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

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Abstract

Purpose: This study aimed to further explore the mechanisms behind the ability of certain linear polyamidoamines (PAAs) to transfect 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 PAA polyplexes 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.

Keywords

Gene delivery; cationic polymers; DNA-complexes; linear polyamidoamines; cytotoxicity

Abbreviations

BPEI: branched poly(ethyleneimine); EtBr: Ethidium bromide; FCCP: Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; H2DCFH-DA: 2′,7′-dichlorodihydrofluorescein diacetate; JC-1: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; LDH: Lactate dehydrogenase; MBA-DMEDA: methylenebisacrylamide/dimethylethylenediamine; MTT: 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; MMP: Mitochondrial membrane potential; PAA: Polyamidoamine; PB: PrestoBlue; PBS: Phosphate buffer saline; pDNA: plasmid DNA; 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.), Imlygic® (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, ref). 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 an issue as 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 (3). A vast range of structural modifications have been designed to improve the biodegradability and toxicity profiles of cationic polymers (4). However, in many cases, a relationship is observed between the level of gene expression achieved and the cytotoxicity of the vectors (5, 6). For instance, increasing the molecular weight of the polymers usually enhances transgene expression but is associated with more pronounced detrimental effects (7, 8).

Mechanistic studies suggest that the transfection efficiency and cytotoxicity of cationic vectors both originate from polymer interactions with biological membranes (5, 6). 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 (8). 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 (9-12). 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/dimethylethylenediamine (MBA-DMEDA) backbone of a molecular weight over 10kDa. 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 (13), with a similar transfection ability than the ISA group of PAAs described elsewhere (ref). 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 25kDa known to be highly efficient as a gene delivery vehicle but also highly toxic (14) 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 (15) (Figure 1). The polymer was ultrafiltered through a 10kDa nominal molecular weight cut-off filter 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, 1mg/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 5mM phosphate buffer saline (PBS). The amount of polymer required to produce the polyplexes was calculated by using the following equation:

Amount of Polymer = Polymer RU Molecular Weight /DNA RU Molecular Weight X Polymer to Nucleotide Ratio X Amount of DNA

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 1µg 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) (2.5µl) and loaded onto agarose gels 1% in 1 x TAE buffer containing ethidium bromide (EtBr, 1µg/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% v/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 ºC and a fixed scattering angle of 90º. 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% CO_2. 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 5×10^4 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-hour 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-minute 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 (1x) was added to each well and plates were incubated for 5 minutes 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 second. 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 50 µL Stop Solution (provided with the CytoTox-ONE™ kit) to each well. The plate was then shaken for 10 seconds and the fluorescence was measured in a TECAN Infinite® M200 plate reader at an excitation wavelength of 560 nm and

emission wavelength of 590 nm. The assay was carried out on two independent occasions (n=3 wells per experiment).

Mitochondrial membrane potential (MMP) measurement

A549 cells were seeded at a density of 4×10^5 cells per well in 6-well plates and incubated for 24 h before transfection. The cells were then treated with various polyplex formulations for 4 h and with FCCP (10 μM) as a positive control for 10 minutes. 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 polyplex 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 485 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 (Figure 2A, lower image). 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 (Figure 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 (Figure 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 (8, 16). Both PAA and BPEI polymers formed polyplexes with a diameter around or below 200 nm (Figure 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 (13, 17).

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 (Figure 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 (Figure 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 (Figure 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 (Figure 4A).

The luciferase activity measured in cells transfected with the PAA polyplexes was not affected by the RU:Nt (Figure 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 (Figure 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 (Figure 4A). However, it is noteworthy that PAA polyplexes formulated at a 5:1 RU:Nt ratio exhibited the same transfection efficiency as BPEI complexes at a 10:1 ratio but without any deleterious effects on the cells (Figure 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 4h 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 (Figure 5 A).

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

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 (18), the dye is converted into a monomeric form (green fluorescence).

As shown in Figure 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. H_2O_2 was used 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 (Figure 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 (19-21).

Bafilomycin A1 caused a dramatic reduction (> 75%) in the luciferase activity measured following transfection with the PAA polyplexes at all RU:Nt (Figure 8B). Similarly, the transfection efficiency of BPEI polyplexes was significantly decreased by ~50% and ~90 % at a 5:1 or 10:1 ratio, respectively (Figure 8B). Interestingly, the drug reversed the cytotoxic effects of the PAA polyplexes and the BPEI-based vectors at the lowest RU:Nt (Figure 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 (22-25). As a consequence, it raises their pH (26, 27), 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 (28).

