Comparing Polyethyleneimine and Poly(beta-amino ester) Nanoparticles as Gene Delivery Vectors in CHO-K1 Cells

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Cell and Tissue Engineering Lab, Spring 2023 10:30 AM

Introduction

Gene therapy presents a promising avenue for treating and potentially curing diseases arising from damage to the genome, such as cancer.[1,2,3] Current treatments aim to alleviate symptoms, targeting the protein/gene expression level effects of these damaged genes rather than the genes themselves.[2] Gene therapy aims to remedy the root cause by delivering genetic material into the affected cells, restoring normal gene expression.[2] Despite its vast potential, gene therapy still faces several challenges that prevent clinical adoption. The most significant impediment remains the safe and effective delivery of genetic material.[3] *In vivo*, bare DNA is rapidly degraded by endogenous enzymes/immune cells, and thus has limited therapeutic value.[2] Hence, efforts have been concentrated on vector-mediated delivery. Early experiments focused on viral vectors since viruses have evolved efficient cell transfection capabilities. However, early viral therapies produced severe side-effects in patients, including life-threatening immune responses and cancers arising from insertion mutagenesis.[1,3] Due to these safety concerns, researchers began to explore non-viral vectors based around nanoparticle (NP) formulations of DNA and various polymers.[1,2,3]

Polymer-based gene delivery offers safety advantages, increased DNA capacity, and reduced manufacturing costs compared to viral vectors; however, most approaches to-date suffer from significantly lower transfection efficiencies.[3] Two barriers are balanced DNA complexing and endosomal escape.[2] Generally, polymers are designed to complex with DNA by including cationic groups (since DNA is anionic) and to promote endosomal escape by including basic groups that can absorb protons pumped into the endosome, buffering the interior, which leads to a buildup of osmotic pressure and eventual rupture of the endosome, allowing the NPs to escape into the cytoplasm.[3] A number of polymers have been explored, and two prominent candidates are polyethyleneimine (PEI) and poly(beta-amino ester) (PBAE). PEI is a branched polymer with a high concentration of amine groups and thus substantial buffering capabilities (greater than PBAE). However, PEI is toxic at high doses.[2] PBAE is a linear polymer that (unlike PEI) contains ester groups between subunits, which are readily cleavable in vivo, making this polymer biodegradable and non-toxic.[4] Both are positive in physiological solutions. Characterizing these polymers and their safety/effectiveness as gene delivery vectors is an important step in the development of gene therapies. The following report compares transfection efficiency and cytotoxicity of various NP formulations of PEI and PBAE in CHO-K1 cells, varying the ratio of polymer weight to DNA dose weight, with measurements taken by microscopy and fluorescence reading.

Methods

Cell Line Details

Cells used in the following experiments were the fourth passage (passaged every 2-3 days) of a CHO-K1 (ATCC; Manassas, VA) cell culture acquired and maintained prior to the start of the experiments. These cells were grown in DMEM/F-12K, HEPES growth media containing 10% fetal bovine serum (heat-inactivated) and both penicillin and streptomycin (to prevent bacterial contamination) in an incubator set to 37 °C / 5% CO_2.

Cell Seeding

The previous passage of CHO-K1 cells was inspected under the microscope to confirm confluency (and absence of overgrowth), followed by aspiration of media, rinse with phosphate buffered saline (PBS), and incubation (same conditions as noted in previous section) with 0.25% Trypsin/EDTA. After confirming cell detachment by microscope, the Trypsin reaction was quenched by addition of the growth media mixture (noted in the previous section) in a ratio of 3 units media to 1 unit Trypsin. The solution was then centrifuged at 1500 RPM for 3 minutes, followed by collection of the cell pellet (discarding the supernatant). Cells were resuspended in media. A sample of the cell solution was mixed with Trypan Blue in a 1:1 ratio, followed by quantification in an automated cell counter. Based on the measured cell density, an appropriate cell concentration for achieving 90% confluency in a 24-well plate after 120 hours (the time between cell seeding and analysis) was calculated, using a cell count-to-confluency relationship (1.47×10^5 cells/cm^2 at 100% confluency) and a doubling time (15.5 hours) measured from earlier passages. Volumes of original cell solution and media were multiplied by 1.1 to account for pipetting error. The new cell solution was added to each well, followed by incubation for 48 hours.

