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Poly(galactaramidoamine) is an efficient cationic polymeric non-viral vector with low cytotoxicity for transfecting human embryonic kidney (HEK293) and murine macrophage (RAW264.7) cells

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Abstract

Poly(galactaramidoamine) (PGAA) is a cationic co-polymer of dimethyl-*meso*-galactarate and pentaethylenehexamine. PGAA electrostatically complexes with plasmid DNA (pDNA) to form nano-sized particles. In this study, we show that PGAA-pDNA polyplexes generate high transfection efficiencies in human embryonic kidney (HEK293) and murine macrophage-like (RAW264.7) cell lines. PGAA-pDNA mediated transfection is a function of the amine:phosphate (N/P) ratio at which the polyplexes are prepared. The maximum expression of luciferase was obtained using polyplexes prepared at an N/P ratio of 40. Polyplexes prepared at increasing N/P ratios did not significantly increase in size but did result in decreasing luciferase expression. Cellular toxicity increased as the N/P ratios at which the polyplexes were prepared increased.

Introduction

Cationic polymers have shown strong potential for delivery of plasmid DNA to cells. Cationic polymers condense pDNA into nano-sized complexes that have greater protection against enzymatic degradation and that are efficiently internalized by cells (1–4). The most commonly used synthetic cationic polymer in non-viral gene delivery is poly(ethyleneimine) (PEI), which generates strong transfection efficiencies across a broad range of cell lines but is also associated with high toxicity (4). Poly(galactaramidoamine) (PGAA; Figure 1) is a recent class of cationic polymers that has shown strong potential for delivery of plasmid DNA but with reduced toxicity relative to PEI (5, 6). PGAA is a co-polymer of dimethyl-*meso*-galactarate and pentaethylenehexamine that is synthesized by AABB step growth polymerization (7). This polymer contains a carbohydrate moiety which reduces the positive charge density of amine groups in the polymer structure, thereby decreasing the overall toxicity. Positively charged amine groups of PGAA display a strong binding and protection capacity with the negatively charged phosphate backbone of DNA (8). The overall strength of the electrostatic binding interaction is dependent on the ratio of the moles of amine groups to the moles of phosphate groups. This amine to phosphate ratio is referred to as the N/P ratio. PGAA has shown robust transfection efficiencies in HeLa, BHK-21 and HepG2 cell lines. PGAA forms polyplexes with DNA at N/P ratios as low as 5 (7, 9). One of the building blocks of PGAA is ethylenimine (EI) and as such, increasing the N/P ratio increases the EI content and would therefore be expected to increase toxicity. In general,

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optimal total protein expression is generated by a careful balance between the toxicity and transfection efficiency of the polymer. Optimizing the N/P ratio at which PGAA is used as a transfection reagent is therefore necessary prior to its utility. The optimal N/P ratio also has the potential to vary from one cell line to another (4).

In this study, we evaluate PGAA as a transfection reagent using N/P ratios of 40, 60 and 80 in human embryonic kidney cells (HEK293 cells). This is a rapidly dividing cell line which gives high transfection efficiency and high protein production (10). Given that transfection efficiencies can vary from cell line to cell line, we also evaluate transfection and cytotoxicity of PGAA in RAW264.7 cells. This cell line is derived from mouse macrophages, has a longer doubling time and is notorious for its difficulty in being transfected.

Materials and Methods

Materials

PGAA (Glycofect™) was obtained from Techulon Inc (Blacksburg, VA). Polyethylenimine (PEI, M_w 25 kDa) was purchased from Sigma-Aldrich (St. Louis, MO). The luciferase assay system with reporter lysis buffer was purchased from Promega (Madison, WI). The Micro BCA™ Protein Assay Kit was purchased from Pierce Biotechnology Inc. (Rockford, IL). The CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI).

Cell lines and Cell culture

HEK293 and RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Both cells were maintained in Dulbecco's Modified Eagle medium (DMEM) and supplemented with 10% fetal bovine serum, 1 mM Sodium pyruvate, 10 mM HEPES, 50 µg/ml gentamycin sulfate. Cells were kept at 37°C in a humidified 5% CO₂ unless otherwise indicated.