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 (Figure 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 (Figure 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 (Figure 9B).

As shown in Figure 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 (5-6), certain members of the PAA class of polymers, such as the MBA-DMEDA structure (13) and the ISA series (10, 12), 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 (13, ref), the optimal RU:Nt ratio for transfection with the PAA polyplexes was 5:1 (Fig.4 B). 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.4 A) but a further increase in polymer content within the polyplexes led to a reduction in luciferase expression (Fig. 4B), likely as a consequence of extensive cell death (Fig.4A). On the other hand, PAA polyplexes exhibited maximum transfection activity when cells maintained around 90% of their metabolic activity (Fig.4A & B). Those contrasting outcomes can be attributed to the intrinsic 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 (Fig.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 (29-31).

It is generally assumed that gene delivery systems have to escape the endosomes before they are degraded in the lysosomes (8). 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 (8). Such a mechanism has nevertheless been recently disproved for PEI vectors (27). 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 (8). Overall, polyplex escape from the endosomes is a very inefficient process (8) 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 (32, 33). 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 (10, 34), although the overall positive charge of the polymers at the lower pH might also contribute to cell membrane destabilisation (35). PLL and PEI, on the other hand, showed haemolytic properties at both a

neutral and acidic pH (10, 34). 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 (35). 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 (14, 37) 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 (37). In contrast, PAA 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 (17). Cytotoxicity data collected in this study also demonstrates PAA escape from the endosomes is not accompanied by 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.

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. Assuming that the acid/base behaviour of MBA-DMEDA is close to that of a PAA with a similar structure obtained by polyaddition of DMEDA with 1,4-bisacryloylpiperazine (38), the pK_a of the two tertiary amine groups of its repeating units can be estimated as 4.9 and 8.4. Accordingly, the polymer will indeed be more protonated and thus, more prone to interacting with biological membranes as the pH approaches its lower *pKa* value.

Overall, the data reported herein indicate that the MBA-DMEDA polymer promotes transgene expression through destabilisation of the endo/lysosomal membranes without this causing a significant toxicity to the cells. They are in line with conclusions drawn from cell-based and in vivo studies with other PAA structures that have shown transfection activity or ability to enhance the cellular effects of bacterial toxins which, similarly to genetic materials need to escape the endosomal compartment before being degraded (10, 17, 34, ref).

Conclusion

This study confirms that PAAs with a MBA-DMEDA backbone are effective at mediating transgene expression *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 *in vivo* but overall, this work suggests that further development of MBA-DMEDA as vectors for gene delivery might be worthwhile.

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Figure legends

Fig.1: Chemical structure of the MBA-DMEDA polymer used in this study

Fig.2: Gel retardation of pDNA polyplexes prepared using (A) PAA (B) BPEI. (A) lane 1 contains naked DNA only as a control, lanes 2-9 contain polyplexes with RU:Nt ratios of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1 and 10:1, respectively and lane 10 contains polymer only as a control. (B) lanes 1 and 11 contain naked DNA only as a control, lanes 2-9 contain polyplexes with RU:Nt ratios of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 10:1, lanes 12-19 contain polyplexes with RU:Nt ratios of 2:1, 5:1, 10:1, 15:1, 20:1, 30:1, 40:1, 50:1 and lanes 10 and 20 contain polymer only as a control. For each gel displayed, the upper image shows the migration of the polymer towards the negative electrode, while the lower image shows the migration of DNA towards the positive electrode.

Fig.3: (A) Hydrodynamic diameter (nm) and (B) Zeta potential measurements for pDNA polyplexes prepared using PAA and BPEI at different RU:Nt ratios (2:1, 5:1 and 10:1) in PBS solution. * indicates a statistically significant difference between zeta potential values determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (***$p < 0.001$; ****$p < 0.0001$)

Fig.4: (A) Metabolic activity and (B) Transfection efficiency measured by a Presto Blue or luciferase assay, respectively, 20h following cell exposure to DNA polyplexes for 4 hours. The data are expressed as mean ± SD (3 replicates, n=2). * indicates statistically significant differences, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (*$p < 0.05$; **$p< 0.01$; ****$p < 0.0001$).