Polymer-Mediated Gene Delivery

Various weight-to-weight (w/w) ratios of polymer (PBAE and PEI) to DNA were tested in the experiments. For PEI formulations, three w/w ratios were tested: 0.5, 1, and 2. For each ratio, the necessary volume of 1 mg/mL PEI in water stock solution was calculated to achieve this ratio relative to a fixed dose of GFP DNA (3.3 μg per 500 μL solution in each well) derived from a 1 mg/mL GFP DNA stock solution. PEI and its associated GFP DNA were diluted in 150 mM NaCl to achieve the desired w/w ratio. PBAE w/w ratios of 50, 75, and 100 were tested. These solutions were made from a 100 mg/mL PBAE stock solution, relative to the same fixed dose of GFP DNA. PBAE and associated GFP DNA solutions were diluted with 25 mM NaAc. Media was then aspirated from the wells, and each of the six NP solutions (having been vortexed and incubated at room temp. for 10 minutes) were added in triplicate to the plate (a total of 18 wells), in a ratio of 1 dose of NP solution to 4 parts media. Two wells were given only media (adjusted to the full volume), two wells were given the dose of DNA+NaCl but no PEI, and two wells were given the dose of DNA+NaAc but no PBAE. The plate was incubated for 4 hours,

then media/NP solution was removed, new media was added, and the cells were replaced in the incubator for 72 hours.

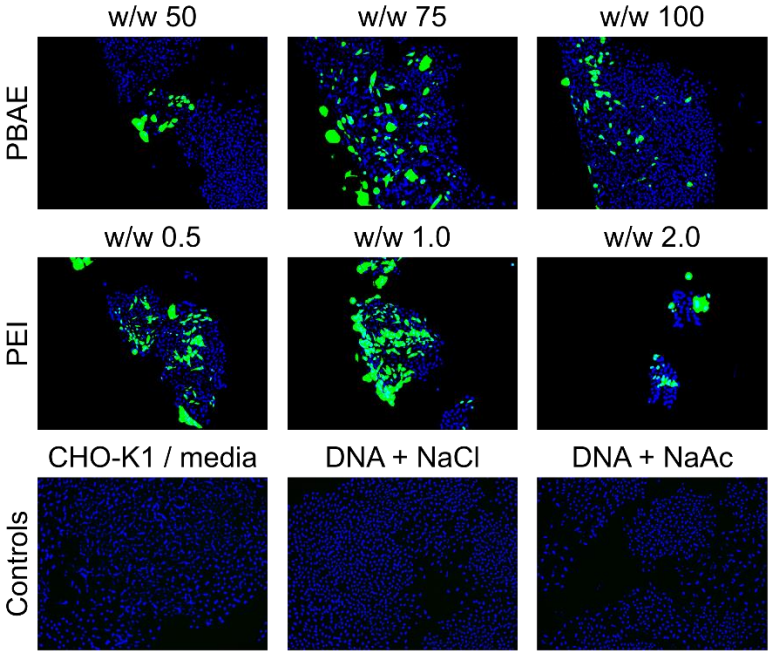
Analysis

Nuclei were stained with Hoescht 33342 dye (Bio-Rad; Hercules, CA), prepared in a 1:250 dye-to-media ratio. Old media was aspirated, followed by addition of the stain solution, incubation for 20 minutes, aspiration of the stain, PBS rinse, and addition of new, pre-warmed media. The cells were then imaged with various microscope filters. A representative slice of each condition was captured with DAPI filter to provide cell count/viability. The same slices were imaged with GFP filter to quantify transfection efficiency. Images of the control were forgotten, requiring the use of another group's (Liu and Venkatesh; 1:00 PM). Additionally, overall GFP fluorescence data was collected with a microplate reader.

Reagents and Equipment

Except for the NP polymers, GFP DNA, and salt solutions (Green Lab; Baltimore, MD), or when otherwise stated, reagents/materials were purchased from Thermo Fisher Scientific (Waltham, MA). A Countess II (Thermo Fisher; Waltham, MA) was used for cell counting. An EVOS M5000 (Life Technologies; Waltham, MA) was used for microscopy. Fiji (ImageJ ver. 1.54b) was used for image analysis.[5] A Synergy 2 (Agilent; Santa Clara, CA) was used for microplate fluorescence reading.

