Comparison of Gene Transfection and Cytotoxicity Mechanisms of Linear Poly(amidoamine) and Branched Poly(ethyleneimine) Polyplexes

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Received: 18 August 2017 / Accepted: 6 December 2017 / Published online: 7 March 2018 © Springer Science+Business Media, LLC, part of Springer Nature 2018

ABSTRACT

Purpose This study aimed to further explore the mechanisms behind the ability of certain linear polyamidoamines (PAs) to transfact cells with minimal cytotoxicity.

Methods The transfection efficiency of DNA complexed with a PAA of a molecular weight over 10 kDa or 25 kDa branched polyethyleneimine (BPEI) was compared in A549 cells using a luciferase reporter gene assay. The impact of endo/lysosomal escape on transgene expression was investigated by transfecting cells in presence of bafilomycin A1 or chloroquine. Cytotoxicity caused by the vectors was evaluated by measuring cell metabolic activity, lactate dehydrogenase release, formation of reactive oxygen species and changes in mitochondrial membrane potential.

Results The luciferase activity was ~3-fold lower after transfection with PAAs than with BPEI complexes at the optimal polymer to nucleotide ratio (RU:Nt). However, in contrast to BPEI vectors, PAA polyplexes caused negligible cytotoxic effects. The transfection efficiency of PAA polyplexes was significantly reduced in presence of bafilomycin A1 while chloroquine enhanced or decreased transgene expression depending on the RU:Nt.

Conclusions PAA polyplexes displayed a pH-dependent endo/lysosomal escape which was not associated with cytotoxic events, unlike observed with BPEI polyplexes. This is likely due to their greater interactions with biological membranes at acidic than neutral pH.

KEY WORDS cationic polymers · cytotoxicity · DNA-complexes · gene delivery · linear polyamidoamines

ABBREVIATIONS

BPEI Branched poly(ethyleneimine)
EtBr Ethidium bromide
FCCP Carboxyl cyanide 4-(trifluoromethoxy)phenylhydrazone
H2DCFH-2′,7′-dichlorodihydrofluorescein diacetate
JC-1 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolyl-carbocyanine iodide
LDH Lactate dehydrogenase
MBA- Methylenebisacrylamide/dimethylacrylamide
MMP Mitochondrial membrane potential
MTT 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
PAA Polyamidoamine
PB PrestoBlue
PBS Phosphate buffer saline
pDNA Plasmid DNA
PEG Polyethylene glycol
PEI Poly(ethyleneimine)
PLL Poly-L-lysine
ROS Reactive oxygen species
RU:Nt Polymer repeating unit to nucleotide ratio

INTRODUCTION

One of the greatest challenges in gene therapy remains the development of safe and effective vehicles to deliver fragile exogenous nucleic acids into host cells. A handful of gene therapies based on viral vectors have been granted market approval in the European Union or United States; i.e.,
Glybera® (uniQure N.V.), Imlygie® (Amgen Inc), Strimvelis™ (GlaxoSmithKline), Kymriah™ (Novartis). Nevertheless, the clinical applications of such vectors is impeded by major concerns surrounding their potential immunogenicity and insertional mutagenesis [1]. To overcome those limitations, non-viral vectors using either cationic lipids or cationic polymers, which can readily complex with the negatively charged phosphate bases in nucleotides, have been investigated intensively as alternative gene delivery systems [2, 3].

Due to the ease of manufacture and low cost of cationic polymers, their development as carriers for gene therapy is attracting a lot of interest, with extensive work being carried out using polyethyleneimine (PEI) or poly-L-lysine (PLL). Safety remains nevertheless an issue as cationic vectors may bind non-specifically to negatively charged blood components and be recognised by phagocytic cells, which could trigger an immune response in addition to decreasing their circulation time in blood [4, 5]. At the cellular level, both PEI and PLL have been shown to trigger staged cytotoxic events. Early deleterious effects occur due to the destabilisation of the plasma membrane by the polymers and delayed toxicity is caused by the induction of the apoptosis process following polymer disruption of mitochondrial functions [6].