Fig.5: (A) Dose-response curve showing LDH released from A549 cells treated with increasing concentrations of BPEI or PAA polymers for 4 h. Data represents the mean mean ± SD (6

replicates, n = 2); (B) LDH released from A549 cells following exposure to PAA and BPEI polyplexes for 4 h. The data are expressed as mean ± SD (3 replicates, n=2). * indicates a statistically significant release compared to untreated cells, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test. (****p < 0.0001).

Fig.6: MMP measured 20 h following A549 cell exposure to PAA and BPEI polyplexes for 4 hours using the JC-1 dye. Cells were treated with 10 µM FCCP as a positive control. The data are expressed as mean ± SD (2 replicates, n=2). * indicates a statistically significant decrease in the % red/green fluorescence ratio compared to untreated cells, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (****p < 0.0001).

Fig.7: Intracellular ROS production measured in A549 cells exposed for 4 hours to PAA and BPEI polyplexes using the H2DCF-DA dye. Cells were treated with 400 µM hydrogen peroxide (H_2O_2) as a positive control. The data are expressed as mean ± SD (3 replicates, n=2). * indicates a statistically significant increase in ROS levels compared to untreated cells, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (**p< 0.01; ***p < 0.001; ****p < 0.0001).

Fig.8: Effect of bafilomycin A1 (500 nM) on (A) the metabolic activity and (B) the transfection efficiency measured by a Presto Blue or luciferase assay, respectively 20h following exposure of A549 cells to PAA and BPEI polyplexes for 4 hours. 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 a two-tailed Student's t-test (**p< 0.01; ****p< 0.0001).

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 20h following cell exposure to PAA and BPEI polyplexes for 4 hours. 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).

