

Efficacy and toxicity of linear polyamidoamine polymers used in gene delivery

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Abstract

Introduction: Interestingly, polyamidoamines (PAAs) are a group of biodegradable cationic polymers that exhibit a transfection efficiency comparable to that obtained with PEI but with a low cytotoxicity compared to other polycationic vectors. However, their cellular adverse effects have 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 with the aim to confirm and better understand the absence of relationship between cytotoxicity and transfection efficiency reported with those vectors.

Methods: Complexes were formed between pDNA and branched polyethyleneimine (BPEI, 25 kDa) or various PAAs including a homopolymer of a molecular weight >10 kDa (HP->10), a PEGylated Triblock Copolymer (CP) and a blend of CP and HP->10 to form a bioreducible cross-linked delivery system. Physicochemical properties of pDNA polyplexes were analysed by using simple techniques including gel electrophoresis, dynamic light scattering (DLS) and zeta potential measurement. The transfection efficiency of pDNA complexes was compared in A549 cells using a luciferase reporter gene assay. The impact of endo/lysosomal escape on transfection

efficiency of pDNA polyplexes was investigated by transfecting cells in presence of bafilomycin A1 or chloroquine, whereas the role of reactive oxygen species in transgene expression was evaluated by using hydroquinone as antioxidant. 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: Gel electrophoresis showed complete incorporation of pDNA by individual and cross linked PAA polyplexes at a similar polymer to DNA (RU:Nt) ratio of (2:1). In comparison, pDNA incorporation was achieved by BPEI at a 3:1 RU:Nt ratio. All polymers were able to condense pDNA into nanoparticles of ≤200 nm in diameter with a positive surface charge. Non-PEGylated PAA polyplexes showed higher luciferase activity than the PEGylated counterparts, but lower than that of BPEI. 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 either enhanced or decreased transgene expression depending on the RU:Nt. No changes were found in the transfection of PAA polyplexes in the presence of hydroquinone compared to BPEI.

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. The presence of excess polymer is considered necessary for improving transfection efficiency of PAA polyplexes. PAA was identified as promising and safe candidate for *in vitro* gene delivery and has potential for future *in vivo* gene therapy.

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List of Abbreviations

ASON	Anti-sense oligonucleotides
BAC-2MP	Bis Acrylamido Acetic acid
BAP-2MP	Bis Acryloyl Piperazine
BB	Plasmid backbone
BPEI	Branched polyethyleneimine
CP	Copolymer [PEG-PAA-PEG]
DCF	Dichlorofluorescein
DiOC6	3,3'-dihexiloxocarbocyanine iodide
DLPC	Dilaurolylphosphatidylcholine
DLS	Dynamic Light Scattering
DMEDA	Dimethyl-ethylene-diamine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DODAP	1,2-dioleoyl-3-dimethylammonium-propane
DODMA	Dioleyloxy-N,N-dimethyl-3-aminopropane
DOGS	5-carboxyspermylglycine dioctadecylamide
DOPE	Dioleoyl-phosphatidylethanolamine
DOSPA	2,3-di-oleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N dimethyll-propan-aminium
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
dsDNA	Double-stranded DNA
DTNB	Dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDL	Electrical double layer
EDTA	Ethylenediamine tetraacetic acid
Et-Br	Ethidium bromide
ETC	Electron transfer chain
FBS	Foetal bovine serum
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

FLuc	Firefly luciferase
G	Dendrimer generation
GFP	Green fluorescent protein
GSH	Glutathione
H2DCF-DA	2′,7′-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl radical
HP	Homopolymer
HP->10	Homopolymer of a molecular weight >10 kDa
HQ	Hydroquinone
IC_{50}	Half maximal inhibitory concentration
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide
kDa	Kilo dalton
LCST	Lower critical solution temperature
LDH	Lactate dehydrogenase
lPEI	Linear polyethyleneimine
MBA	Methylene bisacrylamide
MBA- DMEDA	Methylene-bis-acrylamide-dimethylethylene diamine co-Monomers
MBA-2MP	Methylene Bis Acrylamid- 2 Methyl Piperazine
MBA-MMA	Methylene Bis Acrylamid- Methylamine
MMP	Mitochondrial membrane potential
Mono (Boc)	N-(tert-butoxycarbonyl)
mRNA	Messenger RNA
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)- 2H-tetrazolium
MW	Molecular weight
NAC	N-Acetyl-L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NG30	PAA based on methylene bisacrylamide- dimethylethylene diamine

NLS	Nuclear localization signal
NO	Nitric oxide
NPC	Nuclear pore complex
Nt	Nucleotide
$O_2^{\bullet-}$	Superoxide anion
PAA	Polyamidoamine
PAA	Linear chain Polyamidoamine polymers
PAMAM	Polyamidoamine dendrimer
PB	PrestoBlue
PBS	Phosphate buffered saline
pDNA	Plasmid deoxyribonucleic acid
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLL	Poly-L-lysine
RES	Reticuloendothelial System
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
$RO^{\bullet}$	Alkoxyl
$RO_2^{\bullet}$	Peroxyl
ROS	Reactive Oxygen Species
RU	Repeating units (of the polymer)
RU MWt	Polymer repeating unit molecular weight
RU/Nt	Polymer repeating unit to DNA nucleotide ratio
SH	Thiol
shRNA	Short hairpin RNA
siRNA	Small interfering ribonucleic acid
ssDNA	Single-stranded DNA
TAE	Tris – Acetic Acid – EDTA buffer
TMAG	N-(α-trimethylammonioacetyl)-disodecyl-D-glutamate chloride
V-ATPase	Vacuolar-type H+-ATPase

Chapter one

1 General introduction

1.1 Gene therapy

The principle of gene therapy is based on delivery of nucleic acids which can either restore the function of a deficient gene or inhibit the gene of choice by supressing the protein expression (1). Gene therapy was reported for treatment of inherited conditions such as cystic fibrosis (2), severe combined immunodeficiency disorder (3) or muscular dystrophy (4). Gene therapy is also applicable as a possible treatment for acquired diseases, such as cancer (5), infectious diseases (6), cardiovascular diseases (7) or neurodegenerative diseases (8).

Transfection is a procedure that introduces foreign nucleic acids into cells to produce genetically modified cells. Transfection is a powerful analytical tool for study of gene function and regulation and protein function. The introduced genetic materials (DNAs and RNAs) exist in cells either stably or transiently depending on the nature of the genetic materials (9). For stable transfection, introduced genetic materials that usually have a marker gene for selection (transgenes) are integrated into the host genome and sustain transgene expression even after host cells replicate (**Figure 1.1A**) (10). In contrast with stably transfected genes, transiently transfected genes are only expressed for a limited period of time and are not integrated into the genome (**Figure 1.1B**) (9). Transiently transfected genetic materials can be lost by environmental factors and cell division, so the choice of stable or transient transfection depends on the objective of the experiment. The main purpose of transfection is to study the function of genes or

gene products, by enhancing or inhibiting specific gene expression in cells, and to produce recombinant proteins in mammalian cells (11).

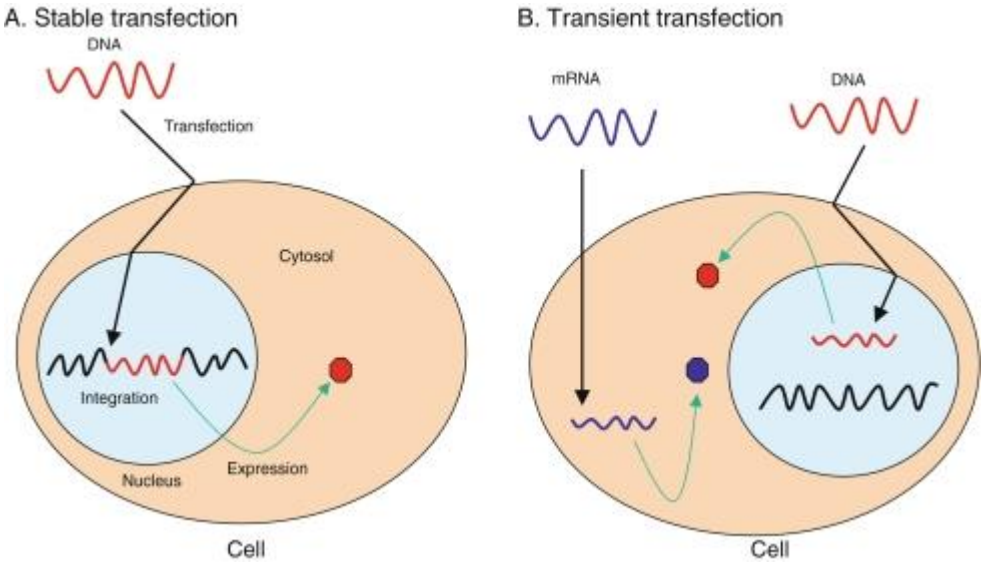


Figure 1.1 Schematic diagrams of two different transfections.
(A) Stable transfection. Foreign DNA (red wave) is delivered to nucleus by passage through the cell and nuclear membranes. Foreign DNA is integrated into the host genome (black wave) and expressed sustainably. (B). Transient transfection. Foreign DNA is delivered into the nucleus but is not integrated into the genome. Foreign mRNA (blue wave) is also delivered into the cytosol, where it is translated. Hexagons are expressed proteins from transfected nucleic acids. Black arrows indicate delivery of foreign nucleic acids (12).

Various types of genetic material have been used in gene therapy such as double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), plasmid DNA (pDNA) (13), messenger RNA (mRNA), miRNA, and RNAi molecules, such as anti-sense oligonucleotides (ASON) (14), small interfering ribonucleic acid (siRNA) and short hairpin RNA (shRNA) (15). Gene therapy that utilizes DNA as a therapeutic agent has been found to act through increasing the expressions of therapeutic proteins by delivering exogenous pDNA which encodes a specific gene. To impart a template for mRNA synthesis, exogenous pDNA should be internalized into the nucleus which ultimately gives rise to protein expression. These plasmids contain several basic components: the transgene expression system (i.e., promoter, gene of interest, and

terminator), regulatory signals, antibiotic resistance marker, origin of replication, and the remaining bacterially derived plasmid backbone (BB). The native plasmid systems are only active in vivo for only 1–2 months, but derivatives of these systems have demonstrated improved expression durations extended to several months and/or years while simultaneously boosting the expression magnitude of the transgene by 10- to 1000-fold (16, 17). For example, plasmids that are devoid of the BB have demonstrated prolonged and sustained efficacy compared with traditional pDNA (18, 19). The extension of activity is due to the reduction of a silencing phenomenon that occurs when approximately 1 kb or more of DNA is placed outside of the transcriptional cargo (between the 5' end of the promoter and the 3' end of the poly A site) (19).

On the other hand, the mechanism of action of RNA-based gene therapy is to reduce protein expression through the delivery of exogenous RNA which knocks down target mRNA in the cytoplasm (20). Typically, siRNA and miRNA are used to facilitate translation inhibition of mRNA transcribed from the targeted gene(s) by directing RNA-induced silencing complex (RISC) activity (21, 22). Although RNA has considerably lower stability (~1 h in plasma) compared with DNA (23), the advantage of RNA-based gene therapy is the associated reduction in immunogenicity and mutagenesis (24). Furthermore, a highly successful gene knockdown using siRNA can reduce the expression of the targeted gene by 80–95% without reducing cell viability (25). In addition, the modulation of target gene expression by RISC does not require nuclear translocation, which is the bottleneck of expression-based systems. However, one drawback is that the expression level of as many as 100 genes may be altered by a single siRNA transcript (26). Alternatively, unlike RNA-based molecules, AONs interfere with mRNA translation through steric hindrance of ribosomal binding in a

sequence-specific manner, improving targeting specificity (27) but at the cost of decreasing knockdown efficiency (20–80%) (28). Despite numerous advantages, off target interactions with nonspecific mRNA sequences, insufficient delivery to the targeted cells, and high manufacturing costs of practice-grade AONs in sufficient quantities impair the widescale adoption of this particular strategy (18, 29).

Recombinant DNA technology has enabled protein-based molecules such as growth factors, zinc finger protein transcription factors, zinc finger nucleases, transcription activator-like effector nucleases, and monoclonal antibodies, to be developed and used as drugs (30). Recombinant DNA techniques are used to clone, express and purify any protein with a known DNA sequence in vitro using cost effective methods (31). Protein-based therapeutics are successful in regenerative applications because they can mimic, activate, or inhibit endogenous pathways, helping the body to heal. Bioengineers can mimic biochemical cues in nature by developing recombinant proteins and engineering their delivery. However, the use of protein-based drugs for regenerative therapeutics is limited by their propensity for instability in vitro and in vivo, presenting a challenge for handling, and implying a need for repeated doses over time and the possibility for unwanted side effects (32).

The avoidance of nucleic acid degradation after entering the target cell is considered fundamental for effective gene therapy. Condensed DNA is more protected against nucleases. However, many factors can prevent the effective transfection of naked DNA through the cell membrane which include susceptibility of DNA to the degradation effect of nuclease in the biological medium (33), in addition to the large size (34) and the negative charge of the DNA macromolecule (35). As a result, a delivery system should be used to transport the therapeutic gene into the targeted cell,

to prevent its degradation by nucleases and to ensure its transcription inside the cell (33, 36).

A number of criteria must be met in the delivery system that will be used in gene therapy:- It should have low host immunogenicity, it should be able to carry different sizes of transgene, the expression of its genetic load should be continuous and regular, it should be highly specific to the target cells and it should have the capability to transfect both dividing and non-dividing cells (37).

1.2 Barriers to pDNA delivery

Gene therapy requires delivery of DNA into the cell nucleus (its site of action). After administration, DNA encoding therapeutic genes must be transported through several biological barriers to be transcribed and translated into therapeutic protein (38-40).

The major extracellular barriers that are encountered in transgene expression are: i) size and charge of DNA, pDNA is a large negatively charged molecule, with high molecular weight reaching up to $3\text{-}4x10^6$ Da (39, 41). Its molecular weight affects cellular internalisation by endocytosis and its charge characteristic inhibits association of DNA with the cell membrane, because the cell surface is predominantly composed of proteins and amphiphilic lipids: sphingolipids, phospholipids and that possess negative charges (42). ii) serum nucleases, which may cause a fast degradation of naked DNA after intravenous injection (43, 44).

The main barriers on the intracellular level are: i) endosomal escape of DNA, which involves translocation of DNA from endosomes to cytosol to avoid DNA trafficking to the lysosomal compartment (45), where a more acidic pH (pH=4.5-5) and numerous enzymes degrade DNA. ii) after release from the endosomal/lysosomal pathway, the DNA has to be able to cross the cytoplasm in order to reach the nucleus of the target

cell (46). It was found that DNA is transported through the cytoplasm in a size dependent manner (47). iii) nuclear entry, the nuclear envelope represents an important barrier for successful gene transfer (48).

It is important to emphasise that naked DNA cannot effectively overcome all of these barriers to reach its site of action. Therefore, gene delivery systems are being developed in order to improve the DNA delivery properties such as; size and charge density, increase DNA stability against enzymatic degradation and increase the efficiency of cellular uptake.

Methods for gene delivery fall into two classes: viral vectors such as retroviruses, adenoviruses and adeno-associated viruses and non-viral vectors for example cationic liposomes and cationic polymers (polyethylenimine, poly-L-lysine and polyamidoamine), which will be briefly introduced in the following sections.

1.3 Vectors used in gene delivery systems

1.3.1 Viral vectors

The ability of pathogenic viruses to encapsulate and deliver their genetic cargo to human cells has been exploited through replacing their genome with therapeutic genes (49). Various types of viruses, including retroviruses (50), lentiviruses (51), adenoviruses (52), and adeno-associated viruses (53) have been employed in gene therapy. A handful of gene therapies based on viral vectors (Error! Reference source not found.) have been granted market approval in China or by Food and Drug Administration (FDA) or European Medicines Agency (EMA) including Gendicine™, an adenoviral vector-based therapy for head and neck cancer; alipogene tiparvovec (Glybera®), an adeno-associated viral vector-based therapy for lipoprotein lipase deficiency; Strimvelis™ which is an retrovirus vector-based gene therapy

product for treatment of severe combined immunodeficiency due to adenosine deaminase deficiency (54); talimogene laherparepvec (Imlygic™), an oncolytic granulocyte macrophage colony-stimulating factor (GM-CSF) gene-carrying herpes simplex virus type 1 for melanoma immunotherapy (55); and Kymriah™ (tisagenlecleucel), human cells genetically modified with a lentivirus, is the first cell-based gene therapy for the treatment of acute lymphoblastic leukemia (56). Although viral vectors are efficient in gene delivery, their clinical application is limited by their drawbacks which include immunogenicity which can be fatal (57, 58), insertional mutagenesis, oncogenicity (59), high cost, difficulty in preparing viral vectors on a large-scale and low loading capacity (35).

Table 1.1 Approved gene therapies based on viral vectors.

Gene therapy product	Advantages	Disadvantages
Glybera®	The virus used for the therapy (adeno-associated virus serotype 1) is not associated with any known disease or pathology in humans (60, 61) Very low immunogenic potential (62)	Cumbersome delivery profile involving multiple injections into the lower extremities (54)
Strimvelis™	No insertional mutagenesis (54) Low immunogenic potential (63)	
Gendicine™	Adenovirus vectors have a theoretical carrying capacity of over 30 kb (64) High efficiency of gene Transfer (65)	
Kymriah™	Once administered, it is present in the blood and bone marrow beyond two years (66) which may be	Life-threatening reaction called cytokine release syndrome, neurological toxicity,

	associated with more durable remission (67).	hypogammaglobulinemia (66, 68)

1.3.2 Non-viral vectors

The disadvantages of the viral vectors have led to the development of non-viral delivery systems as alternatives. Non-viral gene delivery systems can be categorized into two groups: physical methods and chemical methods (69). In contrast to viral vectors, they have larger insert size and lower immunogenicity. However, most polyaminoacids could not be considered as potential biomaterials due to their immunogenicity (70). Moreover, they are cheap, stable and easy to produce in large quantity (58, 71, 72) . On the other hand, their utilization on a large level has been restricted by their low transfection efficiency (73). Chemical vectors are proposed as promising alternatives to viral ones to overcome the drawbacks of the latter. These vectors have three objectives that improve gene transfer into the cell nucleus. They: (i) mask DNA-negative charges, (ii) condense the DNA molecule to make it smaller, and (iii) protect it from degradation by intracellular nuclease. (69). Electrostatic interaction between the negative charge of the DNA molecule and the positive charge of the chemical vectors which include cationic lipids and polymers is required for these processes to be achieved. This type of interaction can lead to the formation of a positive complex known as lipoplex or polyplex, respectively (74).

1.3.2.1 Cationic liposomes

Cationic lipids have been extensively investigated and used as non-viral gene carriers. Numerous cationic lipids have been developed including N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), dioleyloxy-N,N-dimethyl-3-aminopropane (DODMA), 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), 2,3-di-oleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-

dimethyl-l-propan-aminium (DOSPA), 5-carboxyspermylglycine dioctadecylamide (DOGS) (75). The complexes formed between cationic lipids and anionic nucleic acids are called lipoplexes (76). Cationic lipids have three structural components which include a positively charged hydrophilic head group, a hydrophobic tail which can be a cholesterol derivative or saturated and unsaturated chains, and a linker (77) . The hydrophilic head normally consists of one or more amine groups including primary, secondary, tertiary amines and quaternary ammonium salts (78), whereas the hydrophobic group is the non-polar moiety that drives the formation of lipoplexes (79). The linker bond is the chemical part that joins the hydrophilic head to the hydrophobic tail. In addition, its property has a significant role in determining the biodegradability of the cationic lipid and in affecting its toxicity and transfection efficiency (80, 81). However, cationic lipid vectors need to be improved as they still show relatively low transfection efficiency compared to viral vectors, high toxicity and lack of colloidal stability (82, 83).