Amplification and purification of plasmid DNA

Plasmid encoding for the firefly luciferase gene (VR1255) was transformed in *Escherichia coli* DH5α-competent cells and amplified in Lennox L Broth (Research Products International Corp., Mount Prospect, IL) containing 50 µg/ml of Kanamycin Monosulfate (Research Products International Corp., Mount Prospect, IL) at 37°C, overnight. The plasmid was retrieved by centrifugation and purified using the GenElute™ Endotoxin-free Plasmid Maxiprep Kit (Sigma, Saint Louis, MO). Purified plasmid DNA concentration was determined by UV absorbance at 260 and 280 nm using the NanoDrop 2000 spectrophotometer (Thermo scientific, Wilmington, DE).

PGAA /pDNA complexes preparation

PGAA was dissolved in ultrapure DNase/RNase free water. Plasmid DNA was pre-heated to 50–55°C prior to mixing to improve dissolution. PGAA solutions were then diluted to reach the desired amine to phosphate (N/P) ratio. Equal volumes of pDNA solution were added to PGAA solutions which were then vortexed for 20–30 seconds and incubated at room temperature for 30 minutes to form polyplexes.

Particle size measurement

Polyplex sizes were measured using the Zetasizer Nano ZS (Malvern Instruments Inc, Westborough, MA). Size measurements were performed using Dynamic Light Scattering (DLS) at 173° backscatter detection in disposable polystyrene cells (DTS0012). The Z-average size and Polydispersity Index (PDI) was determined by the cumulants analysis.

***In-vitro* gene transfection**

Cells were seeded in 48-well plates at a density of 8×10^4 cells/well 24 hours prior to the transfection. PGAA/pDNA polyplexes prepared at three different N/P ratios, each containing 1 μ g of pDNA/well, were added to the cells in serum free media. Cells transfected with naked pDNA were used as a negative control. After 4 hours, media containing the polyplexes were removed and replaced with complete media (1ml/well) and incubated for a further 48 hours. Nutrient media was replenished every 24 hours. After 48 hours, cells were isolated and treated with 200 μ l of Reporter Lysis Buffer (Promega, Madison, WI) followed by two freeze-thaw cycles to ensure complete lysis. Cell debris was centrifuged down at 13200 rpm for 5 minutes and supernatants were retrieved to measure luciferase expression using the Luciferase Assay System (Promega, Madison, WI). Supernatants were added to the luciferase assay reagent in the ratio of 1:5 and vortexed briefly. Luminescence was measured for 10 seconds using the Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany) and expressed in relative light units (RLU) with respect to control. Supernatants were analyzed for the amount of total protein content using the Micro BCA™ Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL). Luciferase activity was expressed as relative light units (RLU)/mg protein in cell lysate.

Cytotoxicity assay

The cytotoxicity of PGAA was evaluated by MTS assay using the CellTiter 96R Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Cells were seeded in 96-well plate at a density of 1×10^4 cells/well. Twenty-four hours later, cells were incubated in serum free media with 1 μ g of pDNA complexed to PGAA at various N/P ratios. After 4 hours of incubation, 100 μ l of complete media was added to each well. After 24 hours of exposure to treatments, media was replaced with nutrient media. After 48 hours, the media in each well was replaced with 100 μ l of fresh nutrient media and 20 μ l of CellTiter 96® Aqueous One solution Reagent. The plate was incubated at 37°C, 5% CO₂ for 2 hours and the absorbance was recorded at 490 nm using a Spectra Max® plus Microplate Spectrophotometer (Molecular Devices, Sunnyvale, California). Values were expressed as a percentage of the control to which no treatment was added.

Statistical analysis

Data is reported as mean + SEM. One-way analysis of variance (ANOVA), followed by the Bonferronic test to compare multiple pairs was performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com). Differences were considered significant at $p < 0.05$ or less.

Results

Polyplex sizes

The average size of polyplexes as tabulated in Table 1 shows that changing the N/P ratio from 40 to 60 resulted in an increase in nanoparticle size from 198.4 to 243.7 nm. Polyplexes prepared at an N/P ratio of 80 displayed an average size of 229.4 nm. One way analysis of variance (ANOVA) with a Bonferronic post-test showed that there was no significant difference in these sizes. In addition, small and consistent values of PDI suggest that particle aggregation did not vary greatly as the N/P ratio at which the polyplexes were prepared changed.