Figure 1, Almulathanon et al

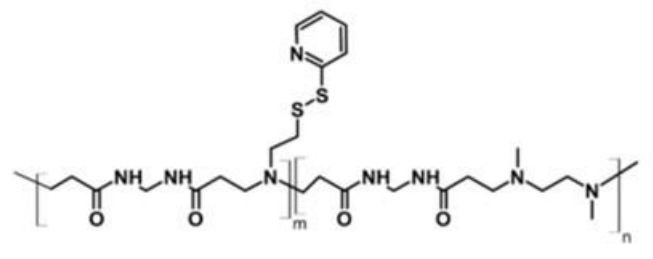

Figure 2, Almulathanon et al

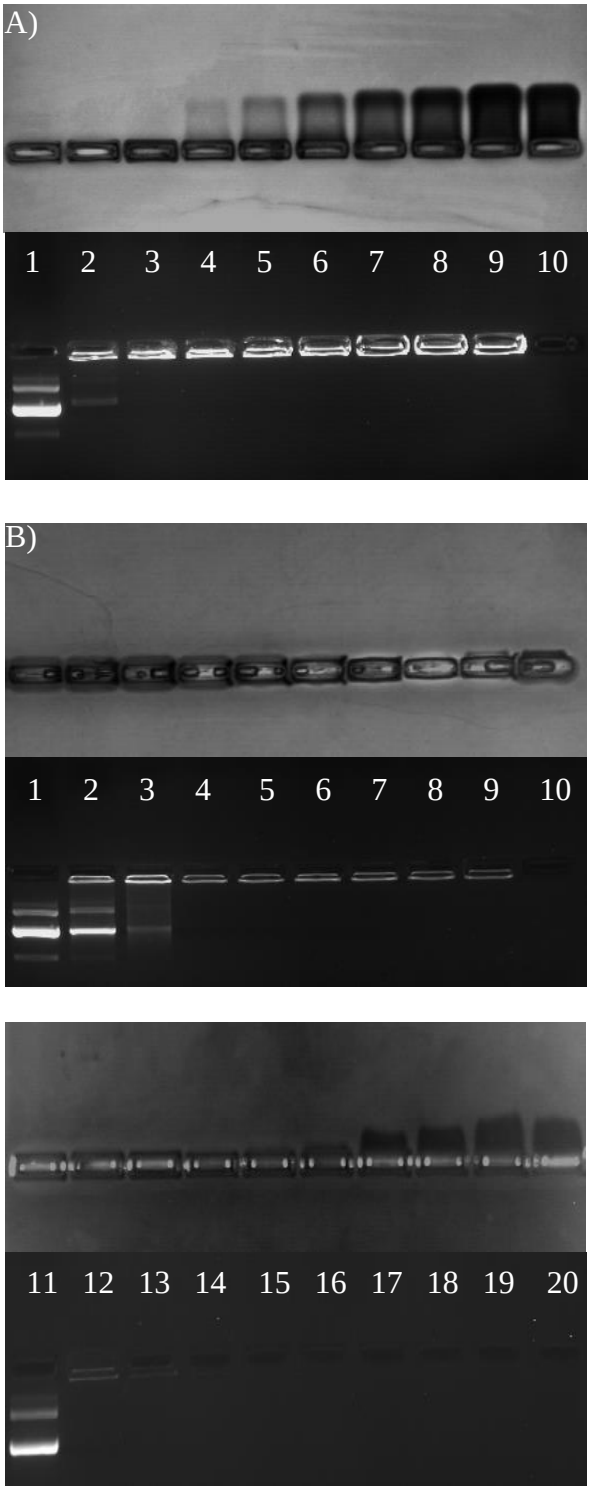

Figure 3, Almulathanon et al

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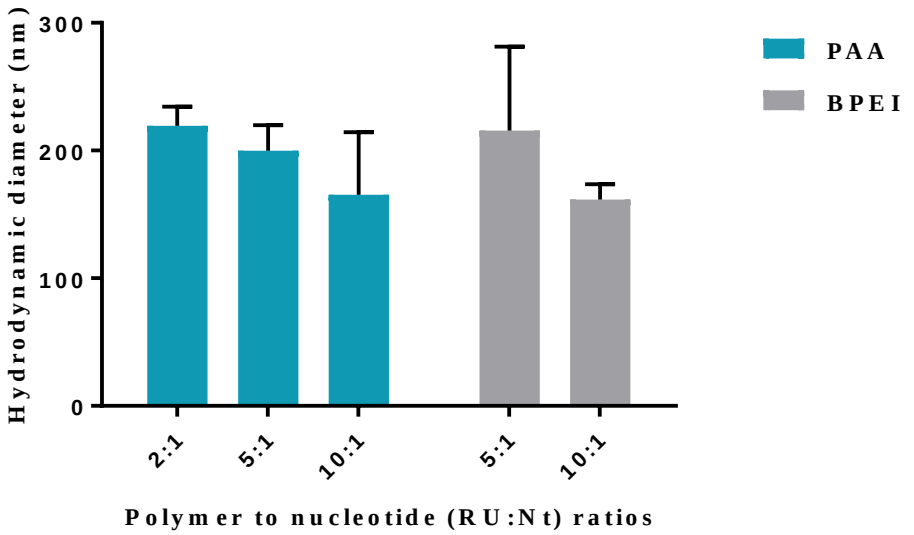

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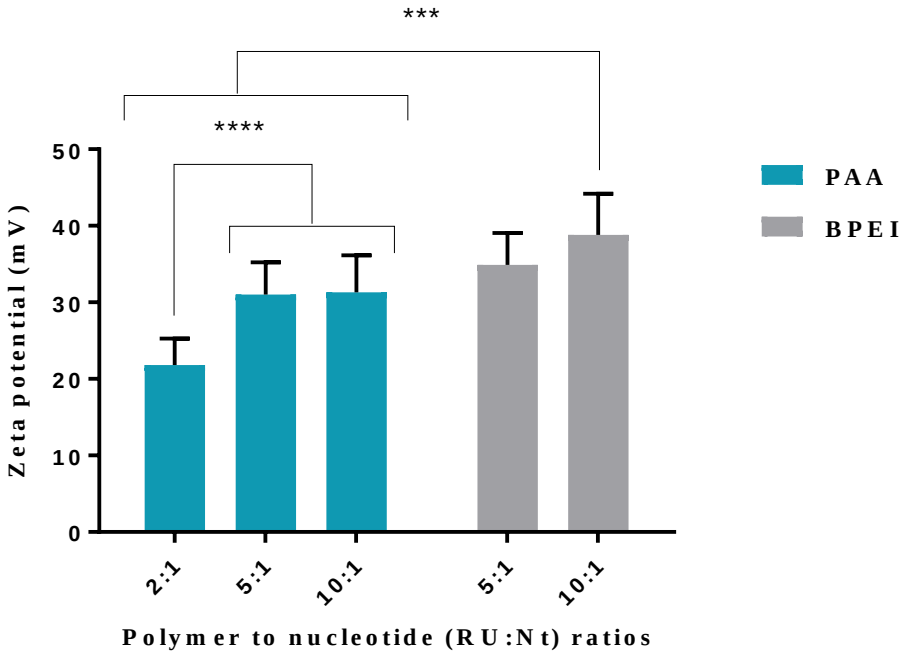