Cationic liposome is an important tool that mediate delivery of DNA (84). Cationic liposomes have been prepared through using cationic lipid such as DOTMA with a neutral co-lipid, dioleoyl phosphatidylethanol amine (DOPE). This mixture of cationic and neutral lipids is known as Lipofectin™ (85). Another cationic liposomal formulation is Lipofectamine™ [DOSPA: DOPE] and is available as commercial transfection agent (86). DOPE has the advantage of facilitating formation of the liposomes and help transition of lipoplexes from a bilayer to a hexagonal conformation within the endosome which in turn facilitates the destabilization of endosomal membranes (87-90) and DNA release in the cytoplasm (91, 92). Therefore, its combination with a cationic lipid has led to improvement in the transfection activity of cationic liposomes (93). However, the use of these reagents *in vivo* is encountered

by their inherent toxicities and low transfection efficiency (94). The mechanism underlying their toxicity is largely induction of apoptosis (95).

1.3.2.2 Cationic polymers

Another class of synthetic vectors that have been extensively studied is the cationic polymers. Cationic polymers are one of the most significant non-viral gene delivery vectors (96) that have been developed and used for nearly four decades (97). These polymers have positively charged groups in their backbone (96) and have the ability to condense DNA molecules by electrostatic interactions into nanometer-sized complexes known as polyplexes (98). Polyplexes, offer several potential advantages over cationic lipids such as small size, higher protection against enzymatic degradation and greater stability (99, 100). Many cationic polymers have been employed as nucleic acid carriers such as chitosan (101), polyamidoamine dendrimers (PAMAM) (102), linear polyamidoamine (PAA) (103), poly-L-lysine (PLL) (97) and polyethylenimine (PEI) (104).

1.3.2.2.1 **Poly-L-lysine (PLL)**

PLL was one of the first cationic polymers employed as a gene delivery vector (97). PLL is a linear polypeptide with the amino acid lysine as repeating unit; thus, it is biodegradable with the potential to condense the DNA and mediate gene delivery both *in vitro* and *in vivo* (105-107). PLL was able to form discrete complexes in water, whereas aggregation occurred after dilution in cell culture medium (108). It is commercially available in different molecular weights. Its efficiency in gene delivery depends on its molecular weight. Low molecular weight PLL formed less soluble complexes with DNA that are rapidly cleared by the liver, whereas DNA condensation and the level of gene expression increased with high molecular weight PLL (109-111).

However, high molecular weight PLLs exhibit high cytotoxicity (83, 107, 112) and produce very low level of gene expression when compared with polyethylenimine (113, 114). Despite their biodegradable peptide structure (115), PLLs and PLL–DNA complexes were found to induce cytotoxicity through plasma membrane damage and mitochondrially mediated apoptosis in different types of human cell lines (Jurkat T-cells, umbilical vein endothelial cells, and THLE-3 hepatocyte-like cell line) (116) due to their cationic surfaces (117), whereas their lower transfection is due to a lack of efficient endosomal escape mechanism (118) as a result of the absence of secondary and tertiary amines in its backbone (119). Moreover, excess of free PLL polymer could not increase the transfection efficiency of the polyplexes (120). It has been found that its transfection efficiency was improved after co-incubation with chloroquine (121, 122) . 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 (123-126). As a consequence, it raises their pH (127, 128), impairs the fusion of endosomes and lysosomes, and inhibits the degradative effects of lysosomal enzymes (129).This increase in the transfection efficiency of PLL after addition of chloroquine indicates that under control transfection conditions, a significant fraction of the vector is trafficked from the endosomes to the late endosomes and lysosomes where it is likely degraded (130).

1.3.2.2.2 **Chitosan**

Chitosan is a natural cationic polysaccharide polymer which consists of two types of repeating units, N-acetyl-D-glucosamine and D-glucosamine, linked by (1-4)-β-glycosidic linkage (131). It can be chemically produced from chitin by the process of deacetylation with widely varying content of N-acetylated D-glucosamine and chain

lengths (132). Chitosan is soluble in dilute acidic solutions at pH less than 6.5 but insoluble in water (133). It is available in low and high molecular weights with various degrees of deacetylation (134). It can be used for biomedical applications due to its biocompatibility, biodegradability and low toxicity (135). The biodegradability of chitosan was found to be related to its susceptibility to hydrolysis by a non-specific proteolytic enzyme, lysozyme, that exists in all human body tissues (136). Transfection efficiency of chitosan polyplexes is influenced by the molecular weight and degree of deacetylation of chitosan. Increase in the degree of deacetylation lead to improvement in the transfection efficiency as a result of higher charge density and therefore higher stability of the polyplexes. Moreover, higher molecular weight chitosan results in more stable polyplexes. However, higher molecular weight polymers may lead to a reduction in the transfection efficiency of the polyplexes because of tight binding of DNA that affect its release in the cytoplasm (137). Furthermore, the highest transfection efficiency of chitosan polyplexes is at pH 6.8-7, whereas transfection decreases at pH > 7.5 due to dissociation of DNA from the polyplexes (138). Despite chitosan having a good biocompatibility, it exhibited low transfection (139, 140) because of slow endosomal escape (141). It has been found that the low buffer capacity of chitosan prevents its escape from the endosome by proton sponge effect compared to other cationic polymers with high buffer capacity, such as PEI (142). Therefore, structural modifications of chitosan are required for successful transfection with chitosan polyplexes (143).

1.3.2.2.3 **Polyethyleimine (PEI)**

Polyethylenimine (PEI) is considered a gold standard non-viral gene delivery vehicle because of its high transfection activity (104). PEI can exist in either linear or branched

form with the molecular weight ranging between 200 Da and 1,500 kDa and its repeating units consist of two aliphatic carbon groups and amino nitrogen (104, 144-148). Linear PEI has only secondary amines, in contrast to branched PEIs which contain primary, secondary and tertiary amino groups. PEIs have protonable amine groups that are responsible for their high cationic charge density and buffering capacity over a wide range of pH (104). The high cationic density of PEI also contributes to the condensation of nucleic acids into polyplexes which protect nucleic acids from enzymatic degradation. Moreover, positively charged polyplexes can bind to negatively charged cell surfaces and be taken up by various endocytic mechanisms as well as cause plasma membrane destabilization (104, 144, 149-152). In the endosomal compartment, PEI based polyplexes may escape by the so-called proton sponge effect (**Figure 1.2**). Because of its many nitrogen atoms, PEI seems to exhibit considerable buffer capacity at the low pH values of the endolysosomes (153). where the unprotonated amines are able to absorb protons. The proton sponge effect is believed to result in excessive V-ATPase mediated H^+ influx, which leads to increased influx of Cl^- and water, and in turn this may lead to osmotic swelling, destabilization and/or rupture of the lysosomal membrane and release of the polyplexes into the cytosolic environment (154). However, this pH-dependent mechanism is heavily debated (155-157) and a recent study making use of lysosomal pH sensors demonstrated that the pH change in PEI containing lysosomes does not occur and thus the proton sponge effect may not be the main route of escape (158).

Regardless of the internalization pathways, toxicity and transfection efficiency of PEI depend on its molecular weight and architecture and directly correlate with the positive charge of the polyplexes (71, 151, 159-161). Higher molecular weight PEI exhibit higher transfection efficiency and cytoxicity than their low molecular weight

counterparts (71, 104, 160-164). Furthermore, linear PEI polyplexes showed less cytotoxicity and transfection efficacy than branched PEI counterparts (165). However, other studies showed that a linear morphology has superior transfection efficiency compared to the branched structure both *in vitro* and *in vivo* (71, 162, 166-168). These discrepancies might be related to the differences in the study design and/or cell types. Moreover, higher transfection efficiency might be due to fewer cytotoxic complications and cell death associated with architecture-dependent cytotoxicity. However, it has been found that the highest transfection efficiency is achieved by cationic polymers that are most cytotoxic (71, 104, 160, 161, 163).

Due to its high cationic charge density and non-biodegradability, PEI is associated with higher cytotoxicity compared to other non-viral vectors (148, 169). Cytotoxicity of PEI triggers cell death (170-172) and a variety of non-specific interactions with the biological environment (146). Without chemical modifications PEI might interact with all cells by non-specific cell attachment, leading to moderate genotoxic effects, necrosis and apoptotic cell death (173) This serious cytotoxicity has been the obstacle to *in vivo* applications as unmodified PEI does not show good transfection efficiency (174, 175).

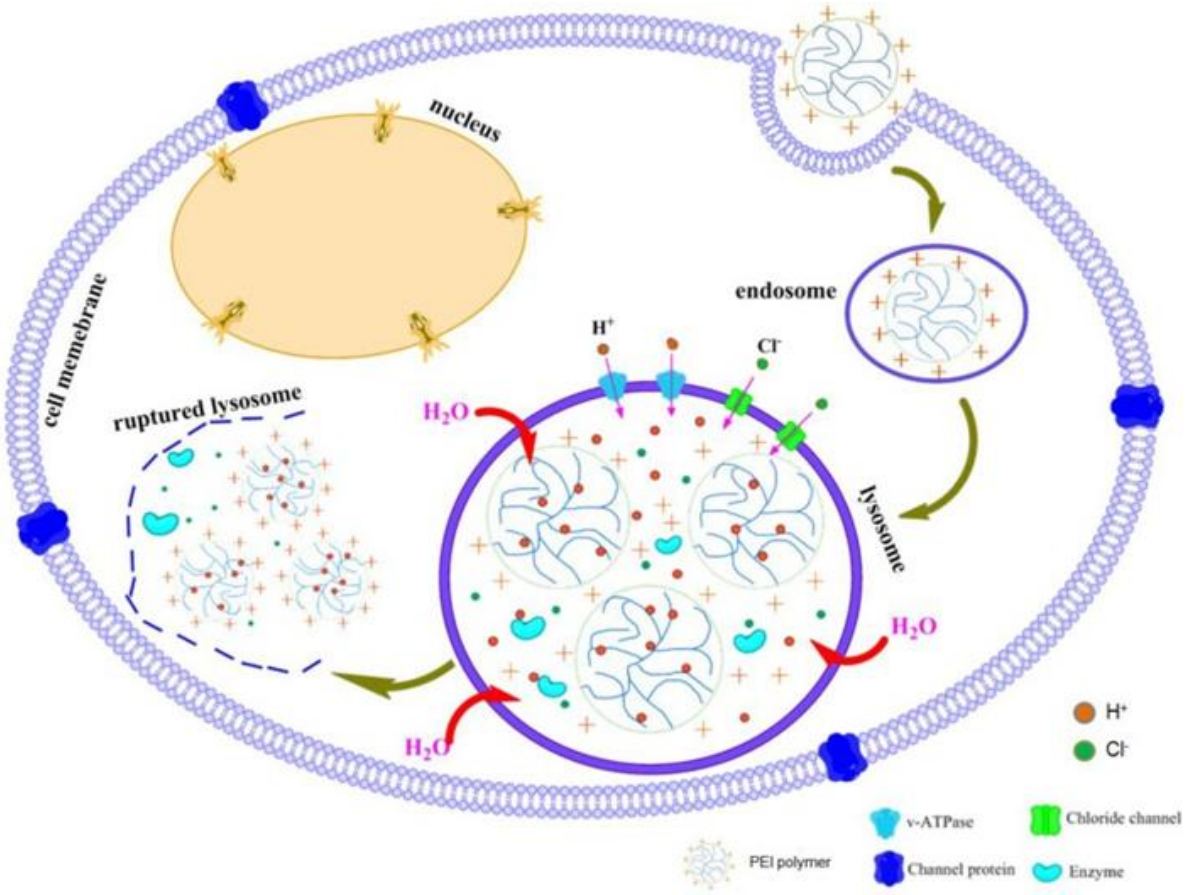


Figure 1.2 Schematic representation of the proton sponge effect of PEI polymer (135).

1.3.2.2.4 **Polyamidoamine Dendrimers (PAMAM)**

Polyamidoamine dendrimers (PAMAM) were first introduced in the mid-1980s (176). They are spheroid, highly branched polymers, with a surface of primary amino groups that gives these polymers a high cationic charge density. The high density of their outer amine groups enables efficient condensation of DNA, leaving the internal tertiary amines available for neutralisation during acidification within the endo-lysosomes, thus enabling more efficient endosomal escape by the proton sponge mechanism (73, 114, 177, 178). PAMAM dendrimers are synthesised by the successive addition of methacrylate or ethylenediamine to ammonia or ethylenediamine core molecules (179, 180). Each round of additions is termed a generation (G) and leads to the molecular weight of the dendrimer being increased and the number of the primary amine surface groups being doubled. The number of generations, therefore, controls the diameter, molecular weight and surface charge of the dendrimers (179). Dendrimers are an attractive architecture for the development of DNA delivery systems due to their well-defined polymer structure and their surface charge properties. Different cell lines were efficiently transfected using PAMAM dendrimers such as liver hepatoblastoma (HepG2), rat embryonal fibroblast (RAT2), mouse embryonal (NIH), and monkey kidney fibroblast (COS-7) cells. The capability of dendrimers to transfect the cells were dependent on the size, shape and amount of dendrimer in the complex (181, 182). It has been found that cytotoxicity and transfection efficiency of PAMAM dendrimers increase with increasing generation (182-184) and the response is well correlated with the number of surface primary amino groups (185). Waite *et al*. showed that the transfection efficiency of PAMAM decreases after reducing the number of primary amines on its surface by acetylation

(186) suggesting the importance of a high number of primary amino groups in providing stable nucleic acid binding (187). However, higher generation PAMAM dendrimers (more than G7) achieved lower transfection efficiency than dendrimer (G6) (188), possibly because of their rigid structure and cytotoxicity (182). PAMAM dendrimers exhibited cytotoxicity through mitochondrial membrane depolarization (189, 190) and generation of reactive oxygen species (ROS) as a result of mitochondrial localisation leading to oxidative stress (191, 192). Moreover, PAMAM dendrimers led to cytotoxicity by causing cell membrane damage, thinning and leakage, endosomal disruption by the proton sponge effect, which can result in endosomolysis and release of polyplexes in to the cytosol (193). Although PAMAM dendrimer has tertiary amino groups which act as a proton sponge in endosomes (192, 194), its transfection efficiency increased after addition of chloroquine, indicating localization of the complexes within the endosome (195) due to limited efficiency to escape from endosomes/lysosomes to release DNA in cytosol. It has been found that the buffer capacity of PAMAM increased with an increase in molecular weight/generation and its proton sponge effect is correlated with cytotoxicity (196).

1.3.2.2.5 **Linear polyamidoamines (PAAs)**

Linear Poly(amidoamine)s (PAA)s are regular linear cationic polymers, which are obtained by stepwise polyaddition of primary monoamines or bis(secondary amines) to bis-acrylamides as shown in **Figure 1.3** (197-199). PAAs contain tertiary amines and amido groups regularly arranged along the macromolecular chain and therefore, the tertiary amino groups in the main chain can be protonated, giving the polymer an overall basic and poly-cationic character with amidic bonds, which are degradable in aqueous solutions (198, 200). Ferruti *et al*. have synthesized and studied the properties

of a number of PAAs and have pointed out their high potential for biomedical applications due to their low toxicity and biodegradability which make them an exceptionally versatile family of polymers (200).

The polymerization reaction allows to introduce many additional functional groups as side substituents to form complexes with nucleic acids. At physiological pH, PAAs being cationic polyelectrolytes can easily interact with nucleic acids. The PAAs are unusual in that the basicity of the aminic nitrogens of the repeating units does not depend on the protonation of the whole molecule. This contrasts with other polycations where the protonation of specific group is markedly affected by the state of neighbouring groups. PAA can undergo marked increase in the degree of their ionization between pH 7.4 and pH 5.5. This is accompanied by a change in the confirmation from a coiled to an extended structure (200, 201). Like PEI and PMAMA dendrimers, PAA can provide buffering capacity within the endosomal compartment and promote the endosomal escape of DNA complexes into cytoplasm (202-204). PAAs also show negligible clearance by the reticuloendothelial system (RES) and are less cytotoxic than PLL and PEI (205, 206). Their gene transfection efficiency is also comparable to Lipofectin and PEI(207, 208). Previous work in the development of linear PAAs was focused on the physicochemical and biological characterization of DNA complexes based on their structures. PAA based on methylene-bis-acrylamide and dimethylethylene diamine co-monomers (MBA-DMEDA) showed the most favourable DNA binding and colloidal properties with less toxicity and more efficient *in vitro* transfection than PLL (205). However, MBA-DMEDA was more cytotoxic than 3 other PAA structures compared in that study. This indicates that biocompatibility including cytotoxicity and transfection efficiency of PAAs are strongly dependent on the structure of PAAs (205, 209).

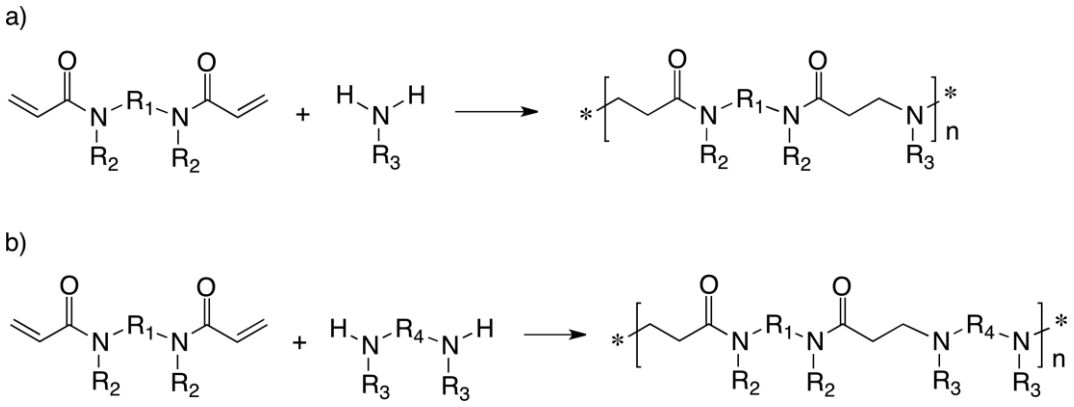


Figure 1.3 Polyaddition of (A) primary monoamines and (B) bis(secondary amines) to bisacrylamide (210).

1.4 Effects of structural variation in the main chain of polyamidoamines

The effect of different charge density and chain stiffness of several PAAs on DNA binding, colloidal stability and transfection activity has been studied by Garnett *et al.* (211). It has been found that the DNA binding capacity of Methylene Bis Acrylamid-Dimethyl ethylenediamine (MBA-DMEDA) or Methylene Bis Acrylamid- 2 Methyl Piperazine (MBA-2MP) with two tertiary amino groups within the repeating unit is higher than Methylene Bis Acrylamid- Methylamine (MBA-MMA) with one tertiary amino group (**Figure 1.4**). On the other hand, the substitution of a piperazine ring for the methylene moiety in the diacrylamide segment has resulted in a substantial reduction in DNA binding capacity (MBA-2MP vs. Bis Acryloyl Piperazine (BAP)-2MP) (**Figure 1.4**). This reduction has been shown to be due to the existence of two piperazine rings per repeating unit which leads to the formation of an inflexible polymer backbone so that the polymer cannot reach the DNA closely. Eventually, it has been concluded that the DNA binding capacity of polymers as well as the colloidal stability and transfection efficacy of polyplexes depend on both the charge density and the structural flexibility of the polymer (212).