A vast range of structural modifications have been designed to improve the biocompatibility of cationic polymers [4]. However, in many cases, a relationship is observed between the level of gene expression achieved and the toxicity of the vectors [7, 8]. For instance, increasing the molecular weight of the polymers usually enhances transgene expression but is associated with more pronounced detrimental effects [5, 9]. Similarly, grafting a hydrophilic non charged macromolecule such as polyethylene glycol (PEG) onto cationic polymers to shield the vector surface charge has been shown to have a positive effect on their in vivo toxicity profile [10] but can negatively affect their transfection efficiency due to decreased cell uptake and/or endosomal escape [11, 12].

Mechanistic studies suggest that the transfection efficiency and cytotoxicity of cationic vectors both originate from polymer interactions with biological membranes [7, 8]. Polyplexes need to overcome several cellular barriers to deliver genetic materials to the cytoplasm or nuclei. It is generally acknowledged that polyplexes enter the cells by endocytotic processes, implying that their escape from the endosomes before degradation in the lysosomes is a critical step in achieving transfection [5]. Permeabilization of the plasma, endosomal or nuclear membranes by the polymer will promote gene expression but also pro-apoptotic events. Dissociating the beneficial effects of cationic polymers from their toxicity therefore constitutes a major challenge.

Linear polyamidoamines (PAAs) are a group of biodegradable cationic polymers that have more recently been explored as gene delivery systems. Interestingly, some of those polymers exhibit a transfection efficiency similar to that obtained with PEI but with a low cytotoxicity compared to other polycationic vectors [13–16]. Their cellular adverse effects have nevertheless only been probed in assays indirectly measuring the cell metabolic activity based on the reduction of tetrazolium salts into coloured formazan derivatives by mitochondrial enzymes such as the MTT, MTS or XTT assays. There is therefore a need for a deeper insight into their cytotoxic profile before their potential as delivery agents in gene therapy can be fully appreciated. Furthermore, the intracellular fate of PAA polyplexes remains unclear to date, prompting questions around the mechanisms underlying their ability to mediate gene transfer.

The present work explored cellular events induced by DNA polyplexes prepared using a linear PAA with a methylenebisacrylamide/dimethylenediamine (MBA-DMEDA) backbone of a molecular weight over 10 kDa. Amongst a series of structurally diverse PAAs, MBA-DMEDA based polymers showed the greatest ability to complex DNA as well as to promote transgene expression [17], with a similar transfection ability as the ISA group of PAAs described elsewhere [18]. Hence, the MBA-DMEDA structure was selected for this study which aimed to confirm and better understand the absence of relationship between cytotoxicity and transfection efficiency reported with certain PAA vectors. Polyplexes made with a commercially available branched PEI (BPEI) of 25 kDa known to be highly efficient as a gene delivery vehicle but also highly toxic [19] were tested alongside for comparison.

**EXPERIMENTAL METHODS**

**Materials**

The PAA used in this study was based on co-monomers of MBA-DMEDA where 21% of the aminic component was replaced by a mono-boc-protected cystamine converted to a dithiopyridyl protecting group [20] (Fig. 1). Due to the low solubility of this polymer, it was not possible to determine its molecular weight and pKₐ values. Nevertheless, the parent polymer without the dithiopyridyl pendant groups exhibited a molecular weight of approximately 10 kDa with a polydispersity $M_w/M_d$ of 1.4, as determined by size exclusion chromatography. Furthermore, its $pK_{a2}$ and $pK_{a3}$ values were measured as 4.89 ± 0.03 and 8.45 ± 0.05, respectively [21]. The polymer was ultrafiltered through a Millipore® ultrafiltration membrane with a 10 kDa nominal molecular weight cut-off (Merck, Darmstadt, Germany) to remove polymer chains with a molecular weight below this value.

The gWiz- LUC plasmid (pDNA; 6.7 kbp) encoding a firefly luciferase reporter gene was obtained from Aldevron
(Fargo, USA). Lactate dehydrogenase (LDH) assay kit (CytoTox-ONE™), bright-Glo™ Luciferase Assay system and Glo Lysis Buffer (1X) were purchased from Promega (Southampton, UK). PrestoBlue® Cell Viability Reagent, DNA Gel Loading Dye (6X) and Tris-Acetate-EDTA (TAE) buffer were obtained from Fisher Scientific (Loughborough, UK). 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFH-DA) and OptiMEM® I Reduced Serum Medium were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) was obtained from Biotium (Fremont, CA, USA). Bafilomycin A1 was purchased from Alfa Aesar (Fisher Scientific, Loughborough, UK). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), BPEI (25 kDa), ethidium bromide (EtBr, 1 mg/ml solution), Coomassie Brilliant Blue R250, agarose powder and all other chemicals or cell culture reagents were purchased from Sigma-Aldrich (Gillingham, UK).