Results



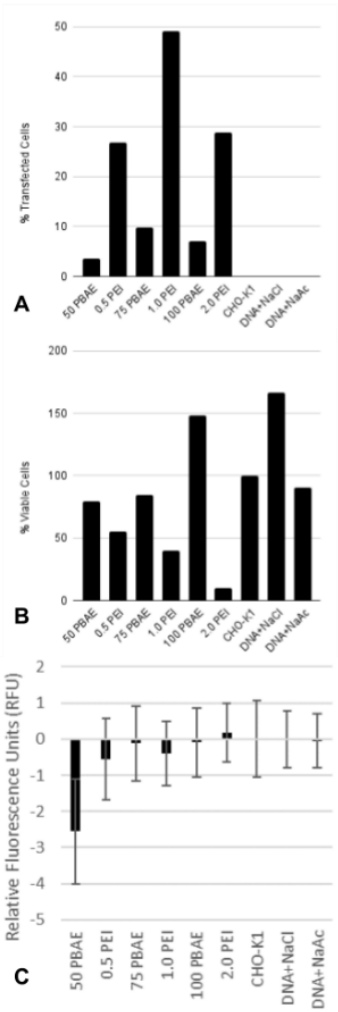


Figure 1 (left): Composite microscope images for each condition. Green channel corresponds to GFP filter (highlighting regions of GFP expression) and blue channel corresponds to DAPI filter (highlighting Hoechst-stained nuclei). Images are processed with Fiji to subtract background, convert to binary, median filter (remove noise), and merge color channels to produce composites. Images are all at same magnification.

Figure 2 (right): Quantitative measures of gene delivery success. (A) Transfection efficiency, calculated from composite images in Fig. 1 by manual counting of the number of nuclei in GFP positive regions in each slice, divided by the total number of nuclei in the slice (automatically counted with Fiji). (B) Cell viability, calculated by dividing the number of nuclei present in each slice by the number of nuclei present in the control. (C) Relative GFP fluorescence measured by microplate reader. Values are normalized by subtracting average RFU of control from all other groups. Standard deviation error bars are displayed.

Composite microscopy images (Fig. 1) illustrate successful GFP expression in all experimental groups and no GFP expression in control groups. For all conditions except PBAE w/w 50, GFP expression was observed in random patches throughout the slice, as would be expected from the homogeneous application of NPs performed in this study. For PBAE, transfection efficiencies of 3.53%, 9.87%, and 7.09% were observed for w/w ratios 50, 75, and 100, respectively (Fig. 2A). For PEI, transfection efficiencies of 26.8%, 49.1%, and 28.9% were observed for w/w ratios 0.5, 1.0, and 2.0, respectively (Fig. 2A). By this metric, PEI outperformed PBAE in all cases, and both PEI and PBAE showed the same trend with varying w/w ratios: the middle condition (1.0 w/w for PEI and 75 w/w for PBAE) displayed higher transfection efficiency than either extreme. A t-test performed over all three w/w ratios between PBAE and PEI groups indicates that the difference in expression between PBAE and PEI is statistically significant ($p = 0.0187 < 0.05$). Additionally, t-tests over all three w/w ratios for PBAE vs. the controls ($p = 0.0204 < 0.05$ for all control groups) and PEI vs. the controls ($p = 0.0080 < 0.05$ for all control groups) also showed significance. Thus, the inclusion of either PEI or PBAE gene delivery NPs produced significant increases in transfection relative to the controls, and the use of PEI produced significant increases in transfection over PBAE (for the surveyed w/w ratios).

Two trends were observed in cell viability calculations (Fig. 2B). With increasing w/w ratio, PBAE viability strictly increased (unexpected), while PEI viability strictly decreased (expected due to toxicity). Additionally, both the PBAE w/w 100 condition and the DNA+NaCl control group displayed higher growth than the CHO-K1/media control group (148% and 166% respectively). Finally, the microplate reader data displayed trends inconsistent with the microscopy. After subtracting the control value from all groups, all but one of the conditions (PEI w/w 2.0) displayed a negative GFP fluorescence value. Except for PBAE w/w 50, the error bars for all groups encompassed zero, corresponding to a very weak signal relative to control, and so statistical analysis was not performed on the fluorescence data (though this data will be discussed in the following section).