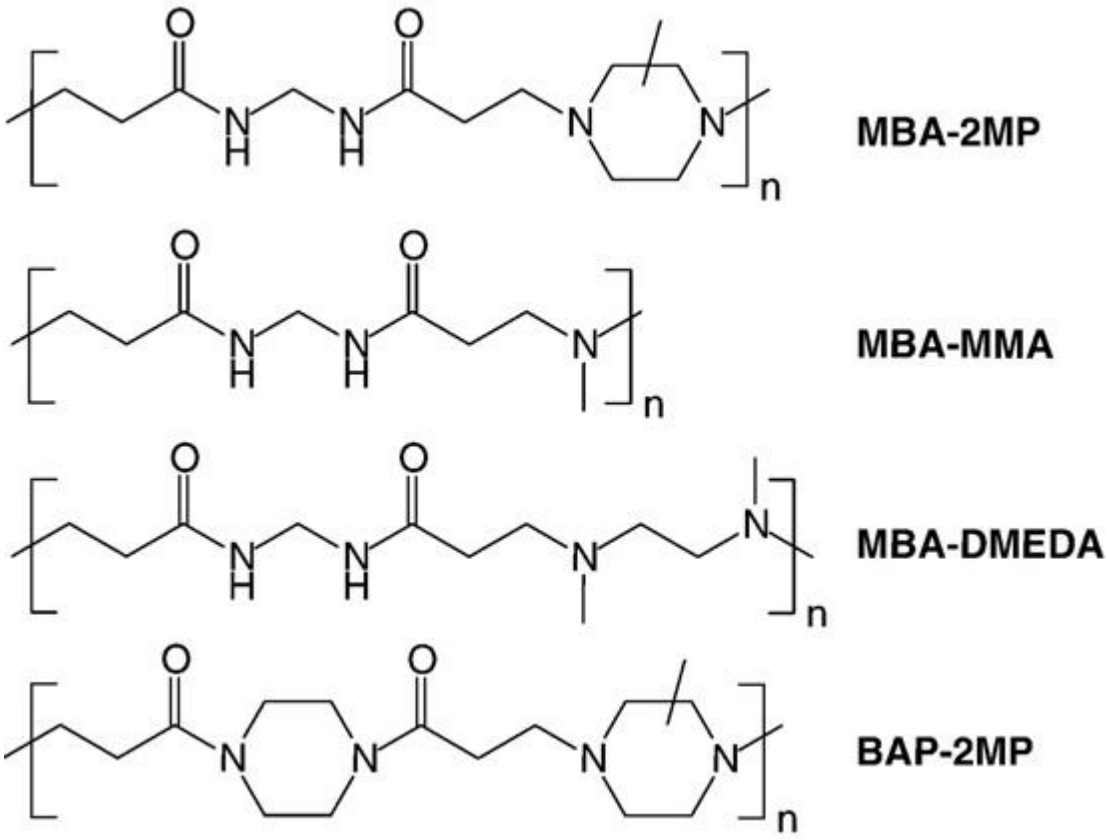


Figure 1.4 Chemical structure of PAAs with different charge density and polymer backbone stiffness (212).

1.4.1 Effects of a carboxylic acid moiety in the bisacrylamide segment of polyamidoamines

After endocytosis, polyplexes located in the endosomes may undergo degradation during the acidification process of endosomes from early to late endosomes and the final fusion with lysosomes. Genes are also easily degraded by enzymes present in the acidic endosomes (pH 5–7) and lysosomes (pH 4.5) (213). One of the main hurdles in gene delivery is the endosomal escape of the polyplexes into the cytosol which is considered necessary for ultimate delivery of their load into the nucleus. Some viruses have endosomolytic effect by using their specific fusogenic peptides which allows their endosomal escape. Disruption of the endosomal membrane can also occur by different synthetic fusogenic peptides such as melittin (214). The susceptibility of these fusogenic peptides to the environmental pH can alter their conformations from

a random coil at neutral pH to an α-helix at acidic pH. Ferruti *et al*. developed a series of amphoteric PAAs possessing a carboxylic acid group in the bisacrylamide unit (**Figure 1.5**). These amphoteric PAAs also display a pH-dependant conformational change upon protonation of the carboxylate and amino groups in these polymers, a behavior similar to fusogenic peptides, as determined by small-angle neutron scattering (215). Therefore, they cause more hemolysis of erythrocytes at pH 5.5 (endosomal pH) than at pH 7.4 (physiological pH) (206). In this series, BAC-2MP (ISA23) shows the greatest hemolysis at pH 6.5, probably due to the highest structural rigidity of this polymer. This polymer also shows "stealth" properties after intravenous administration (204). BAC- 2MP induces a comparable transfection efficiency to that of 25 kDa branched PEI in HepG2 cells and higher than the analog MBA-2MP (**Figure 1.4**) lacking the carboxylic acid groups. These results indicate structural segments that induce pH-dependent conformational changes, as illustrated by the amphoteric PAAs, can be exploited to promote endosomolytic activity, enhanced gene transfection (207).

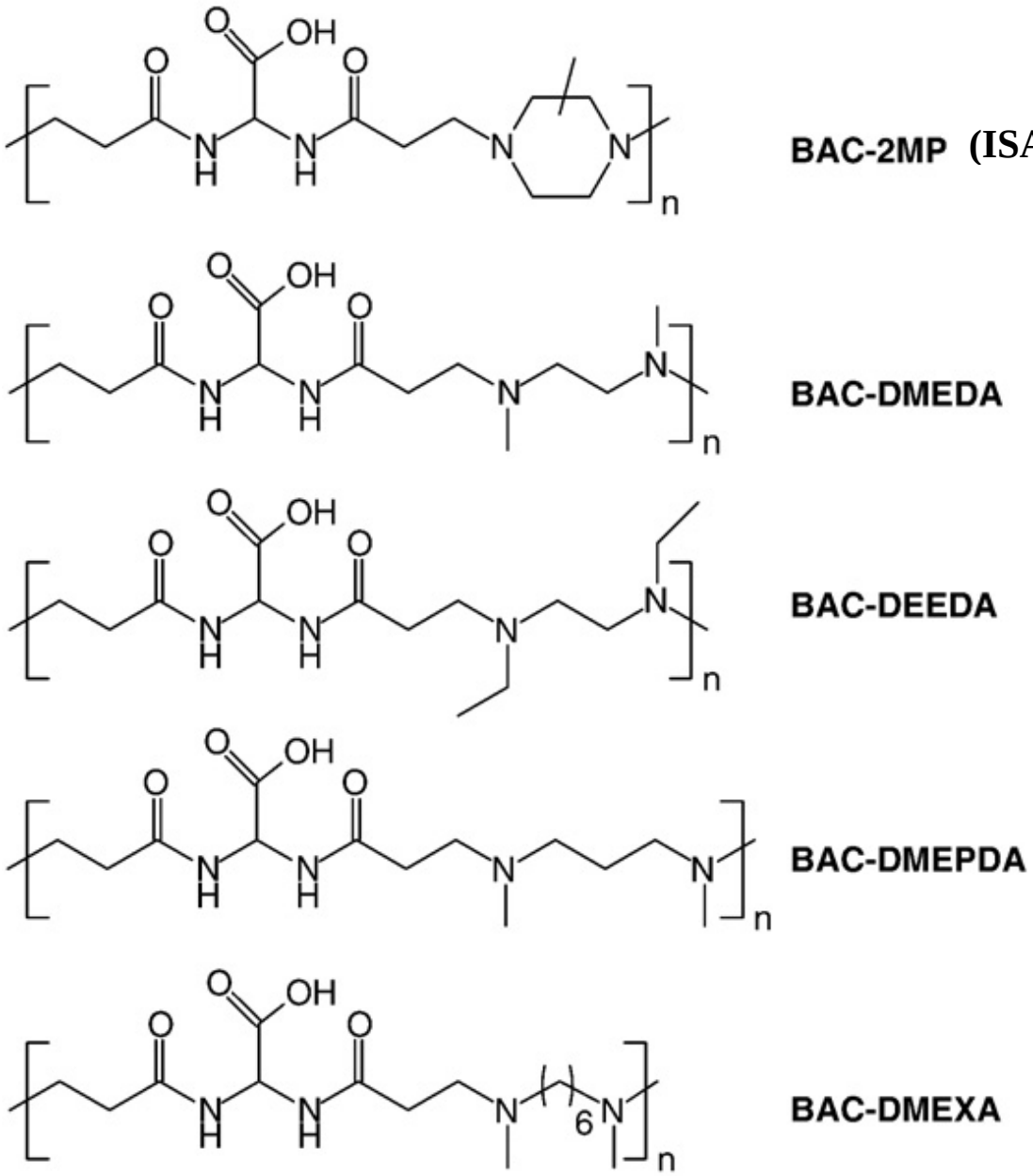


Figure 1.5 Chemical structure of amphoteric polyamidoamines.
In each case, polymers were prepared from 2,2-bis(acrylamido)acetic acid (BAC) by polyaddition reaction with 2-methylpiperazine (ISA 23), 1,2-bis(N-methylamino)ethane (DMEDA-BAC), 1,2-bis(N-ethylamino)ethane (DEEDA-BAC), 1,3-bis(N-methylamino)propane (DMEPDA-BAC), or 1,6-bis(N-methylamino)-hexane (DMEXA-BAC).(212).

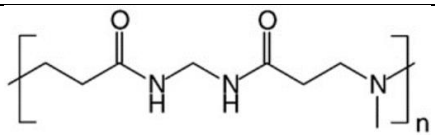
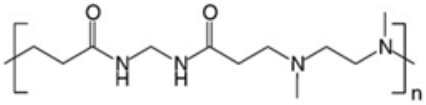
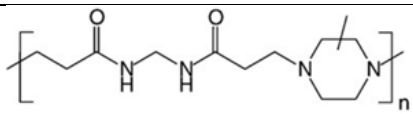
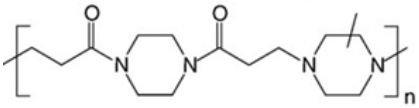
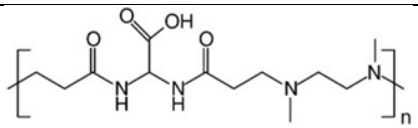
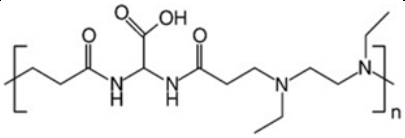
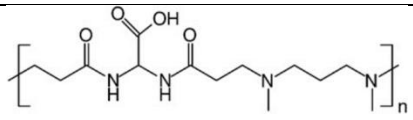
1.5 Linear polyamidoamines physicochemistry

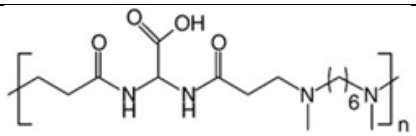
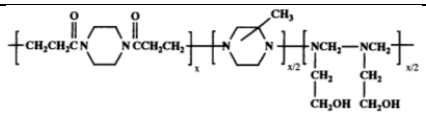
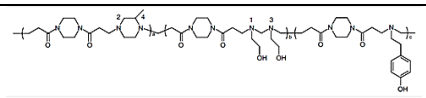
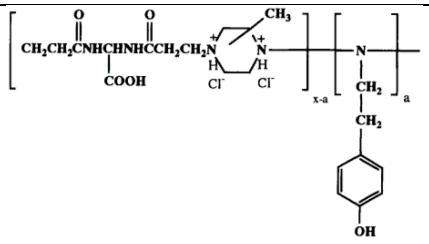
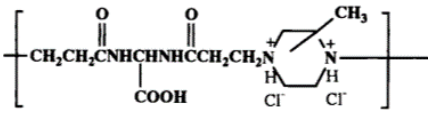
The physicochemical characterization of various PAAs is given in **Table 1.2**. PAA pKa's are, unlike PEI, not influenced by the degree of protonation of neighbouring repeating units (205). The ability to manipulate the biophysical properties of PAAs is very attractive and through careful monomer selection PAAs can be designed to ionize over a physiological pH range (216). Examples of monomers selected to display discrete and reproducible pKa's, are given in **Table 1.2**. This is one of the properties that differentiate PAAs from molecules such as PEI. PAA ionization events may be tailored to facilitate an expansion of the radius of gyration (Rg) of the polymer coil (206, 215) over a pH range of pH 7.4–5.5. This ionization can also mediate a transition in net polymer change from negative at pH 7.4 to positive at an endosomal pH. This is particularly significant as it allows the acidic luminal environment of the endocytic system to act as a physiological trigger during drug delivery. The expansion in the coil's Rg is due to the protonation of amines contained therein. In the instance of ISA23, ISA PAAs are series of linear PAAs that were first synthesized by a scientist called Isabella and are named after her, the polymer gains a net positive charge as the pH drops changing from a near-neutral net change at pH 7.4 to a net cationic charge at pH 5.5. Given the propensity for polycations to destabilize biological membranes, this phenomenon means that PAAs can be synthesized to selectively destabilize biological membranes after endocytic capture, facilitating the cytosolic delivery of macromolecules. Protamine and PLL are known as linear-chain, flexible macromolecules due to their physicochemical properties; they are more effective in neutralization of cell-surface charges than those molecules with rigid structure, which may lead to conformational changes of the cell membrane and therefore its disruption (217). However, viscosimetric titrations in 0.1 M NaCl prove that the conformational

freedom is reduced on protonation. All polymers in neutral form are most likely in a random coil structure due to the great number of accessible conformations with the same energy. After the first protonation of all monomeric units the conformational freedom of the polymer decreases and results in a more rigid structure. It has also been shown that after the first protonation event a pronounced reduction of viscosity at a = 0.5 (a = degree of protonation) was measured (201). A second protonation event led to the electrostatic repulsion between the positively charged onium ions belonging to the same monomeric unit. This effect compels the polymer to adopt a more rigid structure. Therefore, the conformational freedom clearly decreases with the lengthening of polymeric structure (201). PAA conformation and membrane interaction have been characterized using a variety of techniques (206, 218). A recent study investigated the characterization of linear PAAs using small angle neutron scattering (SANS), electron paramagnetic resonance and pulsed-gradient spin- echo NMR. Briefly, these techniques showed ISA23 adopting a compact random coil at pH 7.4 with a Rg of approximately 2 nm. As the pH was lowered, the polymer coil gradually expanded to a maximum of approximately 8 nm at pH 3 before coil collapse (218). Further studies investigating the interaction between ISA23 and 'model' sodium dodecyl sulfate (SDS) micelles showed an interaction between the polymer and the micelle even though, at neutral pH, the polymer was thought to carry a net negative charge (218). The 'model' micelles evaluated in this study (218), were generated using a variety of surfactant mixtures containing bulky malono-bis-N-methylglucamide or extended ethylene oxide head groups as well as 1-palmitoyl-2-hydroxy-sn-glycero-3-phospho- choline, mimicking the cellular endomembrane. This result might indicate a hydrophobic component to the interaction or the preferential protonation of 'clusters' of amines (219). It has also been observed that in terms of

interaction with model membranes, polymer charge was more important than polymer conformation (215). However, when more hydrophobic monomers were introduced into an ISA-like PAA chain, no pH-directed selectivity was observed in relation to a rat red blood cell lysis assay (206). It is possible that this apparent disparity was due to differences in model membrane systems i.e., between model micelles and rat red blood cell membranes. Given that all PAAs with hydrolysable amidic bonds in their main chain are, in principle, degradable in the presence of water, a study examining PAA degradation behaviour in aqueous media at pH 7.5 and 37°C was undertaken (220). These data were acquired by examining both the viscosity of ISA1 and ISA23 and their molecular weight measured by gel permeation chromatography. In summary, ISA1 was seen to have a half-life of approximately 75 h where as ISA23 was seen to have a half-life of approximately 210 h (both measurements were based on viscometry and were performed in 0.2 M phosphate buffer pH 7.5) (220). For use in biomedical applications, this property is considered double edged in that it can be considered advantageous for those PAAs considered as pro-drugs but disadvantageous for PAAs which require the absence of leachable products such as heparinisable biomaterials. The structure of both the aminic and amidic moieties were found to influence the rate of hydrolytic degradation in aqueous media. Furthermore, the degradation rate was strongly influenced by pH (220). However, given the propensity of nonbiodegradable materials that are above the renal threshold to accumulate in biological systems (e.g., poly[ethylene glycol]) (221), PAA biodegradability is desirable if these molecules are to be used as drug carriers in a clinical environment.

Table 1.2 Physicochemical characterization of some PAA polymers.

Polymer	**Code**	**Structure**	**pKa**1	**pKa**2	**pKa**3	**pKa4**	**Mw (Da)**
MBA-MMA	NG29		7.8				9500
MBA-DMEDA	NG30		8.1	4.5			21500
MBA-2MP	NG28		7.1	2.98			8500
BAP-2MP	NG38		7.1	2.98			4930
	NG39		7.1	2.98			15800
	NG37		7.1	2.98			25300
DMEDA-BAC			8.25	4.85	2		29000
DEEDA-BAC			8.65	4.62	2.6		30300
DMEPDA-BAC			8.99	6.85	1.91		11400

DMEXA-BAC			9.53	8.37	2.17		30900
ISA1			8.1	6.9	3.8	3	12300
ISA4			8.1	6.9	3.8	3	15000
ISA22			7.5	3.25	2.1		14900
ISA23			7.5	3.25	2.1		16500

1.6 Linear polyamidoamines biology

1.6.1 Body distribution

Unlike many polycations (217, 222), PAAs may be synthesized to be non-hepatotropic when administered *in vivo* (e.g.,^{125}I-labelled ISA22) (204). This property is thought to be mainly due to the predominantly negative charge of ^{125}I-labelled ISA22 at pH 7.4. This characteristic provides a variety of opportunities for disease targeting, including passive tumor targeting by the enhanced permeability and retention (EPR) effect (204). We have previously demonstrated that ^{125}I-labelled ISA22 was not cleared from the systemic circulation by the liver (<10% recovered dose at 5 h) with 2.5% dose/g (tissue) accumulating in tumor mass after 5 h (204). This is in contrast to ^{125}I-labelled ISA4 (ISA1-*try*), which displayed substantial liver clearance (>80% dose, 1 h), relative to ^{125}I-labelled ISA22 (ISA23-*try*) which evaded liver capture (<10% dose, 1 h) following iv. administration into the caudal vein of the rat (204). It is of note that ^{125}I-labelled ISA4 carries a net positive charge at pH 7.4. Of the ^{125}I-labelled ISA22 recovered from the urine of the rats used, the vast majority of the ^{125}I label was found to be still associated with a macromolecule, for instance eluted in the void volume of a PD10 column. The capacity of ^{125}I-labelled ISA4 to be subject to rapid liver capture has also facilitated the study of the membrane lytic properties of the molecule *in vivo* as will be detailed later. It is well established that particulates are rapidly cleared from the systemic circulation by the reticuloendothelial system (RES; part of the mononuclear phagocyte system located in the reticular connective tissue) (223). RES clearance results from a range of interactions between the particle and serum components including the complement system, resulting in the phagocytosis of the particulate (217). A potential solution to this problem is to form a steric barrier around

particles (or solutes), as has been demonstrated with drug-delivery systems previously, by the inclusion of hydrophilic polymers such as PEG, or HPMA (224). PEGylation greatly reduces the adsorption of serum components to the particles' surfaces, reducing the subsequent uptake by mononuclear phagocytes. Therefore, circulation time of the delivery system is increased, leading to higher drug efficacy (223). This effect has been reported to be analogous to stealth liposomes, becoming up to 100-fold less prone to aggregation by albumin *in vitro* (224). We have previously noted that ISA23 can escape RES clearance after iv. administration allowing tumor targeting by the EPR effect (204). Consequently, it may be possible to use PAAs to provide a 'stealth' imparting corona via its surface conjugation to a particulate. However, unlike high Mr PEG, PAAs are hydrolytically labile, meaning that they can be excreted from the body (204) and will not be subject to accumulation over time.