Preparation of DNA Polyplexes

DNA polyplexes were prepared by the addition of polymer solutions to nucleic acid solutions in 5 mM phosphate buffer saline (PBS). The amount of polymer required to produce the polyplexes was calculated by using the following equation:

\[
\text{Amount of Polymer} = \frac{\text{Polymer RU Molecular Weight}}{\text{DNA RU Molecular Weight}} \times \frac{\text{Polymer to Nucleotide Ratio} \times \text{Amount of DNA}}{X}
\]

with RU = repeating units

Equal volumes of polymer and nucleic acid solutions were mixed together. The polymer- DNA mixture was vortexed, then incubated at room temperature for 30 min upon which the polyplexes spontaneously formed.

Gel Retardation Assay

Polyplexes containing 1 μg of DNA were prepared as described above to obtain polymer to nucleotide (RU:Nt) ratios ranging from 1:1 to 10:1 for PAA and from 1:1 to 50:1 for BPEI. 5 mM PBS (pH 7.4) was added to the polyplex preparation to give a total volume of 20 μl. Complexes were mixed with the DNA Gel Loading Dye (6X) (2.5 μl) and loaded onto agarose gels 1% in 1 x TAE buffer containing ethidium bromide (EtBr, 1 μg/ml). Free DNA and free polymers were also applied onto the same gels. The gels were allowed to run at 80 V for 60 min in TAE buffer. Free, semi or completely bound nucleic acids were visualised using an UV-transilluminator gel imaging system (The Bioimaging Company UK) by using the Syngene software. For visualization of the free polymers, the gels were stained with Coomassie Blue 0.1% w/v in 50% methanol and 10% glacial acetic acid for one hour followed by 24 h in a destaining solution made of 10% w/v methanol and 10% v/v glacial acetic acid. Images were taken under white light using the same imaging software.

Particle Size and Zeta-Potential Measurements

The size of the polyplexes was measured by Dynamic Light Scattering (DLS) using a Viskotek DLS 802 at 25°C and a fixed scattering angle of 90°. Their zeta potential was determined using a Zetasizer Nano Series (Malvern, UK).

Cell Culture

The human lung adenocarcinoma A549 cell line was purchased from the American Type Culture Collection and used over 15 passages. Cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with foetal bovine serum (FBS, 10%), the antibiotics penicillin (100 IU/ml) and streptomycin (100 μg/ml) as well as L-glutamine 20 mM and maintained at 37°C and 5% CO₂. The culture medium was replaced every two to three days and the cells were passaged when they had reached approximately 90% confluence.

Metabolic Activity and Transfection Efficiency

A549 cells were seeded in 24-well tissue culture plates (Nunc, UK) at a density of 5 × 10⁴ cells/well and incubated overnight in 0.5 mL of supplemented DMEM, after which they exhibited approximately 75% confluency. Polyplexes at the selected RU:Nt ratios with a final DNA concentration of 10 μg/ml were prepared and then added to OptiMEM® medium. The cell culture medium was aspirated and cells were rinsed with 0.5 ml of PBS before adding OptiMEM® (0.5 ml) containing the polyplexes. Four hours later, the polyplex suspension was removed and replaced with fresh serum-supplemented DMEM. After a 24-h incubation, the cell metabolic activity and the polyplex transfection efficacy were assessed using a PrestoBlue® (PB) or luciferase assay, respectively.

For the PB assay, the medium was removed and cells were washed with PBS, then 250 μl of PB reagent in Hanks’ Balanced Salt solution HBSS (1:10) was added to each well.
After 30-min incubation, 100 μl of supernatant was transferred into a black 96-well plate (Nunc, UK) and the fluorescence was measured by a TECAN Infinite® M200 plate reader (Männedorf, Switzerland) at an excitation and emission wavelength of 560 nm and 590 nm, respectively. The metabolic activity was calculated as a percentage relative to the activity in untreated control cells (100% metabolic activity).