Figure 4, Almulathanon et al

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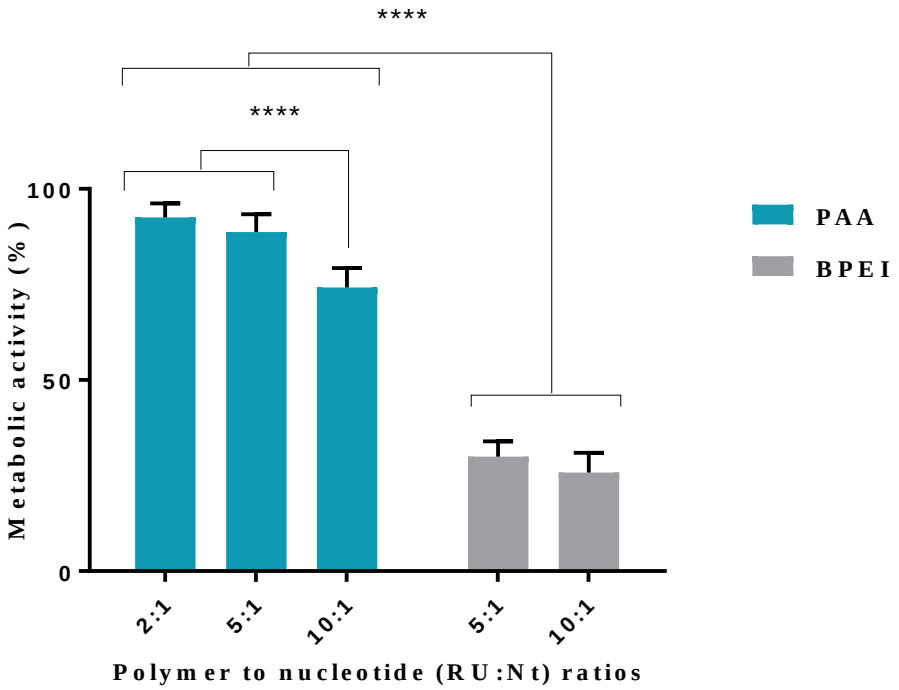

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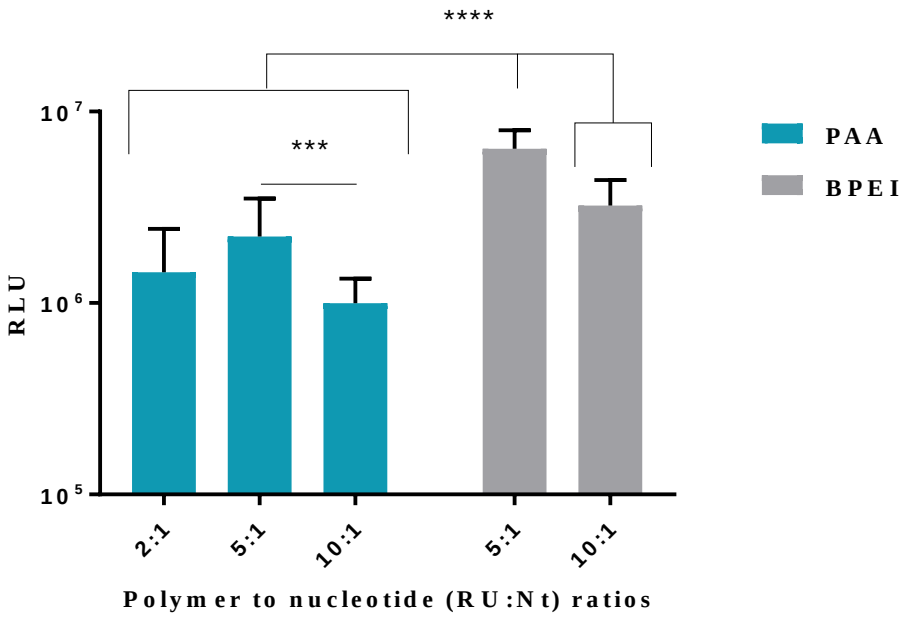