1.6.2 Cellular uptake & intracellular trafficking

Recently, a combination of fluorescent microscopy and subcellular fractionation was used to follow the intracellular uptake and trafficking of PAAs both *in vitro* and *in vivo* (225). The cellular uptake of linear PAAs incorporating a conjugated fluorophore Oregon Green (OG), was measured in B16F10 (highly metastatic murine melanoma) cells. ISA1-OG uptake was approximately 60-fold greater than that seen for ISA23-OG, which was presumed to be due to the positive charge associated with ISA1 relative to ISA23 (at neutral pH). At neutral pH a slightly positively charged PAA (i.e., ISA1), will interact with anionic cell membrane components and thus enhance its entry into the cell via absorptive as opposed to fluid-phase pinocytosis. The colocalization of ISA1-OG with early endosome antigen 1 (EEA1), an early endosomal marker, indicated that the polymer conjugate was within an early

endosome within 5–10 min after having been subject to endocytic capture (**Figure** 1.6) (225) confirming predictions based on the uptake of other fluorescent-labelled polymers (226). Given the body distribution of ^{125}I-labelled ISA4 (ISA1-*try*) (see 'Body distribution'), the intracellular trafficking of ^{125}I-labelled ISA4 through the rat liver was evaluated (225). This study was performed in order to assay ^{125}I-labelled ISA4-mediated intracellular membrane perturbation in response to decreasing pH (i.e., in response to lysosomal translocation) *in vivo*. Furthermore, as the polymer had been radio-iodinated, the results could be readily quantified. Following the iv. administration of the radio-iodinated polymer, radioactivity levels were assayed in various liver subcellular fractions relative to a phosphate buffered saline (PBS) control (the PBS being injected into the caudal vein of separate animals). The distribution of the lysosomal enzyme N-acetyl-ß-D-glucosaminidase (NAG) was monitored in response to polymer administration, NAG release from a membrane-bound compartment being an indicator of PAA-induced vesicle membrane destabilization (225). The PAA-mediated transfer of radioactivity and NAG from the membrane-limited mitochondrial (lysosomal) fraction, to the cytosolic fraction was quantified. These cytosolic translocation events were initially PAA dose dependent but surprisingly, a rapid plateau effect was seen in response to both increasing time and increasing polymer dose. It was concluded that ^{125}I-labelled ISA4 could disrupt the endomembrane system *in vivo*, allowing the translocation of NAG (71,000 g/mol) into the cytosol (**Figure** 1.6). This indicated that the selective membrane lytic effects of PAAs reported by inference previously (227, 228) function *in vivo* as well as *in vitro* (225). It is also possible that ISA4 inhibits vesicle fusion and/or causes an efflux of hydrogen ions, resulting in an increased vesicular luminal pH. This would have the effect of halting the continued destabilization of the membrane by mediating the de-

protonation of the remaining intravascular PAAs. Membrane perturbation (and a rise in intravesicular pH) may inhibit further rounds of membrane fusion, preventing an increase in PAA vesicular concentration (225) until the membrane could seal and the vesicle re-acidify. Seib *et al.* measured the binding, endocytic internalization, and exocytosis of PAMAM dendrimers, generation 2–4 (cationic) in B16F10 murine melanoma cell line along with linear and branched PEI (bPEI) of similar molecular weight (25,000 g/mol). The polymers were fluorescently labelled with OG and analysed via flow cytometry (229). Linear and bPEI displayed similar rates of internalization, however bPEI demonstrated a fivefold higher binding capacity as seen via cell-associated fluorescence with conjugated fluorophore. Seib *et al.* employed methyl-ß-cyclodextrin (MßCD) as a pinocytic inhibitor that has been reported to block the cholesterol-dependent, clathrin-mediated and phosphoinositide 3-kinase dependent micropinocytosis pathways (229, 230). MßCD resulted in 33% inhibition of bPEI accumulation suggesting uptake by cholesterol-dependent and clathrin-independent mechanisms. However, MßCD led to an approximate 173% increase in uptake of linear PEI. A fraction of bPEI binding sites were internalized and the majority of linear PEI binding sites were found to be endocytosed. Internalization rates for dendrimers were found to be generation dependent, (generation 4>3>2), the difference between generations 4 and 3 being suggested to relate to molecular weight, and high number of primary amines on the surface of the macromolecule. With increasing 'charge driven' uptake comes increasing toxicity, which may also be largely attributed to the density of positive charges associated with the polymer.

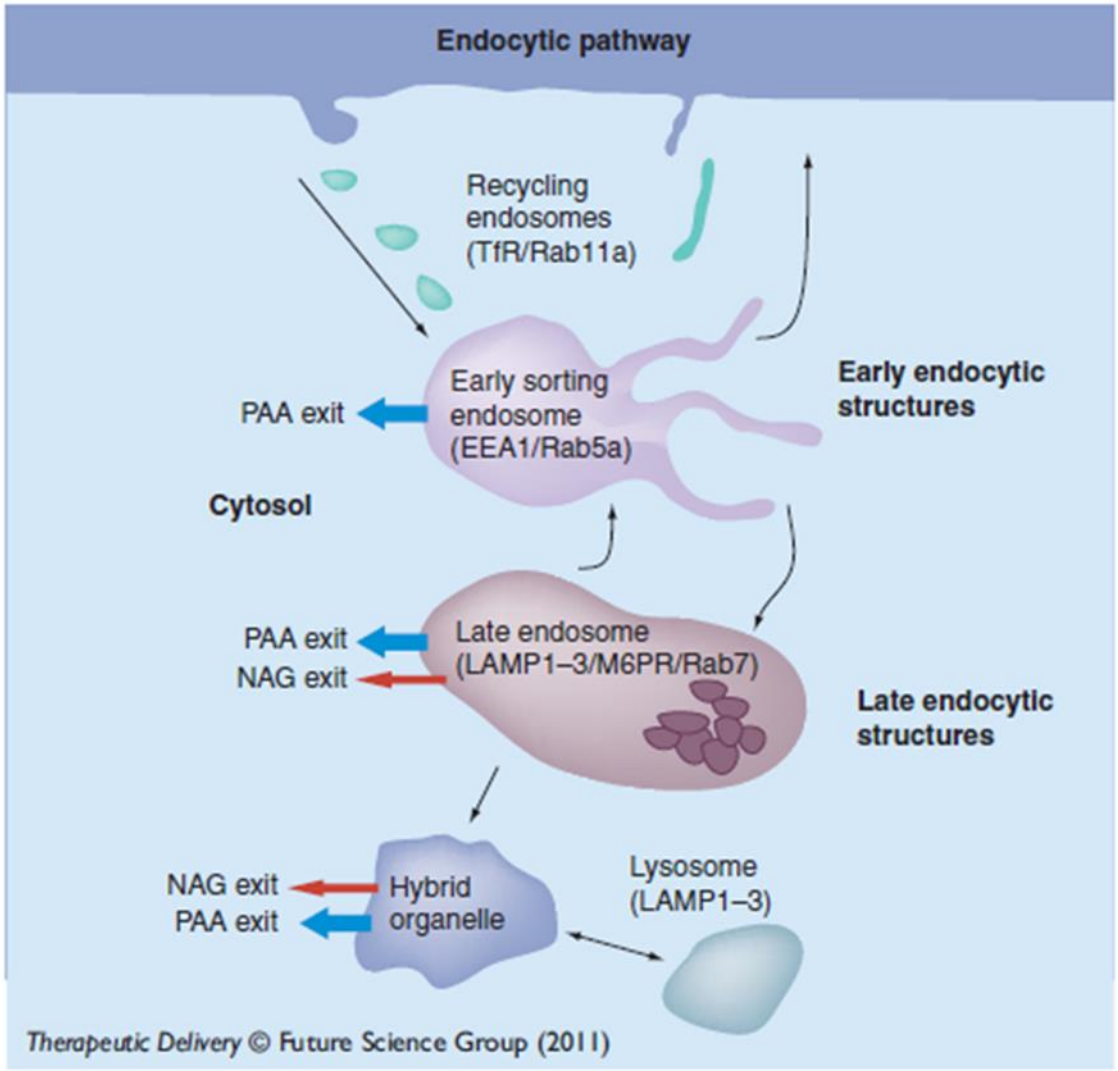


Figure 1.6 The mammalian endocytic and secretory pathways.

1.6.3 Toxicity

An important feature of PAAs is the net charge of the molecule. Acidity is governed by aminic moieties and, in the instance of amphiphilic PAAs (i.e., ISA23) may be balanced via the introduction of an ionized carboxylic acid group (216). Hill *et al.* characterized PAAs that incorporated three different aminic moieties, methylamine (MMA), dimethylethylene diamine (DMEDA), and the cyclic amine 2-methyl piperazine (2-MP) into six structures, with the specific aim of forming PAA–nucleic acid polyelectrolyte complexes for the delivery of DNA (205). Cytotoxicity was tested in two cell lines, namely HepG2 and HL60. NG30 (a methylene bisacrylamide

polymer, containing dimethylethylene diamine [MBADMEDA]), demonstrated cytotoxicity against HepG2 cells similar to that of PLL (22 kDa). Against HL60 cells, NG30 was less cytotoxic than PLL and NG37 was slightly cytotoxic to HepG2 cells (IC_{50} 400 ± 22.5 mg/ml). Polycation toxicity is also dependant on polymer molecular weight and the species of PLL tested in this paper was smaller than the PLL standards used to test ISA1 and ISA23 (Mw ~72kDa). These results support the hypothesis that the toxicity of polycations has a structural basis, as well as being due to charge. It is also evident that the salt form of PAA also contributes to PAA cytotoxicity (231). This study (205) substantiates some of the conclusions we came to when examining the relationship between polymer charge, architecture and biological properties (206) and further exemplifies the fact that PAAs have an advantage relative to other cationic polymers (such as PEI) due to reduced levels of cytotoxicity (209). Whilst the 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay (204, 205, 232) is a very good indicator of polymer toxicity *in vitro*, it is not the only way to monitor cellular toxicity. Measuring the levels of lactate dehydrogenase (LDH) released from cells has also been successfully deployed. LDH is a well characterized cytosolic marker and any rupture of the plasma membrane will cause its release into extracellular fluid (217). Whilst assessing polycation toxicity *in vitro*, Choksakulnimitr *et al.* (217), studied three cell types, brain microvessel endothelial cells (BMECs), macrophages (MØ) and hepatocytes. Choksakulnimitr *et al.* (217) documented neutral polymers such as bovine serum albumin as showing slight LDH release in all of the cell types tested, while the polycations demonstrated a variety of toxicity profiles. As a comparison, protamine and PLL caused gross LDH release, consistent with findings from other research groups. Hill *et al.* (205) found that PLL was cytotoxic against both HepG2 and HL60 cells with a high molecular weight PLL

being more cytotoxic than low molecular weight PLL. In correlation with Hill *et al.* (205) Choksakulnimitr *et al.* (217) reported high cytotoxicity with PLL and protamine in BMECs and MØ's. PLL (Mw 8000) showed less LDH release than PLL (Mw 39800 g/mol). These data support the conclusions published during the course of our studies (204, 232), which also concludes that polycations induce cytotoxicity due to concentration, exposure time and density of cationic residues interacting with the cell and the basic monomer structure (201, 217). However, PAAs were several orders of magnitude less toxic than all of the polycations tested (204-206, 232). It is possible that the differences in toxicity observed between the various cell lines i.e., HL60 and HepG2, were due to the intrinsic biology of these cell lines. This is a finding that is reflected in our own work with CCRF-CEM (human leukemic lymphoblasts) and Mewo (adherent human melanoma) cells (232). CCRF-CEM and HL60 both live in suspension whereas the HepG2 and Mewo cells grow as adherent monolayers. As cells living in suspension are likely to have a greater proportion of their negatively charged plasma membrane in contact with the positively charged polymers, it is not unreasonable to assume that the cells in suspension would sustain more damage to their plasma membrane. This damage might take the form of reduced membrane fluidity and cumulative membrane deformation, eventually leading to membrane rupture. It is possible that a similar mechanism is engaged during the PAA-mediated destabilization of endomembrane, negating the need for a 'proton-sponge' type effect (104, 233, 234). This idea is supported by the observation that PAAs can destabilize late endocytic structures in a cell-free system when applied to what was the cytosolic leaflet of the vesicle (227). Both of these observations would point to a non-proton sponge-driven mechanism responsible for PAA‑mediated membrane destabilization. When measured *in vitro* the IC_{50} of ISA1 is 3.1 ± 0.7 mg/ml and the IC_{50} of ISA23

was >5 mg/ml when measured after the continual exposure of the polymer to the cells over 72h (B16F10 murine melanoma cells) (204). This data describes ISA1 as having relatively low toxicity compared with polycationic vectors such as PEI or PLL (ISA1 is >100-fold less toxic than PEI [Mw 70,000 g/mol] and PLL [Mw 72,000 g/mol]. *In vivo* ISA4 (225) has been administered at doses up to 100 mg/kg iv. in the rat with no acute toxicity being observed. In an attempt to blend the body distribution of ISA22 with the uptake and protein delivery potential of ISA1, random and block copolymers of ISA1 and ISA23 were used to generate hybrid polymers. Resultant polymers had a pI slightly below 7.4. Furthermore, none of the polymers were hemolytic at pH 7.4 whilst demonstrating pH-dependent membrane lytic activity. No cytotoxicity was seen in B16F10 (murine melanoma) cells during a 72 h viability assay, (IC_{50} >2 mg/ml) and only ISA1 and block copolymer B2 (ISA23:ISA1 at 2:1 molar ratio) was able to promote intracellular delivery of gelonin (235). Thus, modifications to structural components facilitated enhanced intracellular delivery of a non-membrane permeant toxin whist further reducing polymer-mediated cellular toxicity (i.e., facilitating a reduction in toxicity relative to ISA1). However, such modifications may not be necessary if the capacity of a molecule like ISA23 to mediate transfection (i.e., DNA delivery) is considered in relation to toxicity.

1.6.4 DNA delivery

Both viral and non-viral strategies have been tested in an effort to effect gene delivery in the clinic and both have encountered significant problems. Viral systems, whilst mediating high transfection efficiencies *in vitro* have proven unstable and catastrophically immunogenic (236). Non-viral systems have largely focused upon the use of cationic material to destabilize cell membranes allowing the cytosolic and nuclear translocation of genetic material (237). When allowed to associate,

polycationic material and polyanionic DNA form compact interpolyelectrolyte complexes (IPEC) via the formation of electrostatic bonds between the negatively charged phosphate groups of DNA and positively charged groups of the polymer (238, 239). Unlike viral delivery systems, polymer gene-delivery systems provide unlimited DNA packing capacity (240). Polymer-based systems can also be synthesized to display a reduced immunogenic profile relative to viral vectors. This is reinforced by data showing that PAAs displayed no observed acute toxicity at doses up to 100 mg/kg after iv. administration in rats (225). As previously discussed PAAs as a group, exhibit low cytotoxicity compared with PEI and PLL and are capable of mediating transfection efficiencies as high as those obtained for commercially available transfection reagents (209, 227). The low toxicity of PAAs may facilitate their use at higher doses than the more toxic PEI or PLL polymers *in vivo*, and it is hoped that this may also be used to counter low transfection efficiencies relative to viral systems. It is anticipated that parameters such as transfection efficiency can also be optimized through careful choice of monomer and this indeed has been recently published (241). This data reinforces the idea that the biological properties of PAAs, such as site specificity (and hence body distribution) (204), hydrolytic degradation in biological fluid (220), and the minimization of toxic side effects (204, 206) can be controlled by modifying the physicochemical properties of the carrier molecule (206, 242). PAA structures (NG28 and 30) have demonstrated the ability to deliver functional plasmid to HepG2 and HL60 cell lines, where others, (PAAs NG29, and NG37–39) could not. NG30- mediated transfection plateaued at 4 μg/ml and showed no decrease in transfection levels despite a continuing decrease in cell viability (205). PAAs with protonated amines bind DNA with higher affinity than those lacking protonated amine groups. The modification of poly(b-amino ester) end groups from alcohol to primary

amine groups also increases polymer binding affinity (243). PAAs ISA1 and ISA23 and their two phenylethylamine- containing analogues ISA4 and ISA22 also have the ability to bind DNA. This was demonstrated by gel retardation inhibiting the electrophoretic mobility of λ Hind III DNA demonstrating IPEC formation. The complexes also demonstrated the ability to prevent degradation by DNase II. Additionally, all ISA family PAAs mediated the transfection of a plasmid encoding b-galactosidase in HepG2 cells. At a concentration of vector:DNA mass ratio of 5:1 ISA23 showed equivalent transfection ability compared with PEI and lipofectACE (227). Relative to other toxic polycations that have become well characterized in terms of their interaction with DNA, PAAs have much to offer. However, there is still much work to be done relative to understanding and manipulating the ability of PAAs to mediate transfection. What is not clear is why the less toxic (relative to ISA1), less positively charged ISA23, which when examined by fluorescent-activated cell sorting demonstrated a lower propensity to intracellular accumulation than ISA1(225), should mediate a higher level of transfection (227). This observation would appear counterintuitive, however, it has been speculated that this is due to the ability of ISA23 to buffer the intravesicular environment. This conjecture needs to be viewed in light of a very well constructed argument that concludes that the degree of hydrostatic pressure generated by the proton-sponge effect would not be great enough to rupture an endocytic vesicle (234). Given that the partitioning of transmembrane proteins in the early endosome often functions as a consequence of partitioning into reticular structures (captured in an extraordinarily elegant SEM by the Gruenberg group (244), it may be seen that there is an excess of membrane, relative to the volume of an early endocytic structure. This would suggest that the amount of swelling necessary to rupture such a vesicle would be very large. In light of the argument presented

previously, and the data showing that PAA on the cytosolic leaflet can mediate vesicular rupture, the proton-sponge hypothesis seems less attractive as an explanation of the mechanics responsible for PAA endomembrane destabilization. Whilst the morphology of lysosome-associated membrane glycoprotein (LAMP) positive late endosomes is significantly different to that of EEA1 positive early endosomes, there are further physiological phenomena that should be considered. These are often referred to as multivesicular endosomes due to the presence of non- LAMP immuno-reactive luminal vesicles. These structures degrade trans-membrane proteins and are a topological adaptation, allowing the lysosomal destruction of (previously) cytosolic domains (245). This may be thought of as a way to 'turn off ' activated receptors once they have been activated, which is an important way to regulate mitogen signaling. Whilst the limiting membranes of late endocytic structures do not display the disparity vis-a-vis surface area to volume ratio that EEA1 positive early endosomes demonstrate, it is important to acknowledge that these are dynamic structures that contain intraluminal membrane. Dynamic intraluminal membrane may 'buffer' luminal volume through back-fusion. This makes the possibility of limiting membrane rupture on account of late endosome hydrostatic pressure less plausible. Consequently, it is possible that there is more than one mechanism responsible for membrane destabilization. There is some compelling evidence that several molecules may use a proton sponge-like mechanism to achieve extravascularization (104) however, it is unlikely that this is the only mechanism used (234). What is apparent is that the proton sponge effect is not employed by PAAs (225). However, the apparently counterintuitive data presented (225, 227) would suggest that further investigation is warranted. This data also provides insight into the relationship between PAA toxicity and transfection efficiency as in the instance of ISA1 and ISA23, (and unlike other

polycations), there seems to describe an inverse relationship between toxicity and transfection efficiency (227). This is also interesting as there remains the possibility of increasing the polymer dose *in vivo*, boosting the apparent transfection efficiency whilst remaining below a toxicity threshold. These experiments need to be performed.