The remaining PB reagent was removed from all wells and cells were washed with PBS again before proceeding to the luciferase assay. 100 μl of Glo Lysis Buffer (1×) was added to each well and plates were incubated for 5 min at room temperature to allow cell lysis. 75 μl of cell lysate was transferred from the 24-well plate into a white 96-well plate (Nunc, UK) and an equal volume of luciferase assay reagent added to the cell lysate in each well. Finally, the luminescence was quantified using a TECAN Infinite® M200 plate reader with an integration time of 1 s. Untreated cells were used to determine the background readings.

In order to assess the impact of endo/lysosomal escape on the rate of transfection, cells were pre-incubated with bafilomycin (500 nM) or chloroquine (100 μM) in OptiMEM® media at 37°C for 30 min. The cells were then transfected with the polyplexes in presence of the drugs. The metabolic activity and transfection efficiency were measured 24 h later following the methods described above. The assays were performed on two independent occasions (n = 3 wells for each experiment).

**Effect of Free Polymers and DNA Polyplexes on Cell Membrane Integrity**

An LDH release assay was performed to assess membrane integrity following cell exposure to the polyplexes. A549 cells were seeded in black 96-well plates at 15 × 10^3 cells/well. After 24 h, cells were treated with a range of BPEI and PAA polymer concentrations (0.1, 1, 10, 100 and 1000 μg/ml) or with selected polyplexes prepared as above. Untreated cells (spontaneous LDH release), cells treated with 4% Triton X-100 (100% LDH release) and culture medium without cells were included as controls. After 4 h incubation, an equal volume of CytoTox-ONE™-reagent was added to each well and the plate was incubated at 25°C for 10 min. The reaction was terminated by addition of 2 ml of a 5 mM solution of FCCP (10 μM) as a positive control for 10 min. The medium was replaced with fresh culture medium, and cells were incubated for an additional 20 h under standard cell maintenance conditions. The medium was removed and 2 ml of a 5 mM solution of JC-1 was added to each well and the cell culture plate was incubated for 30 min in the dark. Cells were harvested and the resulting suspension was measured by flow cytometry using a Beckman Coulter FC500. Red fluorescence (ex: 488 nm, em: 590 nm) was detected in FL2 and green fluorescence (ex: 488 nm, em: 527 nm) was observed in FL1 with 10,000 events captured for each sample. Data were analysed with the Weasel Flow Cytometry Software and are expressed as percentage of red/green fluorescence ratio. The assay was performed on two independent occasions (n = 2 wells per experiment).

**Quantification of Intracellular Reactive Oxygen Species (ROS)**

Cells were seeded at a density of 15 × 10^3 cells/well in black 96-well plates and were incubated for 24 h. Cells were then exposed to H2DCF-DA (10 μM) in PBS for 45 min. The dye was removed and the cells were washed with PBS and then incubated with various polypelex formulations for 4 h or with hydrogen peroxide (400 μM) as a positive control for 2 h. After 4 h incubation, the fluorescence intensity was measured at 483 nm excitation and 535 nm emission with a TECAN Infinite® M200 plate reader. The generation of ROS in treated cells was expressed as a % increase above the ROS level in untreated cells (100% ROS). The assay was performed on two independent occasions (n = 3 wells per experiment).

**Statistical Analysis**

Student’s t-test or one-way ANOVA followed by a Tukey’s test were performed to compare two or multiple datasets, respectively, using Graphpad prism software version 7.0 (San Diego, California, USA). Statistical significance was established when p < 0.05.

**RESULTS**

**Gel retardation Assays**

Gel retardation assay is a simple technique used to assess the ability of polycations to complex nucleic acids. During electrophoresis, unbound polycations move towards the negative electrode and free nucleic acids migrate towards the positive electrode.