Figure 5, Almulathanon et al

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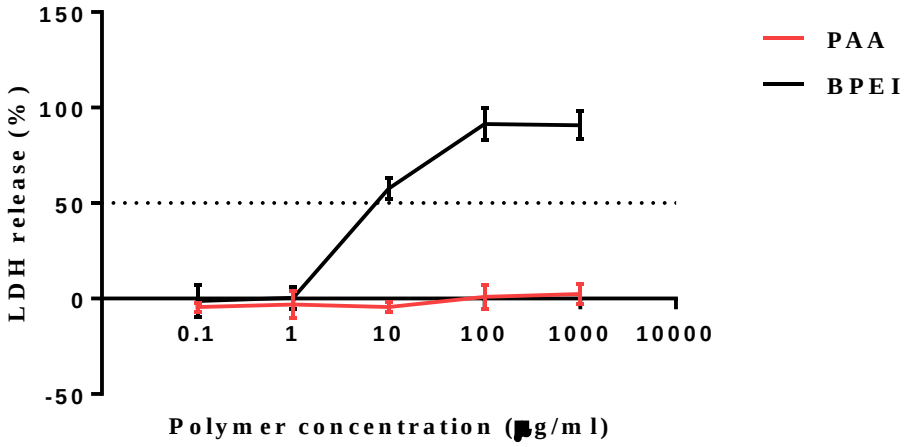

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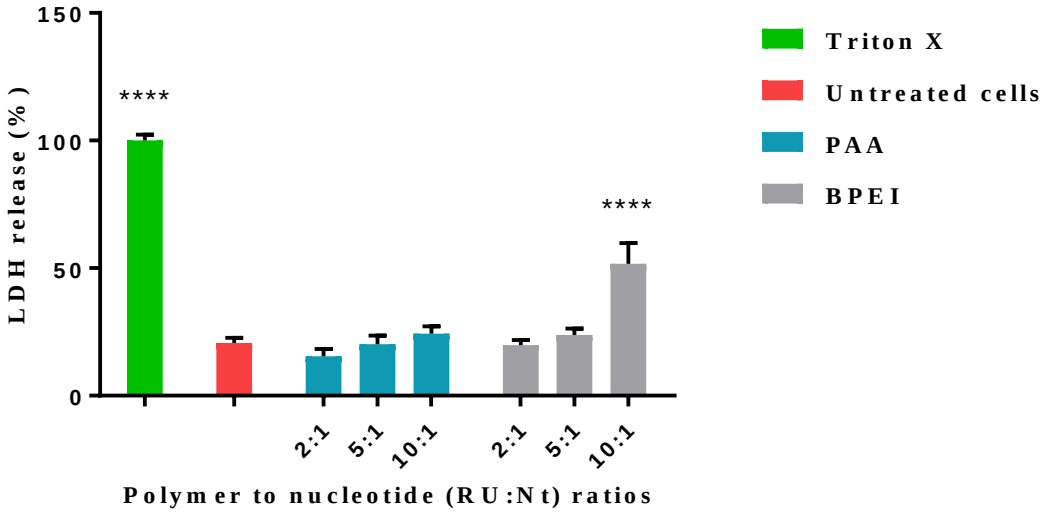