***In-vitro* gene transfection**

The transfection efficiency of PGAA-pDNA polyplexes was evaluated by measuring luciferase expression. Figure 2 shows RLU values obtained from each treatment group

normalized against total protein of the samples. PGAA-pDNA polyplexes generated significantly stronger transgene expression when compared to naked pDNA in both HEK293 (Fig. 2A) and RAW264.7 (Fig. 2B) cell lines. Using polyplexes prepared at increasing N/P ratios results in decreased expression of luciferase. The maximum expression of luciferase expression was obtained with polyplexes prepared at an N/P ratio of 40 which is significantly different from all other treatment groups in both cell lines.

Cytotoxicity assay

After 48 hours of incubation, the viability of cells was found to decrease as the N/P ratio of the polyplexes used increased. PGAA-pDNA polyplexes prepared at an N/P ratio of 40 were significantly less toxic than PEI-pDNA polyplexes prepared at an N/P ratio of 20 (Fig. 3).

Discussion

PGAA is a cationic polymer that consists of carbohydrate groups and four oligoethyleneamines linked together by amide bonds (5). PGAA polymers are degradable and hydrolyze under physiological conditions (11). Previous studies evaluating the optimal construct of PGAA for transfection have shown that the quantity and position of the hydroxyl groups can have a significant impact on transfection efficiency and that galactarate polymers with four ethylenimine units generated the strongest transgene expression. Complexing PGAA with pDNA results in polyplexes that have a net positive charge similar to PEI-pDNA polyplexes that is capable of binding to the net negative charge on the surface of cells. In addition, carbohydrate structures in the PGAA are reported to bind to cell surface proteins through hydrogen bonding interactions. Internalization of the PGAA-pDNA polyplexes is reported to take place through endocytosis with 80% of PGAA-pDNA polyplex internalization blocked if cells were exposed to filipin III (12).

Optimizing the transfection efficiency obtained using cationic polymers complexed with pDNA can be achieved by evaluating varying N/P ratios at which the polyplexes are prepared (1, 2, 4). In this study, we show that PGAA-pDNA polyplexes prepared at an N/P ratio of 40 gives maximum expression of luciferase in both fast growing HEK293 and slow growing RAW264.7 cells. In addition, we show that PGAA-pDNA polyplexes prepared at an N/P ratio of 40 have the lowest toxicity relative to polyplexes prepared at N/P ratios of 60 and 80. PGAA was also found to have toxicity that was significantly lower than PEI-pDNA polyplexes prepared at an N/P ratio of 20. We acknowledge that the difference in toxicity might be reduced if PEI-pDNA polyplexes were utilized at lower N/P ratios. This study provides independent verification of the strong potential of PGAA as a non-viral vector and shows that PGAA (Glycofect™) has strong potential for transfecting murine macrophage-like (RAW264.7) cells and human embryonic kidney (HEK293) cells with low toxicity.

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For reviewers

The new findings presented in this paper are:

1. First demonstration of PGAA transfection efficiency in hard to transfect murine macrophage like cell lines.
2. Determination of the optimal N/P ratio of PGAA-pDNA polyplexes in HEK293 cells and RAW264.7 cells
3. Independent verification of the low toxicity/strong transfection efficiency generated by PGAA polymeric non-viral vectors in previously untested cell lines.

Fig. 1.
Chemical structure of poly(galactaramidoamine)

Fig. 2. Luciferase activity in RAW264.7 cells (2A) and HEK293 cells (2B) that have been treated with poly(galactaramidoamine)-pDNA polyplexes prepared at N/P ratios of 40, 60, 80 in comparison with naked pDNA. Transfection experiments were carried out by incubating HEK293 cells and RAW 264.7 cells with polyplexes for 4 h (reporter gene: VR1255; pDNA: 1 μ g/well). Data is presented as the mean + SEM. *** $p < 0.001$, * $p < 0.05$.

Fig. 3. Cytotoxicity of poly(galactaramidoamine)-pDNA polyplexes in RAW264.7 cells (3A) and HEK293 cells (3B) in comparison with naked pDNA and PEI-pDNA prepared at an N/P ratio of 20 after 48 hours. Cell viability was measured using the MTS assay as described in the experimental section. Data is presented as the mean + SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Table 1

Particle size and polydispersity index (PDI) of polyplexes prepared at different N/P ratios

N/P ratio	Z-average size (r.nm)	PDI
40	198.4	0.427
60	243.7	0.424
80	229.4	0.426