PAA completely abolished pDNA migration at a RU:Nt of 2:1 as indicated by the disappearance of EtBr stained nucleic acids from the gel (Fig. 2a, lower
Nevertheless, EtBr fluorescence could be observed in the loading wells, implying that the dye was still able to intercalate between DNA base pairs and therefore, that tight binding of pDNA by the polymer was not achieved. In addition, free polymer started to appear at a 3:1 RU:Nt with polymer staining becoming more intense as the polymer/pDNA ratios increased (Fig. 2a, upper image). On the other hand, pDNA mobility was completely inhibited by BPEI at a RU:Nt of 3:1 and no discrete polymer or fluorescence staining was associated with the retarded polyplexes below a 30:1 ratio, indicating the ability of the polymer to bind strongly to pDNA to allow a large excess incorporation of polymer (Fig. 2b).

Based on the gel retardation observations, only polyplexes prepared at 2:1, 5:1 or 10:1 RU:Nt for PAA and 5:1 or 10:1 for BPEI were further explored.

**Size and Zeta Potential of the DNA Polyplexes**

It is reported that a DNA polyplex size of less than 200 nm is required for efficient cellular uptake via endocytosis and subsequent transfection [5, 22]. Both PAA and BPEI polymers formed polyplexes with a diameter around or below 200 nm (Fig. 3a).

The zeta potential reflects the surface charge of colloids. This is an important measurement as the surface charge of DNA polyplexes affects their colloidal stability and interactions with the negatively charged cell membranes [17, 23].

The zeta potential of PAA polyplexes was positive (> 30 mV) at all three RU:Nt investigated and was significantly raised when the ratio was increased from 2:1 to 5:1 to then reach a plateau at 10:1 (Fig. 3b). This shows that the free polymer observed in the gel retardation assays at RU:Nt above 2:1 (Fig. 2a) contributes to the polyplex surface charge. BPEI polyplexes also exhibited a positive zeta potential value which, at the 10:1 RU:Nt, was significantly higher than that of the PAA polyplexes. This would be expected from the much higher excess of polymer which is able to bind to the pDNA (Fig. 3b).

**Metabolic Activity and Transfection Efficiency**

The cytotoxicity and gene transfection efficiency of PAA and BPEI based polyplexes were compared by performing a PB metabolic activity assay or a luciferase reporter gene assay, respectively.

Cells exposed to PAA polyplexes at RU:Nt of 2:1 and 5:1 maintained a metabolic activity ~90% of that in untreated cells, while a reduction to approximately 70% of controls was observed when cells were incubated with polyplexes at the highest RU:Nt (Fig. 4a). In contrast, the cell metabolic activity dropped to ~30% of the level measured in control cells after exposure to BPEI polyplexes at the RU:Nt of 5:1 and 10:1 (Fig. 4a).

The luciferase activity measured in cells transfected with the PAA polyplexes was not affected by the RU:Nt (Fig. 4b), indicating that the presence of excess polymer had no impact on the transfection efficiency. With BPEI polyplexes, a peak in
luminescence was obtained at the 5:1 ratio (Fig. 4b). The level of transgene expression achieved with BPEI polyplexes at the highest RU:Nt was greater than that obtained with the PAA vectors, despite their more pronounced cytotoxicity (Fig. 4a).

However, it is noteworthy that PAA polyplexes formulated at a 5:1 RU:Nt ratio exhibit the same transfection efficiency as BPEI complexes at a 10:1 ratio but without any deleterious effects on the cells (Fig. 4).

Effect of Free Polymers and Polyplexes on Cell Membrane Integrity

The LDH assay was used to assess the integrity of the plasma membrane following cell treatment with either the free polymers or the polyplexes. LDH is a cytoplasmic enzyme that is released into the extracellular space when the cell membrane is damaged. The assay was performed after a 4 h exposure in order to capture immediate effects.

LDH released in response to the PAA polymer was negligible at all tested concentrations whereas BPEI showed a concentration dependent effect on the cell membrane with an EC50, i.e., the concentration that caused 50% LDH release as compared to the positive control Triton X, of 8.7 μg/ml (Fig. 5a).

Again, cell exposure to PAA polyplexes did not induce any significant LDH release at any RU:Nt studied (Fig. 5b). In comparison, BPEI based vectors caused an extracellular leakage of the protein at the highest RU:Nt of 10:1 (Fig. 5b).