1.6.5 Protein delivery

Linear PAAs, ISA1 and ISA4 have the capacity to deliver non-membrane permanent toxins to B16F10 cells *in vitro* (228). These non-membrane permanent proteins belong to a superfamily of toxins defined as ribosome-inactivation proteins (RIPs). Ricin toxin, derived from the beans of *Ricinus communis* is a dimeric protein with high cytotoxicity in its native, wild-type form. This heterodimeric toxin contains two protein chains, ricin toxin A chain (RTAC) containing the cytotoxic component, and the lectinic ricin toxin B chain (RTBC) (246). Once endocytosed, RTAC is transported to the lumen of the endoplasmic reticulum (ER) by RTBC. RTAC is then subject to retrograde transport out of the ER into the cytosol. To facilitate this process RTAC and RTBC disassociate via the cleavage of a disulfide linker. RTAC escapes the ER via the ER-associated degradation pathway (ERAD) to the cytosol where it refolds into an active form and cleaves the N-glycosidic bond of the adenosine4324 nucleoside leading to inhibition of protein synthesis (247). RTA alone shows little inherent toxicity relative to the wild-type holotoxin, as cytosolic translocation is very inefficient (248). Conversely, gelonin toxin does not demonstrate lectinic properties and at low concentrations (μg/ml) is not toxic to intact cells (249). A consequence of the inability of these non-membranepermanent toxins to attain cytosolic access and effect cellular intoxication is that they provided a good model to assay PAA-mediated cytosolic protein delivery. The IC_{50} values for ISA1/RTAC and ISA1/gelonin combinations were 0.65 ± 0.05 and 0.55 ± 0.12mg/ml respectively. Similarly when

ISA4 was incubated with a non-toxic combination of RTAC and gelonin the IC_{50} value decreased to 0.57 ± 0.03 and 0.43 ± 0.26 mg/ ml respectively. Conversely, dextran, a neutral polymer and ISA22 and ISA23 were unable to mediate this effect. This was surprising as previously we discovered that ISA23 was as effective as commercial transfection reagents LipofectIN and PEI and more effective than LipofectACE (227). It is possible that the different efficiencies with which the PAAs (ISA1, ISA4 Vs ISA22, ISA23) (225) and toxins enter the cell are responsible for the results reported (228). Gelonin (GenBank accession L12243) carries a positive charge at pH 7.4, making an association between ISA1 or ISA4 unlikely. Coue *et al.* (250) developed a series of novel bioreducible PAAs containing repetitive disulfide linkages (SS–PAA). These water soluble, linear polymers were stable in the extracellular matrix but rapidly degrade within the intracellular environment. Polymers were synthesized by Michael addition of primary amine monomers, 4-aminobutan-1-ol and histamine to N-N'-cystaminebisacrylamide. b-galactosidease was used as a model protein due to negative charge (isoelectric point at pH 4.6). It was demonstrated that SS–PAA complexes containing b-galactosidase nanoparticles destabilized when the pH decreased from 7.4 to intracellular levels of pH 5, demonstrating an endosomolytic effect. However, this experiment was only performed on a negatively charged protein, and further work should be carried out on proteins with varying charge and structure. Yet it does provide further evidence that PAAs are capable of protein delivery to intracellular compartments.

1.7 Barriers for non-viral gene delivery vectors

1.7.1 Cellular uptake

Binding of nanoparticles to and uptake by cells depends on the interaction of their cationic surface charge with anionic cell-surface proteoglycans (251). The main mechanism for the internalization of non-viral vectors into the cells is endocytosis (252, 253). After interaction with the cell membrane nanoparticle internalize into the cells by one or more of various uptake pathways such as clathrin- and caveolae-mediated endocytosis, macropinocytosis, and pathways that are both clathrin- and caveolae-independent. These different pathways vary in the required particle size, the proteins mediating endocytic entry (254) and in the fate of the internalized particles (255).

Clathrin-mediated endocytosis is the major and best characterized endocytic pathway (256). It is a highly regulated and energy-dependent process. It is characterized by formation of vesicles ranging in size from approximately 100 to 150 nm (257). Moreover, polyplexes entering cells by this pathway will experience early endosomes (pH 6) and then will be trafficked into late endosome and lysosomes (pH 5) (258) where the cargo is enzymatically degraded (259).

Caveolae-mediated endocytosis, also called lipid raft-mediated endocytosis (260), involves formation of small vesicles in a cell membrane domain called caveolae that are rich in cholesterol and glycosphingolipids (261) and ranging in size from approximately 50 to 60 nm (262). Polyplexes entering cells by this pathway do not suffer a drop in pH and, instead of being degraded in the lysosomes, can be directly transported to the Golgi and/or endoplasmic reticulum (255). before they are translocated to the cytosol (263).

The type of cell and the polymer used determine internalization of polyplexes and in turn their transfection efficiency**.** Linear polyethyleneimine (LPEI) is mainly taken up via the clathrin pathway in African green monkey kidney (COS-7) and hepatocellular carcinoma (HUH-7) cells, whereas internalization of branch PEI (BPEI) polyplexes occurs by both the lipid-raft-dependent and clathrin-dependent pathways. It has been reported that both these pathways are implicated in HeLa cells, with the former appearing to be more pronounced (152).

1.7.2 Endosomal escape

After cell internalization via the endocytic pathway, nanoparticles will be localized within endosomes. Maturation of the first vesicle, early endosome, to the late endosome occurs by fusing with other sorting vesicles and is accompanied by sudden lowering of pH to 5 due to the action of the ATPase proton-pump. A late endosome is eventually trafficked to lysosome where degradation takes place due to an acidic environment (pH ~4.5) and activation of various degradative enzymes (264). Therefore, escape of nanoparticles from the endosome is considered a main challenge after cellular uptake to avoid lysosomal degradation and to achieve efficient transfection (265). Various strategies were employed to aid nucleic acid release from the endosome. Lipoplexes rely on a lipid mixing mechanism to facilitate nucleic acid release into the cytosol, which proceeds by fusion of lipids with the endosomal membrane leading to local destabilization of endosomal membrane and delivery of nucleic acids into the cytosol (266, 267). In contrast, cationic polymers such as PEI and polyamidoamine dendrimers (PAMAM) have high buffer capacity due to presence of large number of titratable secondary and tertiary amines, therefore, polyplexes can release their cargo into the cytosol by causing endosomal rupture through a hypothesis known as proton sponge effect (102, 104, 154, 268) (**Figure 1.2**). However, This

conjecture needs to be viewed in light of a very well-constructed argument that concludes that the degree of hydrostatic pressure generated by the proton-sponge effect would not be great enough to rupture an endocytic vesicle (234). Moreover, the amount of swelling necessary to rupture such a vesicle need to be very large (269). Consequently, proton sponge effect may not be the only mechanism of endosomal escape in the case of polymers containing protonable amine groups such as PEI and PANAM. Furthermore, Richardson *et al.* suggested that PAA endosomolytic activity was not simply attributable to proton sponge effect. It has been postulated that electrostatic interaction between positively charged aminic groups and the negatively charged endosomal membrane could occur after protonation leading to a physical interaction and subsequent fusion between the polymer and the endosomal membrane which in turn facilitates release of the payload into the cytoplasm (225, 270-273).

1.7.3 Dissociation of polyplexes

After endosomal escape, polyplexes should have the ability to dissociate to allow the release of RNA in the cytoplasm, while DNA require in addition to be translocated from the cytosol to the nucleus (274, 275). Efficiency of DNA release has been shown to be affected by two factors: chain length and polymer molecular weight. It has been found that higher gene expression can be achieved through a short period of time by shorter polycations due to their higher ability to dissociate from the DNA (276). Moreover, transfection efficiency of low molecular-weight PLL-based polyplexes is higher than that of high molecular weight counterparts due to their ability to unpack DNA more readily in the cytosol (274). Vector unpacking can be increased by using stimuli-responsive polymers such as thermo-responsive and redox potential-responsive polyplexes because they enable the efficient and quick release of DNA into the cytosol (277, 278). Temperature responsive polymeric materials have been

extensively used in the construction of new systems intended for gene delivery applications. These polymeric materials containing thermo-responsive units can reversibly exhibit phase transition at certain temperatures, which results in changes in conformation, solubility and hydrophilic–hydrophobic balance. For the polymers becoming soluble upon heating, the transition temperature is defined as upper critical solution temperature (UCST), while for the polymers, which turn insoluble upon heating, the transition temperature is defined as lower critical solution temperature (LCST). The polymers with LCST possess extended conformation below the phase transition temperature, whereas above LCST, they collapse and cannot interact with the surroundings (279). In this regard**,** Kurisawa *et al.* reported a thermoreversible terpolymer vector, poly-(N-isopropylacrylamide (NIPAAm)-co-2-(dimethylamino)-ethy methacrylate DMAEMA)-co-butyl methacrylate (BMA)). It was found that at a temperature above LCST of this copolymer, a tight copolymer/pCMV-lacZ plasmid DNA complex was formed, which was beneficial for the cellular uptake and protection of DNA from DNase attack. While lowering incubation temperature below LCST, the gene dissociated from its carrier, improving gene transfection even with small doses of DNA (280). On the other hand, the redox-responsive gene delivery system is one of the most important stimuli-responsive systems, owing to the differences in the redox environment between intracellular and extracellular conditions. There are several redox processes that occur in the normal physiology of the intracellular environment, such as the oxygen/superoxide (O_2/O_2^-) system, the nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH) system, the glutathione (GSH/GSSG) system and the thioredoxin TrxSS/Trx $(SH)_2$ system. Among these systems, the glutathione disulfide/reduced glutathione (GSSG/GSH) system is the most important, because of its high concentration in intracellular compartments. The

intracellular GSH in tumor cells is much higher (100–1000-fold) than the extracellular levels (281). This difference in the redox environment between the intra-cellular and extra-cellular site favors the development of redox-responsive polymeric systems. The GSH concentration in tumor sites is higher than that in normal tissues. The intracellular GSH concentration facilitates the degradation of many chemical linkages, so gene carriers have been developed to be stable in the extracellular conditions and to release the cargo only after their internalization (282). The most important and most widely-used redox-sensitive linker for a redox-sensitive system is the disulphide linker, which is stable under low reduction conditions and is reduced into thiol groups under high reduction conditions (283). For example, Breunig *et al.* showed that efficient siRNA release was achieved by cleavable disulfide cross-linked PEI (143). In the case of lipoplexes, endosomal escape occurs through interaction and fusion of the cationic lipids with the endosomal membrane which eventually result in the DNA release to the cytoplasm (266). The DNA has to be able to cross the cytoplasm in order to reach the nucleus of the target cell (46). However, the slow mobility of pDNA, in the cytoplasm makes it susceptible to cytoplasmic nucleases (47, 267). The half-life of pDNA in the cytoplasm is 50−90 min (284). The determining factor for the nucleic acid mobility rate through the cytoplasm is the size and spherical structure of the molecule, with circular pDNA being faster than linear structures.(285). The state of DNA compaction with reference to gene delivery is poorly understood, but tight compaction may result in increased mobility and stability against cytoplasmic nucleases (286). It has been shown that polyelectrolyte complexes formed using circular or linear plasmid DNA exhibit different topologies, with linear DNA forming less tightly organized structures than circular DNA (287).

1.7.4 Nuclear internalisation

The nuclear envelope represents one of the major barrier for gene delivery. Transfection of dividing cells is easier than nondividing one due to the destruction of their nuclear membrane during mitosis which will allow incorporation of exogenous DNA to the host genome (288, 289). On the other hand, nuclear entry in nondividing cells can occur only through the nuclear pore complex (NPC) located in the nuclear envelope. Particles up to about 9 nm (e.g., 40- 60-kDa proteins) can freely diffuse through these pores (289). In contrast, transport of larger molecules through nuclear pores is signal mediated, involves shuttle molecules, and requires energy. The basic peptide derived from the simian virus 40 large tumor antigen (PKKKRKV) is a nuclear localization signal (NLS) that mediates binding of the cargo to an adaptor protein importin-α (290), which is then bound by the karyopherin-ß (Kapß) family member, importin-ß. This trimeric complex then translocates through the NPC into the nucleus where it is dissociated by small GTPase, RanGTP (288). . The intracellular barriers discussed above are outlined in (Error! Reference source not found.) below.

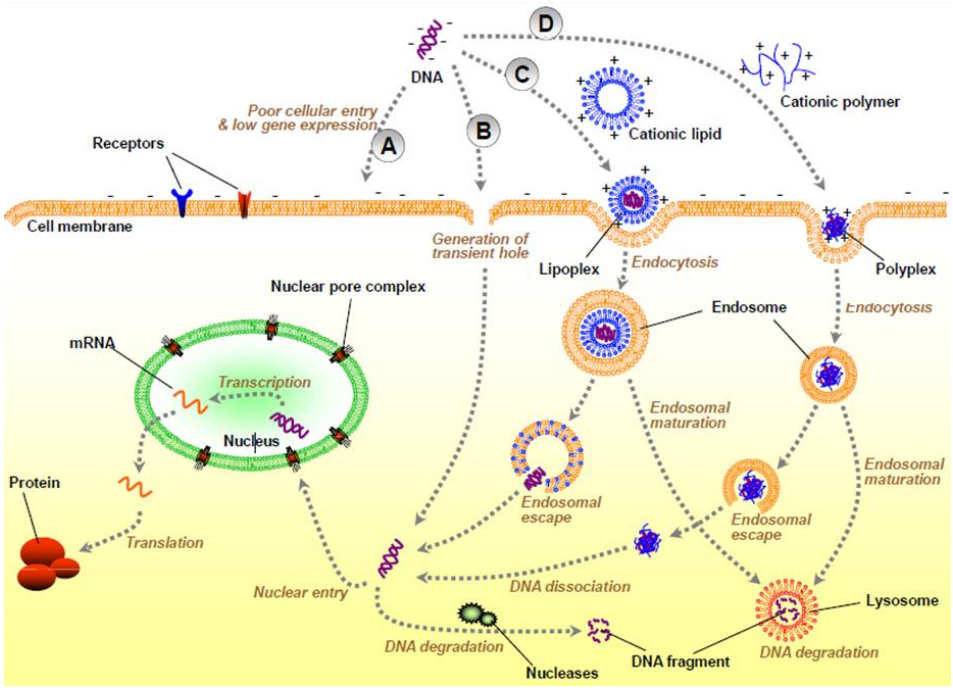


Figure 1.7 Intracellular barriers and process of non-viral gene delivery.
Naked DNA has poor ability to traverse cell membrane (A). However, it can be facilitated via physical method by generating transient holes on cell membrane (B), or via chemical method (cationic lipid (C) or cationic polymer (D)) by enhancing cellular uptake (94).

1.8 Clinical trials involving nonviral gene delivery systems

The number of clinical trials of gene therapy for treating genetic diseases has increased worldwide in recent years. However, none of the nonviral vectors have been approved by the Food and Drug Administration (FDA) (275). Allovectin-7, which consisted of a cationic lipid mixture and a plasmid DNA, has been used successfully in a phase II clinical trial against metastatic melanoma. However, this treatment had been abandoned because it failed to increase the overall survival in a phase-3 clinical trial for treatment of advanced metastatic melanoma (275, 291). Nonetheless, various lipid-based formulations continued to be developed in the clinic. Cationic liposomes consisting of N-(α-trimethylammonioacetyl)-disodecyl-D-glutamate chloride (TMAG), Dilaurolylphosphatidylcholine (DLPC), and DOPE have been used in patients with malignant glioma (292). On the other hand, polymer based siRNA

delivery systems consisting of cyclodextrin polymer and PEG steric stabilization agent have been used in a phase-1 clinical trials against cancers and showed potent antiproliferative activity across multiple types of cancer cells (293, 294). A gene delivery carrier for immunotherapy of epithelial ovarian cancer has been successfully developed by using polyethylene glycol-polyethylenimine-cholesterol (295).

1.9 Effects of polyethylene glycol (PEG) incorporation into vectors on transfection efficiency and cytotoxicity

As previously mentioned, the highly positively charged complexes account for their desirable electrostatic binding to the cell membrane, which leads to efficient internalisation and high level of gene expression *in vitro*. However, *in vivo* administration of highly positively charged systems was found to be the origin of many undesired non-specific interactions with blood components such as blood cells and serum proteins, which result in a rapid clearance of the complexes from the blood circulation by the reticuloendothelial system (RES) (296). This usually occurs by phagocytosis, which takes place after a process known as opsonisation. Opsonisation is the process by which certain blood components such as opsonin proteins adsorb onto the surface of the foreign particles or organism, thereby making it more visible to the phagocytic cells. The degree of opsonisation relies mainly on the surface characteristics of a particulate system, with serum protein adsorption occurring more with charged particles of a hydrophobic nature (297, 298). Therefore, one of the promising approaches to avoid clearance of the complexes from the blood circulation and to increase their systemic circulation time is by incorporating hydrophilic moieties, for examples polyethylene glycol (PEG) and poly[N-(2-hydroxypropyl) methaacrylamide] (pHPMA) into the complex structure in order to modify the surface characteristics and to shield the surface charges (299-301).

PEG is a non-ionic, hydrophilic polymer, well known for its biocompatibility, flexibility and non-immunogenicity. PEG has many features that can be used in gene delivery systems. The PEG layer does not permit interparticulate aggregation because of the formation of a sterically stabilised coating around the particles and thus improving the stability of the system, whilst simultaneously increasing the solubility (296, 299). PEGylated copolymers having polycationic groups can interact with nucleic acids in the same manner as in standard cationic polymers, causing the neutralization of the phosphate backbone of nucleic acids. PEG cationic copolymers then attain an orientation on the outside of the nucleic acid complexes where their chains extend towards the bulk solution resulting in sterically stable and small nanoparticles (302). This kind of property of copolymers is advantageous over homopolymers, because the PEG moieties of the copolymers form a steric barrier system against the undesired attachment of biomolecules. Unlike homopolymer systems, PEG-copolymers cause the displacement of the water molecules surrounding PEG moiety, and results in less or no aggregation of the polyplexes in biological compartments. The presence of PEG at the end of the cationic molecules prevents the adherence of biomolecules to the polyplexes and their elimination via the RES is also reduced. This shows that PEG attachments can protect the polyplexes from the intra- and extracellular environment with a strong shielding functionality (303). On the other hand, cytotoxicity of cationic polymers can be reduced by masking of their surface positive charge with a hydrophilic layer such as PEG (304). It has been found that surface modification of PAMAM dendrimers by PEGylation lead to a significant reduction in their *in vitro* and *in vivo* cytotoxicities and haemolysis (305). Despite reported advantages of a polycation-hydrophilic segment for DNA delivery, formation of large particles with decreased cellular membrane interaction and poor cellular

uptake could be caused by PEGylation due to reduction of the positive charge (306). For instance, it has been reported that the transfection efficacy of PEG-g-PEI was decreased when the proportion of PEG side chain increased (307). It is possible that the presence of PEG, which is known to sterically hinder interactions between particles, provides a steric barrier around these complexes thus preventing interaction with the cell membranes, and therefore the transfection efficacy is decreased (308).This problem was recognized as the PEG dilemma (35, 309) where high proportion of PEG result in less toxicity and lowered the transfection activity. Conversely, low proportion of PEG led to high toxicity and transfection efficiency (310). Beyond this there is also the issue of pharmacokinetics and pharmacodynamics, which is also influenced by change density, with positively charged material being sequestered to the liver very rapidly, or in the instance of charged particulates such as interpolyelectrolyte complexes, accumulating in the lungs or spleen (232, 233, 311). However, efficient gene transfer with PEGylation has been reported in some studies (312, 313). The efficiency of PEG–PEI conjugates has been found to be strongly affected by both the degree of PEGylation and the MW of PEG chains (312).Therefore, careful selection of an appropriate hydrophilic polymer balance is a key to development of effective gene delivery systems.

