexes and the cells without any treatment, respectively (Fig. 4).

4 Notes

1. For in vitro assays, pDNA was first dissolved at 1 mg/mL in dH_2O and stored at −20 °C and then diluted in dH_2O to the final concentration of 0.2 mg/mL.
2. Polymers were dissolved at 100 mg/mL in DMSO and stored at −20 °C avoiding light. For in vitro assays, polymers were further diluted in 25 mM sodium acetate buffer (pH = 5.2) to the final concentration of 1 mg/mL.
3. YOYO-1 was stored at −20 °C avoiding light.
4. HeLa cells were passaged at a subcultivation ratio of 1:4 and the cell-culture medium was renewed from twice to thrice per week.
5. The amount of polymer was based on the designated polymer/DNA weight ratio and the amount of DNA in each well. For example, in a 96-well plate and the weight ratio is 10, the formulation should

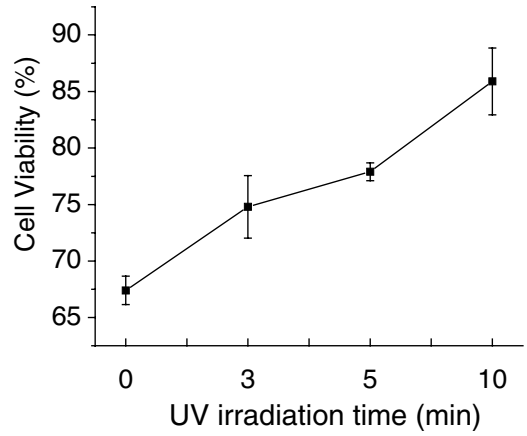

Fig. 4 Viabilities of HeLa cells transfected with polyplexes at polymer/DNA weight ratio of 50 and irradiated with UV light ($\lambda = 365$ nm, 20 mW/cm^2) for various time ($n = 3$)

be the addition of 5 μL of 0.2 mg/mL polymer solution to 0.5 μL of 0.2 mg/mL pDNA solution.

6. The amount of DNA was based on the number of seeded cells and experiments. For a 96-well plate, the dose of pDNA in each well is 0.1 μg/well, while for a 24-well plate, the dose of pDNA is 0.5 μg/well.
7. Slightly pipette the mixture and vortex it before doing experiments since the polyplexes may aggregate in the bottom of the tube.
8. The diluted EtBr was incubated in the dark environment. Prepare as control pure EtBr solution by mixing 1 μL of dH_2O and10 μL of 10 μg/mL EtBr solutions.
9. Use non UV-irradiated polyplexes as controls.
10. The labeling process is by mixing YOYO-1 and DNA together. YOYO-1 is a "turn-on" dye. Free YOYO-1 doesn't have fluoresce. No purification step is needed according to the manual protocol of YOYO-1.
11. Prepare polyplexes with various polymer/DNA weight ratios tuning the volume of the polymers (*see* Subheading 3.1, **step 5**).
12. Use cells without any polyplexes treatment as controls. The uptake, transfection, and toxicity experiments were designed at least in triplicates.
13. Add PBS into each well and shake the plate slightly to remove the polymers and DNA bound to the cell membranes.
14. Prepare working solutions by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1 (v/v) Reagent A:B) based on the description in the protocol.
15. Time could be between 2 and 4 h depending on the density of cells.

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