Effect on the Mitochondrial Membrane Potential

The ability of the polyplexes to trigger apoptosis was determined through measurement of the mitochondrial membrane potential (MMP) in a JC-1 assay using FCCP as a positive
control. The JC-1 probe is a cationic lipophilic dye which accumulates in the mitochondria where it forms aggregates (red fluorescence) at a high MMP. When MMP decreases during the early stages of apoptosis [22], the dye is converted into a monomeric form (green fluorescence).

As shown in Fig. 6, PAA polyplexes caused no detectable loss in MMP at any of the tested RU:Nt whereas a decrease in the red/green fluorescence of the JC-1 dye, indicative of a drop in MMP, was quantified after cell exposure to the BPEI polyplexes.

Intracellular Formation of Reactive Oxygen Species

The generation of intracellular reactive oxygen species (ROS) after cell exposure to the polyplexes was probed using the H2DCF-DA dye. This is non-fluorescent but it is converted into the highly fluorescent 2′,7′-dichlorofluorescein upon oxidation in the cell cytoplasm. H2O2 was used as a positive control in the assay.

Both types of polyplexes triggered ROS production but while the effect was observed at RU:Nt of 5:1 and 10:1 when those were prepared with BPEI, it was only apparent at the highest ratio with the PAA polyplexes (Fig. 7).

Inhibition of Endosomal Acidification

Bafilomycin A1 was co-incubated with PAA and BPEI polyplexes during transfection to investigate whether an influx of protons into the endosomes favours transgene expression. Bafilomycin A1 is a specific inhibitor of the Vacuolar-type H+-ATPase (V-ATPase) and therefore, prevents endosomal acidification [25–27].

Bafilomycin A1 caused a dramatic reduction (> 75%) in the luciferase activity measured following transfection with the PAA polyplexes.
polyplexes at all RU:Nt (Fig. 8b). Similarly, the transfection efficiency of BPEI polyplexes was significantly decreased by ~50% and ~90% at a 5:1 or 10:1 ratio, respectively (Fig. 8b). Interestingly, the drug reversed the cytotoxic effects of the PAA polyplexes and the BPEI-based vectors at the lowest RU:Nt (Fig. 8a).

**Inhibition of Endo/Lysosomal Functions**

To assess the effect of lysosomes on transgene expression, A549 cells were transfected with PAA or BPEI polyplexes in presence of chloroquine. Chloroquine is a weak base that diffuses across membranes in its unprotonated form but upon
protonation in the acidic environment of the endosomes and lysosomes, becomes trapped in the organelles [28–30]. As a consequence, it raises their pH [32, 33], impairs the fusion of endosomes and lysosomes, and inhibits the degradative effects of some endosomal/lysosomal enzymes depending on their activity profile at a higher pH [34].

Co-incubation of the cells with PAA polyplexes at a 2:1 RU:Nt and chloroquine resulted in a 3-fold increase in luciferase activity (Fig. 9b). In contrast, the drug significantly decreased luciferase expression by 2- or 3.5-fold when the polyplexes were prepared at a 5:1 or 10:1 ratio, respectively (Fig. 9b). Addition of chloroquine led to a 2.5-fold enhancement in the transfection efficiency of BPEI polyplexes at a 5:1 ratio, whereas no change in luciferase expression was observed at the 10:1 ratio (Fig. 9b).

As shown in Fig. 9a, upon treatment with chloroquine, the metabolic activity of cells transfected with BPEI polyplexes at the 5:1 RU:Nt was increased as compared to that in cells exposed to the polyplexes alone. However, the drug had no effect on the metabolic activity when BPEI polyplexes at the highest ratio or when PAA polyplexes were used for transfection.

**DISCUSSION**

While it has been suggested the ability of conventional cationic polymers to promote transgene expression is linked to their cytotoxicity [7, 8], certain members of the PAA class of polymers, such as the MBA-DMEDA structure [17] and the ISA series [14, 16], do not seem to follow such a trend and exhibit a relatively high transfection efficiency with minimal detrimental effects on the transfected cells. This study aimed to increase understanding of PAA promising behaviour in gene delivery by comparing cellular events occurring upon transfection with DNA polyplexes prepared using either a MBA-DMEDA based PAA with a molecular weight over 10 kDa or BPEI of 25 kDa.