1.10 Effects of disulfide bonds on transfection efficiency and cytotoxicity

The complexation of PEGylated cationic copolymers with DNA affords complexes with a PEG corona that improves colloidal stability of the complexes owing to reduced interactions with blood components, resulting in prolonged circulation (299, 314). However, PEGylated complexes showed significantly decreased transfection efficiency compared to positively charged complexes formed from cationic homopolymers, because of their lower surface charge. A strategy to improve stability

and reduce cytotoxicity of the polymers while enhancing their transfection efficiencies is to incorporate reducible disulphide bonds into the polymer backbone to form bioreducible polycations facilitating redox-sensitive polymer breakdown (315, 316). These bioreducible complexes protect their contents from extracellular enzymes by forming stable complexes that can uptake by the cell via the endocytosis pathway (317). The usage of disulphide bonds as cleavable linkers in drug or gene delivery systems has been reported in several publications (318-320). A disulphide bond (-S-S-) is a covalent linkage, which arises as a result of the oxidation of two sulphydryl groups (SH) from two cysteine residues or other sulphydryl-containing materials (321, 322).

There are two distinct characteristics that render disulphide bonds attractive for the design of gene delivery systems, which are the disulphide bond stability in plasma and its reversibility within cells (323). It is well-known in fact that disulphide bonds are stable under extracellular physiological conditions but are rapidly cleaved in a reductive intracellular compartment due to the presence of a relatively high concentration of glutathione (GSH, 0.5–10 mM), the most abundant reducing agent in mammalian cells (324, 325), and thioredoxin reductase, leading to the destabilisation of complex and release of DNA (112, 326, 327) while the extracellular space is less reducing with only 2–20 μM of GSH (324, 325). The existence of these differences in reductive potential has been exploited for triggered intracellular delivery of a wide range of bioactive materials, such as DNA (328, 329), siRNA (330), antisense oligonucleotide (331), proteins and low molecular weight drugs (323).

Disulphide bonds can be incorporated into gene delivery systems following two different strategies. The first strategy is the introduction of the disulphide linkages in the polymeric main chain or in the side chain (inter-chain disulphide linkages or

disulphide-containing cationic polymers) (332-334). The second strategy is the formation of intra-chain disulphide linkages between two different components (intra-chain cross-linked) (335). Polymer based nucleic acid complexes having disulphide bonds (-S-S-) can be rapidly cleaved after cellular uptake to facilitate the release of the entrapped DNA or siRNA from the complexes. Thus, degradation of long polymers linked via disulphide bonds yields shorter polymer fragments resulting in low toxicity by avoiding accumulation of high molecular weight polycations in the cellular compartment. Alternatively, breakdown of disulphide linkages in cross-linked systems triggers the rapid intracellular release of DNA or siRNA leading to increased transfection efficiency (336).

A variety of disulphide (-S-S-) containing polycations have been developed for non-viral DNA and siRNA gene delivery, including disulphide based peptides (337), chitosans (338), low-molecular weight PEI (339), PMAM dendrimers (340), and disulphide containing PAA (341).

1.11 Aims and objectives of the thesis

The combined findings from the earlier work by Hill (205) and Jones (211) identified the potential of the poly(amidoamine)s based on a methylene-bis-acrylamide dimethylethylene diamine repeating unit (MBA-DMEDA) as delivery systems for nucleic acids as they showed good biological activity for transfecting DNA. However, their cellular adverse effects have 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. As a result, there is 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. Therefore, the current study aimed to investigate the mechanisms underlying the cytotoxicity and transfection efficacy of various PAAs including a homopolymer of a molecular weight >10 kDa (HP->10), a PEGylated Triblock Copolymer (CP) and a blend of CP and HP->10 to form a bio-reducible cross-linked delivery system and compare those with the cellular effects caused by commercially available BPEI (25 kDa).

The objectives of this study involve

- Formulation and characterization of pDNA polyplexes using BPEI and various PAAs polymers. Complexation and presence of free polymer was tested by agarose gel electrophoresis, while the size and zeta potential of the polyplexes was determined by DLS and zetasizer respectively. The buffer capacity of polymers was evaluated by acid-base titration using pH meter.
- Cytotoxicity caused by the polyplexes was evaluated by measuring cell metabolic activity, lactate dehydrogenase release, formation of reactive oxygen species and changes in mitochondrial membrane potential.
- The transfection efficiency of DNA polyplexes 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, while the effect of reactive oxygen species (ROS) formation on transfection efficiency was evaluated by using hydroquinone as antioxidant.

Chapter two

2 Materials and methods

2.1 Materials

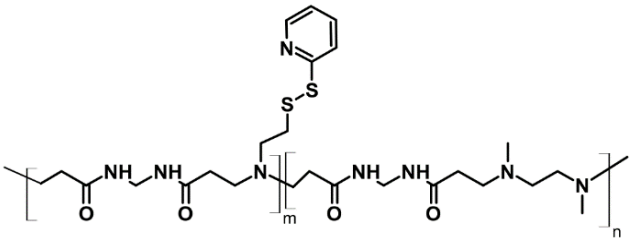
2.1.1 Cationic polymers

Branch polyethyleneimine (25 kDa) was purchased from Sigma-Aldrich (Poole, UK) and used as a reference control. The strucures of PAA polymers used in this study including homopolymer (HP) with molecular weight greater than 10 kDa (HP->10) and PEGylated tri-block copolymer was shown in Figure 2.1 and their properties in Table 2.1. These PAAs were provided by Prof Paolo Ferruti, Department of Organic and Industrial Chemistry, University of Milan, Italy. HP and CP that were used in this study were based on MBA-DMEDA (methylene bis acrylamide and dimethylethylenediamine co-monomers). CP has cystamine repeating units (RU) with BOC (t-butyloxycarbonylamino) protected pendent sulfhydryl group (2.4 kDa) distributed at either ends of PAA block (11kDa) and a PEG molecule (1.7 kDa) on each terminus.

Table 2.1 Properties of polyamidoamines

Polymer	Mw (Da)
HP	>10
CP	19.2

A)



B)

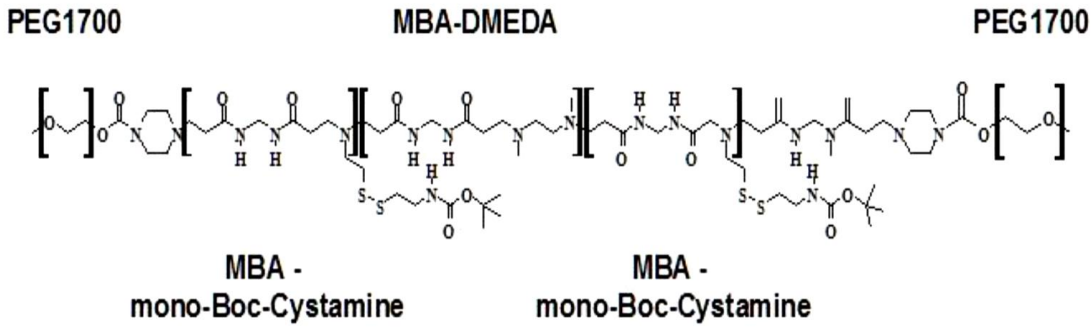


Figure 2.1 Chemical structures of PAA A) HP->10kDa and B) CP

2.1.2 Chemicals, solvents, buffers and reagents

Nuclease free water was obtained from Ambion. gWiz- LUC plasmid (pDNA; 6.7 kbp) encoding firefly luciferase reporter gene (**Figure 2.2**) was obtained from Aldevron (Fargo, USA). Lactate dehydrogenase (LDH) assay kit (CytotoxONE™), bright-Glo™ Luciferase Assay system and Glo Lysis Buffer (1X) were procured from Promega (UK). Tris-acetate-EDTA (TAE) buffer was obtained from Fisher Scientific (UK). 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFH-DA), PrestoBlue® (PB) cell viability reagent and OptiMEM® serum-reduced medium were purchased from Invitrogen. 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) was obtained from Biotium. Bafilomycin A1 was purchased from Alfa

Aesar. Accutase® solution, Dithiothreitol (DTT), N-Acetyl-L-cysteine (NAC), hydroquinone (HQ), Ellman's reagent (5-5'-Dithiobis, 2-nitrobenzoic acid or DNTB), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), ethidium bromide (EtBr, 1mg/ml solution), Coomassie Brilliant Blue R250, agarose powder and all other chemical reagents were purchased from Sigma-Aldrich (UK).

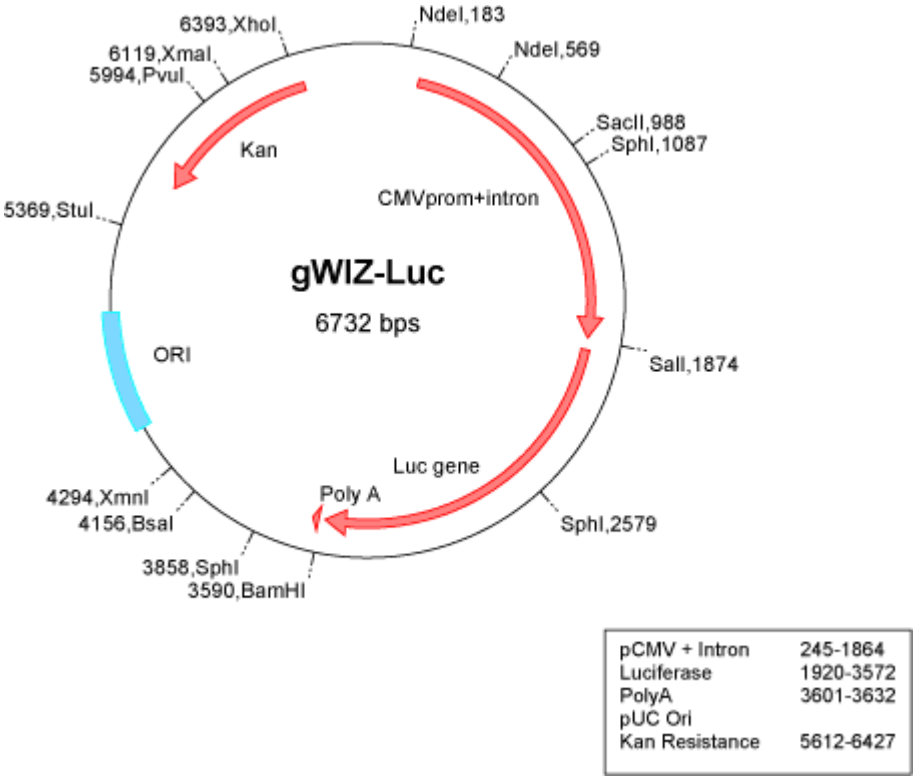


Figure 2.2 Map of the reporter plasmid gWiz-Luc.

2.2 Reduction of PEG-PAA-PEG copolymer (CP)

The PEGylated copolymer (CP) was supplied with the pendant thiol groups on the triblock copolymers protected by a BOC group. CP requires reduction to remove BOC group to allow formation of free thiol that are expected to form disulphide bridges by crosslinking with adjacent thiols or by reacting with HP that contains dithiopyridine

pendants. The reaction between a free thiol and a dithiopyridine results in the formation of a disulphide bond while releasing a thiopyridine group. To achieve reduction of the disulphide bonds (-S-S-) to the free thiol, the following method has been adopted. HPLC water was deoxygenated by passing nitrogen through it for 30 minutes. CP (150 mg) was dissolved in deoxygenated water (8ml) in a sealed vial covered with parafilm and stirred using a magnetic stirrer for 10 minutes until the polymer was completely dissolved. Nitrogen was also passed through a sodium hydroxide solution (1M) in a separate vial for 6 minutes and NaOH solution was poured drop wise into the polymer solution to adjust the pH to 7.4. The polymer solution was kept for 30 minutes on the magnetic stirrer, then a reducing agent like DTT (60mg) was added and stirring was kept for at least one hour. The above solution was then transferred to a viva cell 250 (Viva science, Sartorius group, U.K).

Nitrogen was set at 4 bar pressure and the cell was filled to a volume of 250ml. Deoxygenated water was passed through three times and the reduced polymer was frozen with liquid nitrogen and lyophilized for 48 hours. Thereafter, the yield of the reduced polymer was calculated on the basis of mass recovery:

$$\textbf{Yield (\%)} = \frac{\text{Final amount of polymer}}{\text{Theoretical yield of polymer}} \textbf{ X } 100$$

where,

Theoretical Yield of the Polymer

= Initial mass of polymer (g) – Mass of leaving group (g)

where,

Mass of leaving group (g)

= Moles of disulphide RU in the polymer (mol)

x MW of leaving group (g/mol)

where,

MW of leaving group,

- mono-Boc (t-butoxycarbonylamino) ethyl mercaptan, (C7NO2H14S-) = 176 g/mol

2.3 Determination of total sulfhydryl groups (–SH) using Ellman's reagent

Ellman's test is the standard protocol for the determination of free thiol groups. Ellman's reagent (DTNB) (10 mM) was dissolved in reaction buffer (0.1 M Phosphate Buffer with 1Mm EDTA, pH 8.0). To determine the amount of thiol group in the sample, Ellman's reagent (20 µl) was added to 100 µl of the reduced CP solution (1mg/ml) and then the volume completed to 1000 µl with reaction buffer. The mixture was incubated in the dark for 15 minutes at room temperature. Afterwards, the absorbance value of the mixture was measured using a TECAN Spark® 10M multimode microplate reader (Männedorf, Switzerland) at 412 nm. Blank solution was also measured as background. The percentage of thiol recovery was calculated using the following formula:

Percentage of thiol recovery =

$$\left(\frac{\textbf{Actual moles of thiol obtained from Ellman's Assay (mol)}}{\textbf{Theoretical moles of thiol repeating units from the polymer (mol)}} \right) \text{X } 100$$

The moles of disulphide RU in the polymer can be obtained by, Disulphide RU in the polymer

$$= \frac{\text{Amount of polymer (g)} \times \left(\frac{\text{Percentage of PAA}}{100}\right) \times \left(\frac{\text{Percentage of thiol RU}}{100}\right)}{\text{MW of PAA thiol RU after reduction (g/mol)}}$$

2.4 Preparation of DNA polyplexes

In all experimental settings composition of polyplexes was characterized by the molecular weight ratio of the repeating unit of polymer to nucleotide (RU:Nt) ratio in the mixture. DNA polyplexes were prepared by mixing equal volumes of polymer and pDNA solutions in 5mM phosphate buffer saline (PBS) and incubated for 30 minutes at room temperature to form stable complexes. Two component systems are complexes made by mixing one polymer, either PEGylated or Non-PEGylated PAA, with the pDNA. pDNA is an essential part of the polyplexes, hence counted as one component. Two component systems contain only one polymer in the polymer solution, whereas three component systems contain a mixture of two polymers. Here we explain the formulas involved in the calculation of required amount of polymer for mixing with pDNA.

2.4.1 Two component complexes

The complexes were prepared according to the polymer RU to pDNA nucleotide (RU/Nt) ratio. For two component systems, the required amount of polymer for a given RU/Nt ratio and known amount of pDNA was calculated as follows,

Amount of HP >10 required =

$$\frac{\text{HP >10 RU Molecular Weight}}{\text{DNA RU Molecular Weight}} \times \left(\frac{\text{RU}}{\text{Nt}} \text{ ratio}\right) \times \text{Amount of pDNA}$$

or

Amount of CP required =

$$\frac{\text{CP RU Molecular Weight}}{\text{DNA RU Molecular Weight}} \times \left(\frac{\text{RU}}{\text{Nt}}\ \text{ratio}\right) \times \frac{1}{\text{PAA ratio}} \times \text{Amount of pDNA}$$

2.4.2 Three component complexes

Three component complexes were prepared from a polymer blend solution, containing PEGylated (CP) and non-PEGylated (HP >10) polyamidoamine polymers mixed in a required HP: CP ratio, where the total polymer PAA repeating units were determined taking both the polymers in the mixture into account. HP: CP ratio was represented as Required Parts of HP PAA RU: Required Parts of CP PAA RU, [e.g: 1:1; 2:1; 3:1]

$$\textbf{HP ratio} = \left(\frac{\text{Required parts of HP}}{\text{Parts of HP} + \text{Parts of CP}}\right)$$

$$\textbf{CP ratio} = \left(\frac{\text{Required parts of CP}}{\text{Parts of HP} + \text{Parts of CP}}\right)$$

Therefore, amount of HP required for a given RU/Nt ratio can be calculated by,

Amount of HP =

$$\frac{\text{HP >10 RU Molecular Weight}}{\text{DNA RU Molecular Weight}} \times \left(\frac{\text{RU}}{\text{Nt}}\ \text{ratio}\right) \times (\text{HP ratio}) \times \text{Amount of pDNA}$$

Amount of CP =

$$\frac{\text{CP RU Molecular Weight}}{\text{DNA RU Molecular Weight}} \times \left(\frac{\text{RU}}{\text{Nt}}\ \text{ratio}\right) \times \left(\text{CP ratio}\right) \times \frac{1}{\text{PAA ratio}} \times \text{Amount of pDNA}$$

2.5 Physicochemical characterization

2.5.1 Gel retardation assay

Polyplexes were prepared by adding the required amounts of polymer (HP->10, CP, cross linked system and BPEI) to 1µg of DNA and 5 mM PBS (pH 7.4) was added to give a total volume of 20 µl. Different polymer to nucleotide (RU:Nt) ratios ranging from 1:1 to 10:1 and various HP:CP ratios (1:1, 2:1, 3:1) were prepared. Mixing was accomplished by vortexing briefly and samples were incubated at room temperature for 30 minutes. Then complexes were mixed with the loading buffer (2.5ul) and loaded onto a 1% agarose gels in 1xTAE buffer containing EtBr (1ug/ml). Free DNA and free polymers were also applied on the same gel. The gels were allowed to run at 80 V for 60 minutes in TAE buffer. Free, semi or completely bound nucleic acid was viewed using an UV-transilluminator gel imaging system (The Bioimaging Company UK) by using Syngene software. For visualization of the free polymers, the gels were stained with 0.1% Coomassie Blue staining solution (0.1% w/v Coomassie Blue staining in 50% methanol and 10% glacial acetic acid) for one hour and left in a destaining solution (10% v/v methanol and 10% v/v glacial acetic acid) for 24 hours. Images were taken using white light and the same imaging software.