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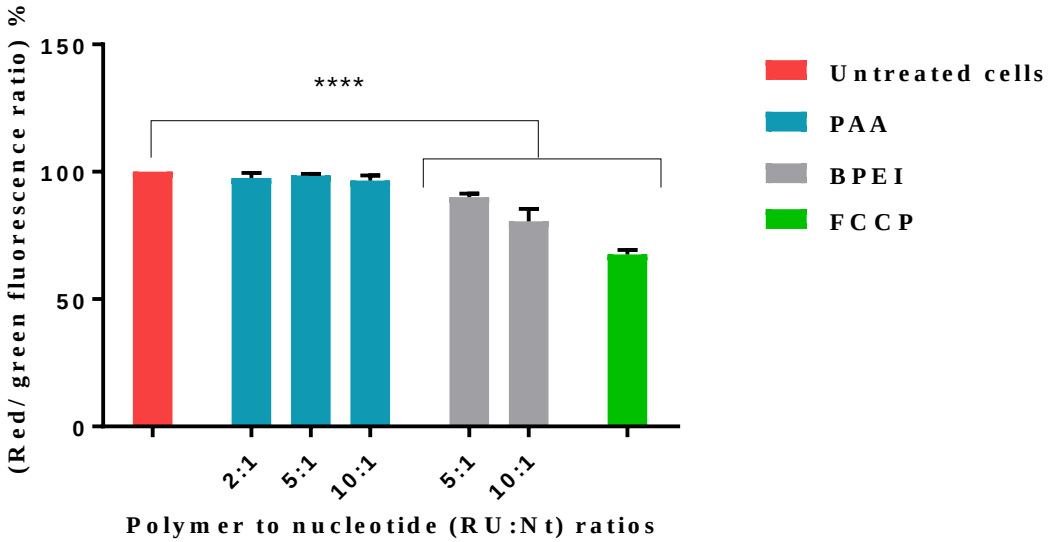


Figure 7, Almulathanon et al

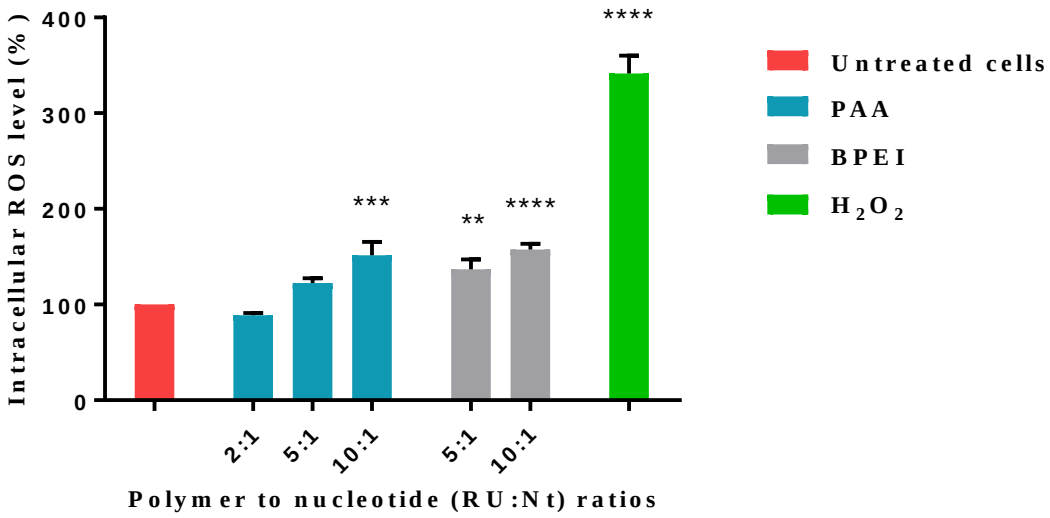
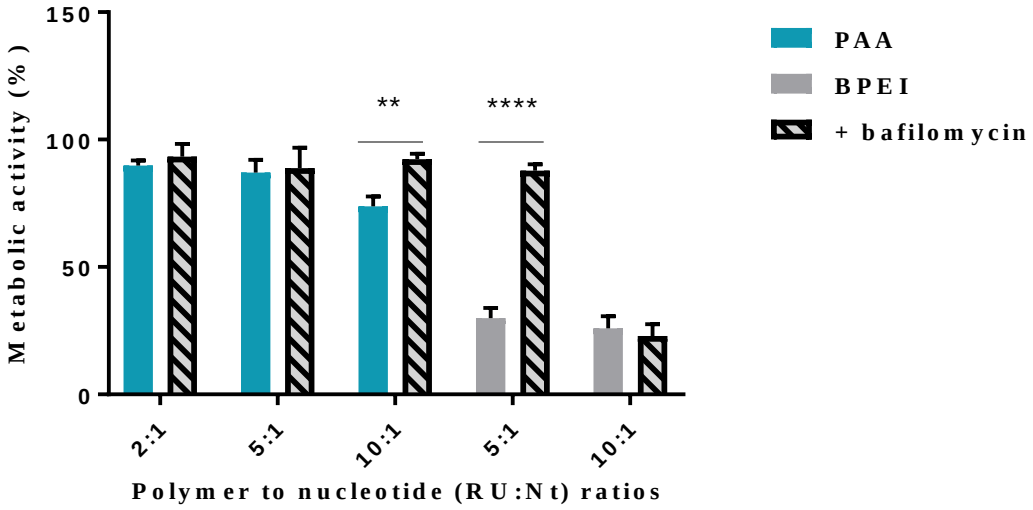