As reported previously [17, 18], the optimal RU:Nt ratio for transfection with the PAA polyplexes was 5:1 (Fig. 4b). Nevertheless, at that ratio, a higher expression of the luciferase gene was observed when A549 cells were transfected with BPEI polyplexes than with their PAA counterparts. BPEI mediated efficient transfection despite the cell metabolic activity dropping to 30% of the control (Fig. 4a) but a further increase in polymer content within the polyplexes led to a reduction in

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**Fig. 9** Effect of chloroquine (100 μM) on (a) the metabolic and activity and (b) the transfection efficiency measured by a Presto Blue or luciferase assay, respectively 20 h following cell exposure to PAA and BPEI polyplexes for 4 h. The data are expressed as mean ± SD (3 replicates, n = 2). * indicates a statistically significant difference from the control condition in absence of inhibitor, as determined by two-tailed Student’s t-test (**p < 0.01; ***p < 0.001).
H+-ATPase (V-ATPase) pump inhibitor bafilomycin A1 dem- 
gene expression. Their contrasting outcomes can be attributed to the in-
trinsic properties of the polymers since both types of polyplexes had a comparable size and zeta potential at the
5:1 RU:Nt (Fig. 3). Interestingly, the excess of PAA polymer observed at that ratio (Fig. 2) did not appear to contribute to the transfection efficiency or cause detectable cytotoxic effects. Nevertheless, an increase in free polymer at the higher 10:1 ratio, as evidenced by a more intense staining on the agarose gel (Fig. 2), had a negative impact on transgene expression, probably due to damage caused to the cells (Figs. 4 and 7).

The absence of LDH release in the extracellular environment immediately post exposure of the cells to both types of polyplexes at a 5:1 RU:Nt (Fig. 5) indicates that direct permeabilisation of the cell membrane is not a major mechanism driving gene expression and/or cytotoxicity. This is also in agreement with the polyplexes entering the cells via endocytotic processes, as reported for similar gene/protein delivery vehicles in various cell types [35–37].

It is generally assumed that gene delivery systems have to escape the endosomes before they are degraded in the lysosomes [3]. A ‘proton sponge effect’ triggered by the protonation of the polymer amine groups once inside the acidic environment of the endosomes and eventually causing the rupture of the vesicles has long been described as a possible mechanism of polyplex endosomal escape [5]. Such a mechanism has nevertheless been recently disproved for PEI vectors [38]. An alternative hypothesis has therefore been formulated suggesting that protonated polymers interact with the inner membrane of the endosomes and thus create pores allowing the release of the polyplexes into the cytoplasm [3]. Overall, polyplex escape from the endosomes is a very inefficient process [3] and any factor affecting this might have a significant impact on the ability of cationic polymers to mediate transgene expression.

Transfection of A549 cells in presence of the Vacuolar-type H+-ATPase (V-ATPase) pump inhibitor bafilomycin A1 demonstrated that acidification of the endosomes was critical for the induction of luciferase expression by both BPEI and PAA polyplexes (Fig. 8). Bafilomycin had nevertheless a greater impact on the transfection efficiency of PAA/DNA complexes (Fig. 8). PAAs have been reported to be subjected to an expansion of their coil structure at low pH as a result of electrostatic repulsion between protonated nitrogens [39, 40]. Such a conformational reorganisation has been suggested to account for the dramatic enhancement in the polymer haemolytic activity observed at pH 5.5 as compared to pH 7.4 [14, 41], although the overall positive charge of the polymers at the lower pH might also contribute to cell membrane destabilisation [42]. PLL and PEI, on the other hand, showed haemolytic properties at both a neutral and acidic pH [14, 41]. Although the PAA used in the present study was structurally different, this can explain why the polyplexes were more dependent on a drop in pH to evade the endosomes than their BPEI counterparts. The BPEI repeating unit has a much lower molecular weight than that of PAAs (~40 vs ~210 Da) and features four independent protonable groups, whose protonation is affected by the protonation state of neighbouring sites [43]. As a result, at any pH, the positive charge density of BPEI and thus, the polymer interactions with biological membranes are higher than for PAAs.