2.5.2 Particle size measurements by Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) measures the mean intensity of scattered light from particles, within a detection aqueous media, that arrive at the detector (342). This

technique requires a single scattering regime sample, in which the sample needs to be diluted to obtain singly-scattered photons (342). Laser light is initially passed through a diluted single-scattering suspension. When the photons smash with particles that are moving in the suspension, they are scattered singly with a different angle depending on the particles' sizes. Thereafter, the overall scattered photons which are passed through a pinhole will be detected using a photonmultiplier tube (PMT) detector. Ultimately, they are analysed using an autocorrelator and computer, which provide a size and size distribution of the particles in the suspension. The DLS technique is non-invasive, easy to use, gives immediate results, and inexpensive. It is used to measure the particle sizes from a few nanometers to 1-2 micrometers (342).

pDNA polyplexes were prepared at a DNA concentration of 20μg/ml in 5mM of PBS solution (pH 7.4) based on different RU:Nt and HP:CP ratios. Aliquot of the polymer with the required ratio were added into the DNA solution to obtain the desired polymer (RU) to nucleotide (Nt) ratios. The solutions were mixed by gentle agitation to avoid bubbles and polyplex formation was allowed to occur for 30 minutes. 20 μL of the sample was transferred to a DLS quartz cuvette and then diluted with 30 μL of 5mM of PBS solution (pH 7.4). The size of the polyplexes was determined by Dynamic Light Scattering (DLS) using a Viskotek DLS 802 at 25ºC and a fixed scattering angle of 90º.

2.5.3 Zeta potential measurement

A zeta potential is a potential represented at the solid/liquid interface (**Figure 2.3**) (343).

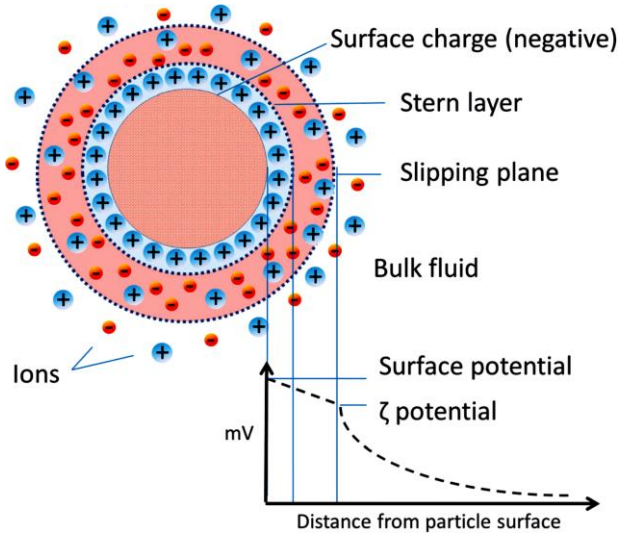


Figure 2.3 Schematic representation of zeta potential (343).

The net charge density of particles in contact with an aqueous media, mostly water, in the presence of an electrical field generates an electrical double layer (EDL) (344). This EDL occurs from, for example, positively charged ions staying preferentially close to negatively surface-charged particles as described in **Figure 2.3**. The EDL consists of a stern layer and a diffusion layer. The stern layer, where the counter ions are, carries ions which are absorbed to the charged surface of the particles. The diffusion layer, where an electroosmosis exhibited, is an outermost layer of the EDL, and the outer edge of this layer is called a slipping plane or a shear plane. The ions which are out of the slipping plane can move freely in the solution, while the ions which are bound or within this plane will move following the particles. Thus, the potential measured at the slipping plane is termed the zeta potential (344).

Zeta-potential was measured with a Zetasizer Nano Series (Malvern, UK). Folded capillary cells were filled with pDNA polyplexes solution containing 10 µg of pDNA in 1 ml. The measurement was made at 25 ºC, in 5 mM PBS at pH 7.4.

2.5.4 Buffering capacity of the polymers

The buffering capacity of PAAs (HP->10, CP) and BPEI polymers was evaluated by acid−base titration. Therefore, a known amount of each polymer that contain 0.06 mmol of protonable amine groups was dissolved in 5 mM PBS. The pH of the polymer solution was initially set at 12 by using 1 M NaOH and then acidified to pH 4 using 0.1 M HCl (20 µL). pH values of the solutions were measured by using a pH meter which is an instrument that measures acidity or alkalinity of a solution expressed as pH (345). The buffer capacity was calculated by using the following equation:

Buffer capacity (%) = $100(\Delta V_{HCl} \times 0.1M)/ Nmol$

wherein ΔV_{HCl} is the volume of HCl solution (0.1 M) required to bring the pH value of the polymer solution from 7.4 to 5.1, and Nmol (0.06 mmol), is the total moles of protonable amine groups in the polymer.

2.6 Biological characterization

2.6.1 Cell culture

The lung adenocarcinoma A549 cell line was obtained from Sigma-Aldrich (UK). This cell line has been selected because it can be transfected easily compared to bronchial cell lines such as Calu-3 cells. A549 cells (passage 5-20 from initial passage number) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with foetal bovine serum (FBS, 10 %) and the antibiotics (penicillin (100 IU/ ml) and 100µg/ml streptomycin). All these materials were obtained from Sigma-Aldrich (UK) and were filtered before addition into the medium to keep it in sterile conditions.

Cells were grown in 75 cm^2 flasks (Corning, UK) and maintained in an incubator at 37°C with 5 % CO_2 and a humidified atmosphere. The culture medium was changed

every two to three days through addition of 10 ml of fresh DMEM to avoid depletion of essential nutrients. Cells were passaged when the confluence reached approximately 85 % -90%. First of all, the medium was eliminated and the cells were washed with 5 ml phosphate buffer saline (PBS). Trypsin/ Ethylenediamine tetraacetic acid (EDTA) (1X) was added to the flask which was then incubated for 5 minutes at 37 °C, 5% CO_2. Once the cells had detached from the flask, they were centrifuged and then resuspended in fresh medium in a new 75 cm2 flask (Corning, UK) with a split ratio of 1:5.

2.6.2 Effect of free polymers on cell metabolic activity

The MTT reagent was used to determine the metabolic activity of A549 cells after exposure to the individual polymers. This reagent relies on the metabolic activity of mitochondrial enzymes which reduce it into insoluble purple formazan. Active mitochondrial dehydrogenases of living cells will cause this conversion, whereas dead cells do not cause this change. Formazan is insoluble in water, but can be solubilized using DMSO and the absorbance of the dissolved material is indicative of the number of living cells in culture. The appropriate cell density to be used in the MTT assay was first determined. A549 cells, in DMEM containing FBS (10%), were seeded at a density ranging from 1500 to 50000 cells per well in 96-well plates (Corning, UK). They were then incubated for 48 hrs to allow for their attachment on the plate. Cells were washed with PBS and 50 µL of MTT dissolved in serum free media (0.5 mg/ml) was added to each well. MTT/medium was added to wells without cells as a control. After 2 h of incubation, the cells were washed again with PBS, wells were emptied and 100 µL dimethylsulfoxide (DMSO) was added per well. The absorbance was read at 570 nm using a TECAN Spark® 10M multimode microplate reader (Männedrof, Switzerland).

For the cytotoxicity tests, cells were seeded (10,000/ well) and incubated for 24 hr for attachment. The polymer solution was prepared by dissolving the polymer in 5 mM PBS and then mixed with the transfection medium, OptiMEM. The cells were treated with different concentrations (0.1, 1, 10, 100 and 1000 μg/ml) of BPEI, HP and CP for 4 hrs and then replaced by DMEM complete medium. Untreated cells (100% MTT metabolism) and cells treated with 4% Triton X-100 (0% MTT metabolism) were included as negative and positive controls, respectively. After 24 hrs, the cells were washed with PBS and an MTT assay was carried out as described above. Dose response curves were plotted for each polymer, demonstrating metabolic activity relative to the control versus polymer concentrations. The cytotoxicity of the polymers was compared through calculation of the concentration causing a 50% drop in the metabolic activity of the cell population (IC_{50}).

2.6.3 Determination of the appropriate cell density for the Presto Blue assay

The PrestoBlue™ (PB) assay (Invitrogen, Life Technologies LTD) is one of the frequently applied assays for examining cell viability and proliferation *in vitro*. PB reagent consists of a blue-coloured non-fluorescent compound, known as resazurin. This compound is present in an oxidized form in the reagent. Viable cells have the capacity to reduce and convert resazurin into a pink fluorescent compound (resorufin) by the activity of metabolic enzymes in their cytosol (1-3). The intensity of the fluorescent resorufin can be measured, and this reflects the quantity of viable cells.

To generate a standard curve for the PB assay, the A549 cells were cultured at various densities ($2 \times 10^4 - 10 \times 10^4$) into a 24-well plate (Nunc, UK) in triplicate. They were grown in Dulbecco's modified Eagle's medium as described above. The cells were placed at 37 °C for 20-24 hours to allow them to attach. PB was prepared at a 1:9 ratio

with Hanks' Balanced Salt solution (HBSS), and kept unexposed to light due to the reagent being light sensitive.

Old media was aspirated, and 0.25 ml of the reagent was added into each well and the plate was placed into the incubator at 37 °C for 30 minutes. 100 µl of supernatant was transferred into a black 96-well plate (Nunc, UK), protected from light. Finally, the intensity of the reduced form of the reagent was read on a TECAN Spark® 10M multimode microplate reader (Männedorf, Switzerland) at an excitation and emission wavelength of 560 nm and590 nm, respectively. The data was collected and the fluorescence was plotted against cell number.

2.6.4 Effect of pDNA polyplexes on cell metabolic activity

A549 cells were seeded in 24-well plates (Nunc, UK) at a density of 5×10^4 cells/ well and incubated for 24 hr in 0.5 mL of supplemented DMEM, after which they exhibited approximately 75% confluency. The cell culture medium was aspirated and cells were rinsed with PBS before adding OptiMEM (0.5 ml) containing the polyplexes at the selected RU:Nt ratios. Four hours after transfection, the polyplex suspension was removed and replaced with fresh serum-supplemented DMEM. After a 24-hour incubation, the cytotoxicity was assessed by performing a Presto Blue assay. The medium was removed, and cells were washed with PBS, then 250 µl of PB reagent in HBSS (1:9) was added to each well. After 30 minutes incubation, 100 µl of supernatant was transferred into a black 96- well plate (Nunc, UK) and the fluorescence was measured by a TECAN Spark® 10M multimode microplate reader (Männedorf, Switzerland) at an excitation and emission wavelength of 560 nm and 590 nm, respectively. Untreated cells were considered as control with 100% metabolic activity.

The metabolic activity was calculated as a percentage relative to untreated control cells.

2.6.5 Effect of free polymers and pDNA polyplexes on cell membrane integrity

CytotoxONE™ -reagent was utilized to assess the membrane integrity after cell exposure to the polymers or the polyplexes through measuring the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. The reagent reacts with the released LDH which results in the conversion of resazurin into resorufin. Resorufin is a fluorescent material and its production is proportional to the amount of LDH released. A549 cells were seeded in black 96-well plates at 15×10^3 cells/well. Polyplexes were prepared by mixing the tested polymers with DNA (10 μg/ ml) at the selected RU:Nt ratios (2, 5, 10:1) and then diluted with OptiMEM medium. After 24 h, the cells were treated with the same concentrations of free polymers used for the MTT assay and with the selected ratios of various polyplexes. 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 hrs incubation, an equal volume of CytotoxONE™ -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 to each well. The plate was then shaken for 10 seconds and the fluorescence was measured by a TECAN Spark® 10M multimode microplate reader (Männedorf, Switzerland) at an excitation wavelength of 560 nm and emission wavelength of 590 nm. Dose response curves demonstrating LDH release (%) relative to the controls versus polymer concentration were plotted for each free polymer.

2.6.6 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 the selected polyplex formulations prepared as described above. Four hours after transfection, the medium in the wells was replaced with fresh culture medium, and the cells were incubated for an additional 20 h under standard incubation condition. Negative control consisted of untreated stained cells. For positive control, cells were incubated with FCCP (positive control) for 10 minutes at a final concentration of 10 μM. After 24 h, the media was removed and 2 ml of a 5 mM solution of the JC-1 dye was added to each well and the plate was incubated for 30 min at 37 °C in the dark. After that, cells were harvested by accutase (Cell Detachment Solution) and transferred to a test tube for measurement. The cell samples were analysed by flow cytometry using a Beckman Coulter FC500. Red fluorescence (ex: 488 nm, em: 590 nm) was detected in channel FL2 and green fluorescence (ex: 488 nm, em: 527 nm) was observed in channel FL1 with almost 10,000 events collected for each sample. Data were analyzed with the Weasel Flow Cytometry Software. Data were expressed as percentage of Red/Green fluorescence ratio. The assay was performed on two independent occasions (n=2 wells per experiment).

2.6.7 Determination of intracellular formation of 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 a cell-permeable fluorescent probe, H2DCF-DA (10 μM) in PBS for 45 min under standard incubation condition. The dye was removed, and the cells were washed with PBS and then incubated with various polyplex formulations prepared as described above for 2 or 4 h and with hydrogen peroxide (H_2O_2) (400 μM) in OptiMEM as a positive control for 2 h. After 2 or 4 h

incubation, the fluorescence intensity was measured at 485 nm excitation and 535 nm emission with a TECAN Spark® 10M multimode microplate reader (Männedrof, Switzerland). 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).

Cells were seeded at a density of 400 × 10^3 cells/well in 6-well plates and were incubated for 24 h. Cells were then treated with various polyplexes for 2 or 4 h and with H_2O_2 (100 μM) in PBS as a positive control for 30 min. After 2 or 4 h incubation, cells were washed with PBS and then exposed to the fluorescent probe H2DCF-DA (10 μM) in PBS for 45 min under cell culture standard incubation conditionsacuutase. The dye was aspirated off and the cells were washed with PBS and then harvested by accutase and transferred to a test tube for measurement. The cell samples were analysed by flow cytometry using a Beckman Coulter FC500. Green fluorescence (ex: 488 nm, em: 527 nm) was measured in channel FL1 with almost 10,000 events collected for each sample. Data were analyzed with the Weasel Flow Cytometry Software. 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=2 wells per experiment).

2.6.8 *In vitro* transfection efficiency of pDNA polyplexes

The transfection efficacy was assessed after performing a Presto Blue assay as described above. The remaining of the PB reagent was removed from all wells and cells were washed with PBS again. Then, 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 measured by a TECAN Spark® 10M multimode microplate reader (Männedorf, Switzerland) with an integration time of 1 second. Untreated cells were used to determine the background luminescence.

2.6.9 Effect of bafilomycin A1 on transfection efficiency and cytotoxicity

Cells were seeded at 5×10^4 cells/well in 24-well plates and were incubated for 24 h, yielding a cell density of about70–80% confluency. The cells were then washed with PBS and treated with bafilomycin A1 at a final concentration of 500 nm in OptiMEM media for 30 min at 37 °C. After that, the cells were transfected with the polyplexes and the metabolic activity and transfection efficiency were measured by following the same methods as described above.

2.6.10 Effect of antioxidant on generation of reactive oxygen species

Cells were seeded at a density of 15×10^3 cells/well in black 96-well plates and were incubated for 24 h before. Cells were then incubated with 10 µM H2DCF-DA for 45 min. To study the effect of antioxidant on ROS generated by the polyplexes, cells were pre-treated with the antioxidant hydroquinone (HQ) diluted with OptiMEM (0.5 µM) for 30 minutes, followed by a co-treatment with polyplexes at the selected ratios for 4 h. Hydrogen peroxide (H_2O_2) was used at a concentration of 800 µM as a positive

control for 2 h and untreated cells as a negative control. The fluorescence intensity was measured at a 485 nm excitation wavelength and 535 nm emission wavelength with a TECAN Spark® 10M multimode microplate reader (Männedorf, Switzerland). The resulting oxidative stress level was expressed as a percentage increase in ROS production in relation to that of untreated cells.

2.6.11 Effect of anti-oxidant on transfection efficiency and cytotoxicity

A549 cells were seeded in 24-well plates at a density of 5×10^4 cells/well and incubated for 24 hr in fresh complete medium. In an attempt to determine whether the ROS was involved in PAA polyplexes mediated gene delivery and cytotoxicity, cells were pre-treated with the antioxidant HQ (0.5 µM) before being exposed to the polyplexes as described above. The metabolic activity and transfection efficiency were measured by following the same method described earlier.

2.7 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 $p < 0.05$. Each set of experiment was conducted on 2-4 samples per group, and the values represent mean ± standard deviation (SD) for repeated experiments. The results were analysed by either student's–t test for comparing two groups or one-way analysis of variance (ANOVA) test, followed by a Tukey or Dunnett post-hoc test for comparing more than two groups. Differences between groups were considered to be significant at $p<0.05$. All data were analysed statistically using Graphpad prism software version 67.0 (San Diego, California, USA). The figures were also obtained using the same software.

Chapter three

3 Formulation and characterization of PAA -DNA polyplexes

3.1 Introduction

During the development of non-viral gene delivery systems, it is essential to carry out physicochemical characterisations to ensure they are suitable for biological investigations. The first criterion to take into account is the ability of the potential non-viral gene delivery system to bind and form complexes with DNA effectively. Furthermore, the physicochemical properties of the resulting DNA complexes, such as surface charge and particle size can have a major influence on the efficiency of the system to deliver gene into the cells. Cationic polymers are essential for binding DNA and condensing it via electrostatic interactions into small-sized particles or compact polyplexes. Moreover, the surface charge of a gene delivery system is a significant parameter affecting its ability to interact with cell surfaces. Since cell membranes are generally negatively charged, the overall positive charge of a delivery system can facilitate its association with cell membranes and thus potentially enhance cellular uptake and transfection efficiency (120, 151, 346). Interaction with cells andcellular uptake can also be enhanced by using of targeting ligand on the nanoparticle surface such as folic acid and transferrin which specifically bind to a particular receptor (347).

Physical stability of polyplexes can be achieved via either electrostatic or steric stabilization to avoid aggregation of nanoparticles during *in vitro* applications (348). In addition, charges on particles contribute to their colloidal stability. i.e., preventing aggregation of DNA complexes. However, an excessive net-positive surface charge may inhibit DNA release at the appropriate stage inside the cell and ultimately result in low levels of gene expression (15). Moreover, positive charge polyplexes can be

aggregated under physiological salt concentrations due to the screening effect of salt which can increase hydrophobicity of polyplexes by reducing the solvatation effect of their surface charges (348).

To overcome instability issues without encountering problems associated with high surface charges, a sterically stabilized polyplex formulation has been developed through a crosslinking system (Error! Reference source not found.) (335). Formation of cross-linked pDNA-PAA polyplexes occurred by mixing a pDNA with a polymer blend solution containing PEGylated copolymer (CP) and non-PEGylated homopolymer (HP) PAA polymers. The concept of crosslinking system involves self-assembling of negatively charged pDNA with the positively charged polymers (HP and CP) in addition to the formation of disulfide linkage between reduced thiols from the copolymer with other active thiols from copolymer or with thio-pyridine molecules from the HP which are in close proximity (335). Electrostatic attraction acts as glue between the polymer and pDNA, bringing several polymer strands and pDNA together. As the core of the polyplex becomes relatively hydrophobic and compact, the PEG tends to preferentially assemble at the surface of the nanostructure in the aqueous medium where it can be mobile. The PEG chains would limit the size of the complexes due to stearic hindrance and also prevent aggregation due to charge neutralization (223). Active thiols from the CP would form di-sulphide bridges with other active thiols from CP or thio-pyridine molecules from the HP which are in close proximity. This new crosslinking might result in compaction of the nano-structure, which intern might promote further crosslinking, compaction and stabilization.