Figure 8, Almulathanon et al

A)



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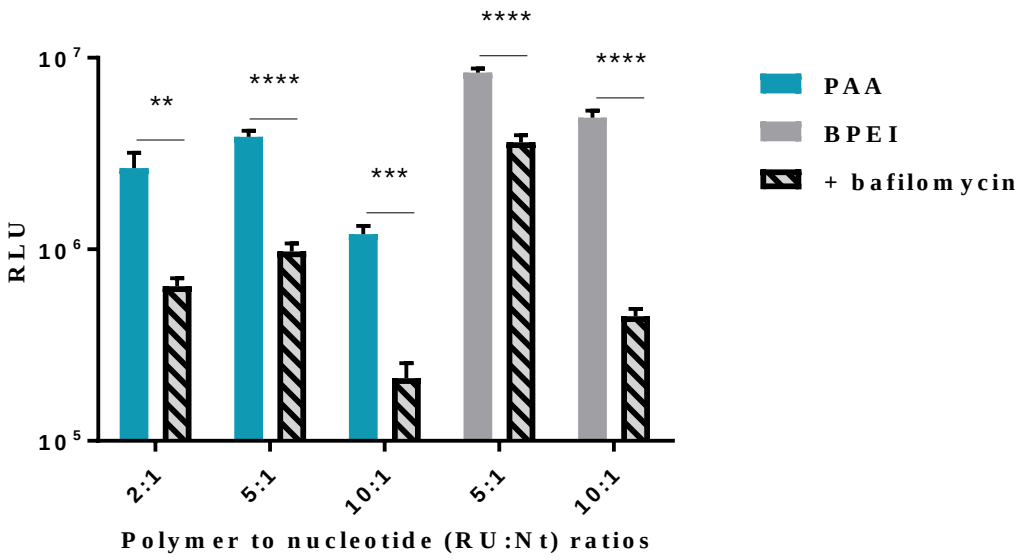
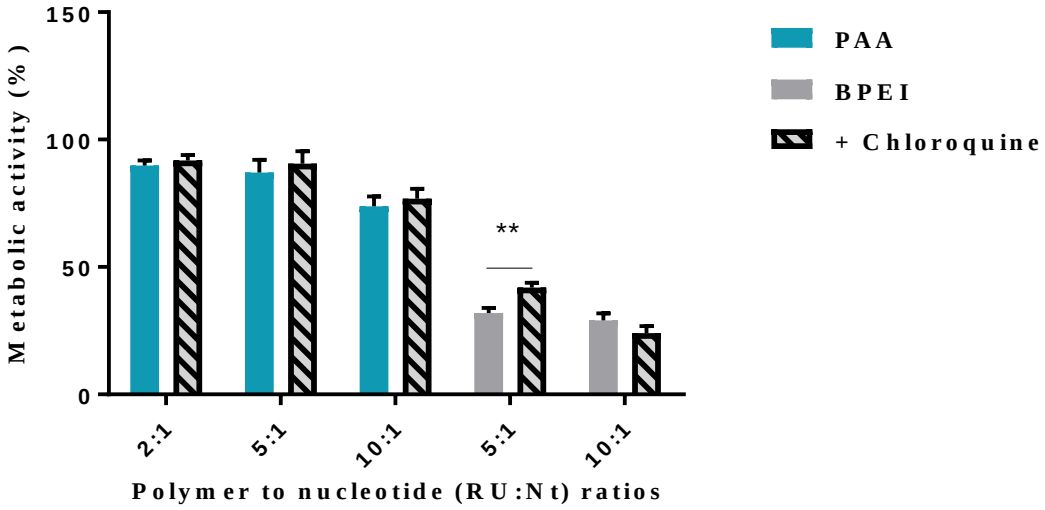


Figure 9, Almulathanon et al

A)



B)