Interestingly, co-incubation of A549 cells with BPEI polyplexes at the optimal RU:Nt ratio and bafilomycin reversed the reduction in metabolic activity caused by the polymeric DNA complexes alone, indicating that their cytotoxic effects mainly originate from their endosomal escape. BPEI polyplexes caused a significant reduction in MMP in the cell line (Fig. 6), similarly to previous observations in Jurkat T and HeLa cells [19, 44] and stimulated the production of ROS (Fig. 7). These phenomena are likely consequences of the polymer disruption of the mitochondrial membrane and/or a significant polymer induced leakage of endosomal content into the cytoplasm and will eventually lead to apoptosis [44]. In contrast, PAAs polyplexes did not affect the MMP (Fig. 6) and only increased ROS cytoplasmic levels at the highest RU:Nt ratio (Fig. 7), probably because the polymer is unable to interact with the membrane of organelles once released in the neutral cytoplasmic environment. This is supported by the absence of LDH release upon cell exposure to the polymer at pH 7.4 (Fig. 5a). Furthermore, while leaky endo/lysosomal vesicles were isolated from hepatic cells after injection of the ISA1 PAA structure to rats, the polymer was unable to permeabilize naïve vesicles when it was added to the suspension medium, in contrast to PEI [23]. Cytotoxicity data collected in this study also demonstrates PAA escape from the endosomes is not accompanied by acute adverse effects the cell is unable to compensate for, which suggests that any pores created in the endosomal membrane by the cationic polymer must be transient. A chronic transfer of the endosomal content into the cytoplasm upon repeated cell exposure to the PAA vectors may nevertheless perturb their normal physiology and eventually induce long-term toxic effects.

Transfection in presence of chloroquine enhanced luciferase expression mediated by PAA polyplexes at a 2:1 RU:Nt and BPEI polyplexes at a 5:1 ratio (Fig. 9), which indicates that under control transfection conditions, a significant fraction of the vectors are trafficked from the endosomes to the lysosomes where they are likely degraded. In the BPEI polyplex case, improved transfection coincided with a reduction in cytotoxicity and therefore, could also be a result of an increase in cell survival following partial inhibition of endo/lysosomal escape. Surprisingly, chloroquine had a negative impact on the transfection efficiency of PAA polyplexes at
higher RU:Nt without affecting their cytotoxicity (Fig. 9). It therefore seems that these vectors benefit from their progression into the late endosomes/lysosomes. It is possible that the excess polymer associated with the complexes requires a lower pH than in the early endosomes for membrane permeabilisation and/or DNA decomplexation. The \( pK_a \) of the two tertiary amine groups of the repeating units of the polymer used in this study can be estimated as 4.9 and 8.4 (see ‘Materials’). Accordingly, it will indeed be more protonated and thus, more prone to interacting with biological membranes as the pH approaches its lower \( pK_a \) value.

Although the data reported herein would need to be confirmed in a range of cell culture models, overall they indicate that the MBA-DMEDA polymer promotes transgene expression through destabilisation of the endo/lysosomal membranes without this causing a significant acute toxicity to the cells. They are in line with conclusions drawn from cell-based and \textit{in vivo} studies with other PAA structures that have shown transfection activity and/or ability to enhance the cellular effects of bacterial toxins (which, similarly to genetic materials need to escape the endosomal compartment before being degraded) [14, 23, 41, 45].

**CONCLUSION**

This study confirms that PAAs with a MBA-DMEDA backbone are effective at mediating transgene expression \textit{in vitro} without significant cytotoxicity at the concentration required for optimal transfection. It also shows that the transfection efficiency of those PAA polymers rests upon pH-dependent releases from both the early endosomes and late endosomes/lysosomes which, in contrast to BPEI vectors, do not induce pro-apoptotic events. Efficacy and safety need to be evaluated \textit{in vivo} but overall, this work suggests that further development of MBA-DMEDA as vectors for gene delivery might be worthwhile.

**ACKNOWLEDGMENTS AND DISCLOSURES**

A.A.Y. Almulathanon was funded by the Ministry of Higher Education and Scientific Research (MOHESR) in Iraq.

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