Discussion

Prior to the experiments, it was hypothesized that PBAE would produce higher transfection efficiencies since it is biodegradable and less cytotoxic compared to PEI, allowing a larger mass of polymer to be used, increasing its ability to absorb protons/facilitate endosomal escape and thus increasing the number of cells that successfully uptake and express GFP DNA. The data, however, supports rejection of this hypothesis, as PEI produced a statistically significant increase in transfection efficiency over PBAE. A portion of the hypothesis was supported by the experiments; PBAE displayed

higher cell viabilities than PEI in all conditions, agreeing with the literature.[4] In fact, as PBAE w/w increased, so did viability. This result is difficult to explain from the literature and may simply be an experimental artifact (since only three measurements were taken, one for each w/w ratio, it is difficult to draw broader conclusions).

The inclusion of polymer-negative controls (DNA+NaCl and DNA+NaAc) confirm that the polymer is necessary for gene delivery, as these groups displayed no GFP expression. Within each polymer's w/w ratios, PBAE w/w 75 and PEI w/w 1.0 achieved the highest transfection efficiencies. The first result agrees with the literature, however, the second does not (an optimal PEI w/w of 3.0 is commonly cited).[4,6] A possible explanation may be differing sizes of PEI molecule used between the present experiments and past work. The molecular weight of PEI used in the present experiments was unknown, and so it is difficult to compare the results to other work.

Interestingly, for all experimental conditions, the cells grew along the edges of the wells, rather than being evenly distributed. The cell morphology suggested healthy growth (elongated, adherent cells), and it seems probable that this occurred due to experimenter error. In thin liquid layers, like those present in the wells of the plate used, a meniscus forms due to capillary action along the edges. This can lead to a higher concentration of cells along the edges of the well and is often observed in cell cultures.[7] However, gentle shaking/mixing of the plate could have prevented aggregation along the edges of the wells, producing a more even culture. This edge effect also produced some anomalous data. Examining the PBAE w/w 75 condition in Fig. 1, a large region of GFP expression contains no nuclei. This region contained crowding of cells against the edge of the well. Under DAPI filtering, this region was so bright, that nuclei could not be resolved and were removed during image processing, leading to decreased measured transfection efficiency. This effect may have also factored into the inconsistent microplate reader data. Since cells tended to aggregate along the edges, the empty center likely depressed the averaged fluorescence over each well. Another anomalous point in the fluorescence reading was the dramatic negative value of PBAE w/w 50, which was attributed to an extremely low value in one of the wells (A1), which skewed the mean. Other groups also observed anomalous readings from that well, suggesting an issue with the instrument.

The presented experiments successfully compared the transfection efficiency and cytotoxicity of various weight-to-weight ratios of PEI and PBAE nanoparticle-based gene delivery schemes. The results suggest a significantly higher efficiency from PEI-based NPs, but a much higher cell viability from PBAE-based NPs. The study contained some anomalies that may be cause for continued investigation, particularly the apparent increasing viability with increasing PBAE w/w ratio and the observed aggregation of cells along the edges of the 24-well plate. Overall, this report presents a framework for the testing of NP gene delivery vehicles, which is essential to the development of life-saving gene therapies.

References

[1] E. L. Scheller and P. H. Krebsbach, "Gene therapy: design and prospects for craniofacial regeneration," *Journal of dental research,* vol. 88, no. 7, p. 585–596, 2009.

[2] J. W. Salameh, L. Zhou, S. M. Ward, C. F. Santa Chalarca, T. Emrick and M. L. Figueiredo, "Polymer-mediated gene therapy: Recent advances and merging of delivery techniques," *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology,* vol. 12, no. 2, p. e1598, 2020.

[3] J. J. Green, *Cell and Tissue Engineering Lab: Gene Delivery*, 2020.

[4] G. Niu, Z. Jin, C. Zhang, D. He, X. Gao, C. Zou and Z. Hu, "An effective vaginal gel to deliver CRISPR/cas9 system encapsulated in poly (β-amino ester) nanoparticles for vaginal gene therapy," *EBioMedicine,* vol. 58, 2020.

[5] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld and B. Schmid, "Fiji: An open-source platform for biological-image analysis," *Nature Methods,* vol. 9, pp. 676-682, 2012.

[6] P. A. Longo, J. M. Kavran, M. S. Kim and D. J. Leahy, "Transient mammalian cell transfection with polyethylenimine (PEI)," *Methods in Enzymology,* vol. 529, pp. 227-240, 2013.

[7] J. Zhao and H. Lu, "Meniscus Effect on the In Vitro Dosimetry of the T25 Flask Under 2.45 and 5.25 GHz Exposures," *IEEE Microwave and Wireless Components Letters,* vol. 23, no. 2, pp. 105-107, 2013.