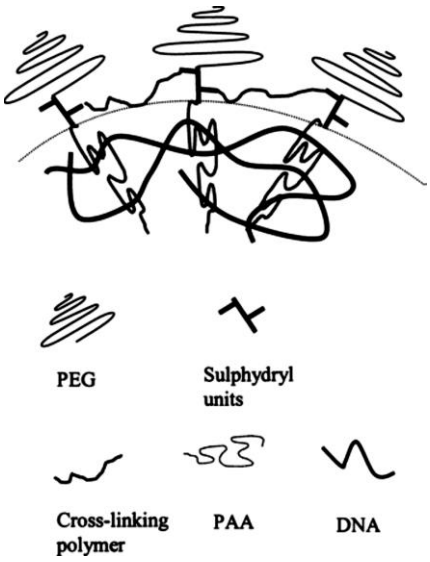


Figure 3.1 Cartoon depicting the cross-linking between the PAA homopolymer (HP) and PEGylated copolymer (CP) designed to occur at the surface of the complexes through placement of the thiol (sulphydryl) blocks near the terminal PEG segments (335).

The particle size of DNA complexes plays an important role in cellular uptake. It is widely accepted that a small particle size is beneficial for efficient delivery of DNA into cells. It has also been found that endocytic vesicles have specific sizes and therefore large size complexes (>200 nm) may not be taken up by some pathways such as clathrin (349), so both particle size and surface charge play key roles in cellular uptake via endocytosis (15, 350). Not only is endocytosis more efficient with particles size smaller than 200 nm, but the rate of movement in the cytoplasm was also found to be particle size dependent (351). The immobility of the large particles, however, is likely due to macromolecular crowding in the cytoplasm (352) as a result of presence of macromolecules such as proteins and ribosomes which typically occupy 20–30% of the total volume of the cytoplasm (353). Macromolecular crowding provides the cytoplasm with strongly viscoelastic properties which renders diffusion in this fluid strongly anomalous (354).These polymers should also have a sufficient buffer capacity to facilitate endosomal escape of the polyplexes and thus, avoid degradation

by lysosomal enzymes or recycling out of the membrane (355). The buffer capacity of titratable amine groups can effectively facilitate endosomal rupture, inducing efficient gene expression. An example of the importance of buffer capacity is that polyethylenimine (PEI) has an advantage over PLL in transfection due to its high buffering capacity (355).

The aim of this chapter was to investigate the physicochemical properties of pDNA polyplexes. It includes initial preparation of both two-component (HP-pDNA, CP-pDNA and BPEI-pDNA) polyplexes and cross linked pDNA polyplexes (HP-CP-pDNA) made from three-components at different polymer repeating unit to pDNA nucleotide ratios (RU/Nt) and HP RU to CP RU ratios (HP: CP). All these different polyplexes were characterized by gel-retardation assay, size measurements (DLS), zeta potential and buffer capacity of the individual polymers. The suitable formulations that will be used in the *in vitro* testing were then selected depending on the following criteria (a) efficient pDNA incorporation (b) amount of free polymer in the pDNA polyplexes; and (c) size of the polyplexes.

3.2 Results

3.2.1 Yield of reduced polymer

The Boc-protected thiol group could be easily removed by using DTT. The recovery of PEGylated CP after reduction and purification was presented by percentage mass recovery and percentage free thiol. Mass recovery was 70 % of the expected yield, while the thiol yield was 34 %.

3.2.2 Gel retardation assay

Gel retardation assay is a simple technique used to understand the binding ability and complexation behaviour of polycations with nucleic acids at increasing ratios of polymer to nucleotide. Polymer appears on the cathodic side (Upper portion) of gel stained by Coomassie Blue staining, while migration of pDNA can be seen (lower portion of gel) by ethidium bromide (EtBr) staining. During electrophoresis, unbound or excess polycations move towards the cathode and free nucleic acids migrate towards the anode.

Individual PAAs (HP->10, CP) showed similar binding profile. They completely retarded pDNA migration at a RU:Nt ratio of 2:1 as indicated by the disappearance of EtBr stained migrating nucleic acids from the gel (**Error! Reference source not found.**). Nevertheless, tight binding was not achieved as fluorescence could be observed in the well as a result of intercalation between pDNA and EtBr. In addition, free polymer started to appear at a 3:1 RU:Nt ratio and continued to increase as the polymer/ pDNA ratios increased. Complete neutralization of PAA polyplexes occurred only at RU:Nt ratio of 2:1 where there was no electrophoretic mobility and polyplexes remained within the loading wells. In contrast, pDNA mobility was completely inhibited by BPEI at a RU:Nt ratio of 3:1 and no discrete polymer or

fluorescence staining was seen associated with the retarded polyplexes, indicating the ability of the polymer to totally condense pDNA (Error! Reference source not found.).

The binding properties of pDNA polyplexes prepared by using a blend of HP and already reduced PEGylated CP as a cross-linked system was investigated at different polymer to pDNA (RU:Nt) ratios (2:1, 5:1, 10:1) and various HP:CP ratios (1:1, 2:1, 3:1). All formulations showed complete pDNA binding at all RU:Nt and HP:CP ratios, where complete DNA complexation occurred (Error! Reference source not found.). In addition, a gradual decrease in brightness of fluorescent bands for pDNA complexes can be clearly observed with increasing RU:Nt ratios and the pDNA polyplexes started to become positively charged at a 5:1 RU:Nt ratio and showed a tendency to slightly move away from the loading wells towards the cathode at a RU:Nt ratio of 10:1 regardless of the HP:CP ratios. However, free polymers started to appear in the gel at all HP:CP ratios for both 5:1 and 10:1 RU:Nt ratios.

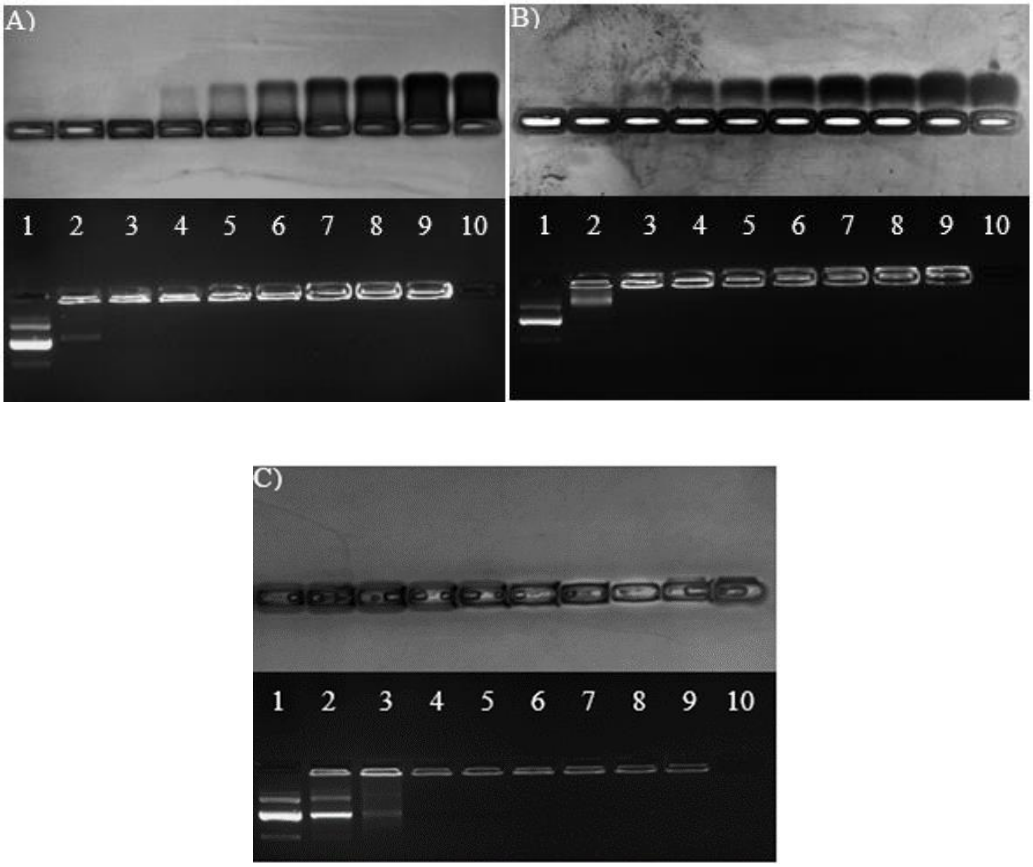


Figure 3.2 Gel retardation assay of pDNA polyplexes prepared by using (A) HP->10 (B) CPand (C) BPEI.

For each image lane 1 contains naked DNA only as a control, lane 2-9 contain polyplexes with different 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. Upper images show the migration of the polymers towards the cathode, while lower images show the migration of DNA towards the anode.

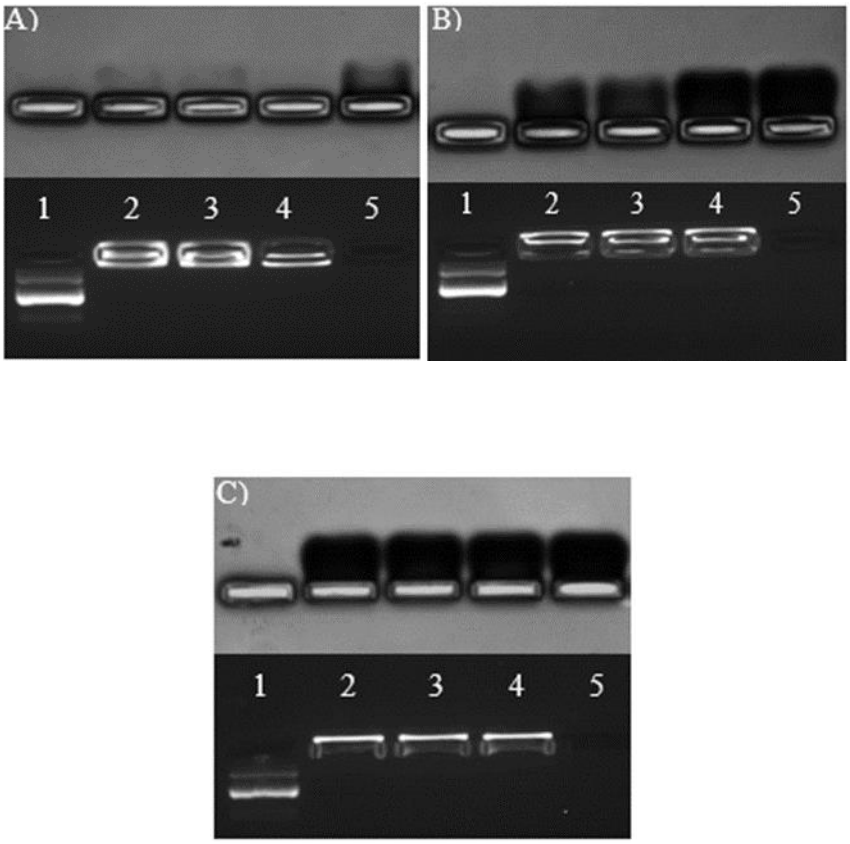


Figure 3.3 Gel retardation assay of cross-linked pDNA polyplexesprepared by using a polymer blend of HP->10 and CP.
In each gel image lane 1 contains naked pDNA only and lane 5 contains free polymer mixture only as a control, while lanes 2-4 contain polyplexes prepared with homopolymer to copolymer (HP:CP) ratio of 1:1, 2:1 and 3:1 respectively at RU:Nt ratios of (A) 2:1, (B) 5:1 and (C) 10:1. Upper images show the migration of the polymer towards the cathode, while lower images show the migration of DNA towards the anode.

3.2.3 Size of the DNA polyplexes

To investigate whether PAA polyplexes have the ability to condense DNA efficiently, their sizes were measured as a function of RU:Nt ratios using DLS analysis. Based on the results obtained from gel retardation assay, only DNA polyplexes that achieved complete binding were selected for size measurement. All polymers formed polyplexes with a diameter around or below 200 nm (Supporting information of DLS analyses is provided; see appendices for more details). There was a general trend towards a decrease in particle size with increasing RU:Nt ratios although this was only found to be statistically significant for the crosslinked system (Figure 3.4 Hydrodynamic diameter (nm) measurements for HP->10, CP, BPEIand cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).).

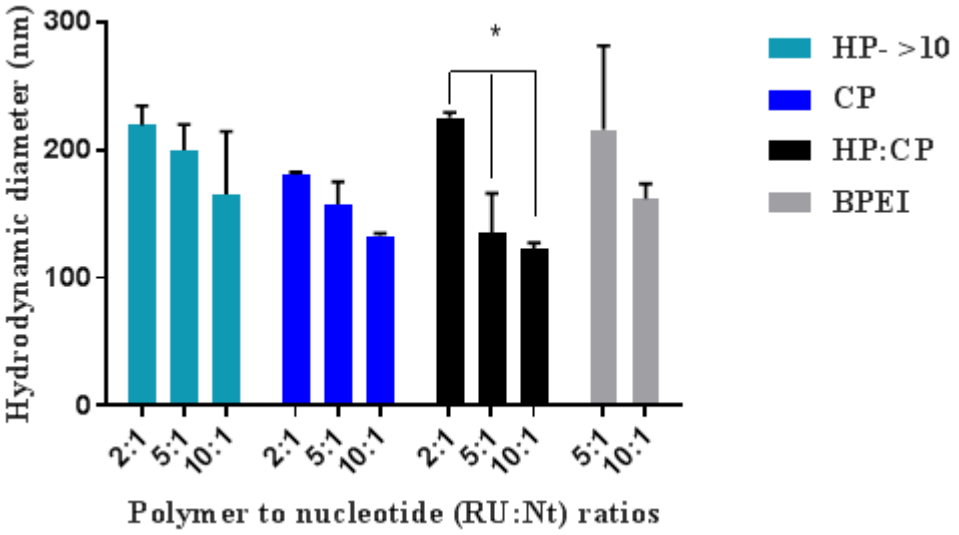


Figure 3.4 Hydrodynamic diameter (nm) measurements for HP->10, CP, BPEIand cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).
Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. Each measurement represents the mean ± SD of 10 readings obtained from duplicate samples. * indicates statistical significance ($p<0.05$) among RU:Nt ratios of each polymer, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test.

3.2.4 Zeta potential of the DNA polyplexes

The surface charge of DNA polyplexes will affect their colloidal stability and interaction with the negatively charged surface of cells (209). Therefore, the zeta potential of pDNA polyplexes at various RU:Nt ratios was measured to assess their surface charges. All polyplexes exhibited positive zeta potential at the selected ratios. Moreover, there were no significant differences observed between PEGylated and non-PEGylated PAAs. However, HP->10 polyplexes at the RU:Nt ratios of 5 and 10 displayed comparable positive surface charge to that of BPEI, whereas magnitude of charges for both CP and cross linked are always lower than the corresponding polyplexes produced with BPEI (Figure 3.5 Zeta potential measurements for HP->10, CP, BPEIand cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).).

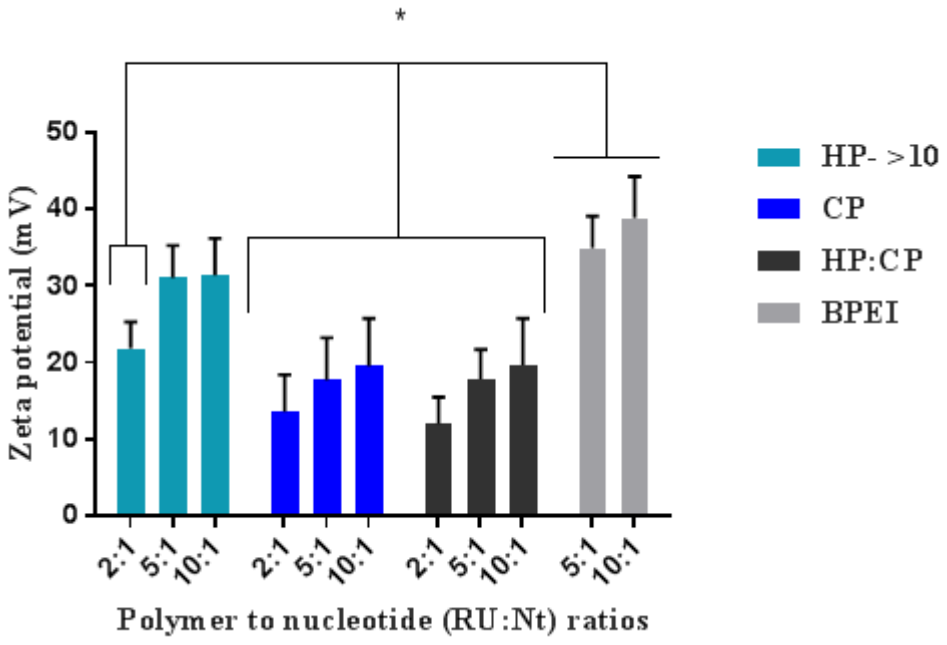


Figure 3.5 Zeta potential measurements for HP->10, CP, BPEIand cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).
Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. Each measurement represents the mean ± SD of 12 readings

obtained from duplicate samples. * indicates statistical significance ($p<0.05$) among RU:Nt ratios of each polymer, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test.

3.2.5 Buffering capacity of the polymers

The proton-buffering effect of PAA polymers was evaluated through acid−base titration. The buffering capacities of the polymers were expressed as the percentage of amine groups becoming protonated between pH 7.4 and 5.1. Both PEGylated (CP) and non-PEGylated PAA (HP) showed comparable buffering capacity, 40% and 36% respectively, but lower than that of BPEI (90%) which displayed a more gradual slope in the pH range of 7.4 to 5.1 (**Error! Reference source not found.**).

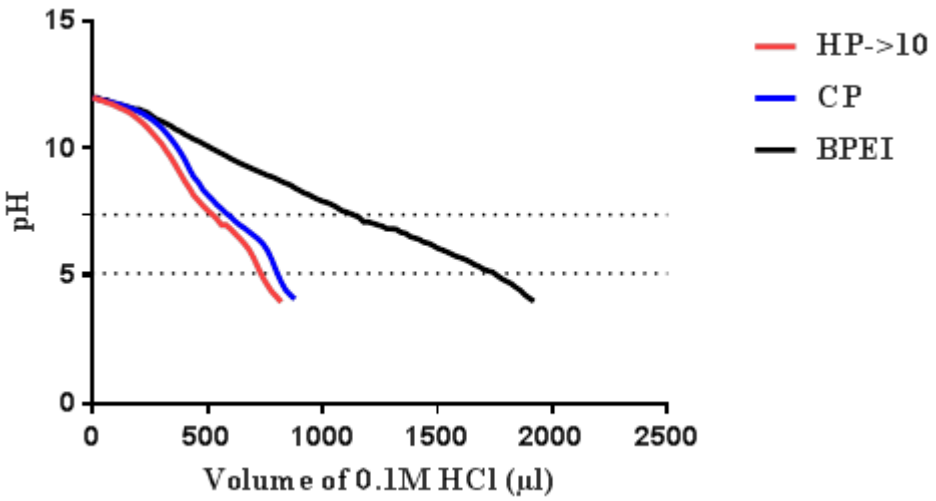


Figure 3.6 Acid−base titration curves for HP->10, CP and BPEI (1 mg/ml) in 5 mM PBS.
Alkaline solution of polymers were prepared and titrated against HCl (0.1 M) from pH 12 to 4 at room temperature. Solutions were adjusted to pH 12 with 0.1 M NaOH, and 5 µL aliquots of 0.1M HCl were subsequently added and the pH measured.

3.3 Discussion

Interaction of cationic polymers with DNA can occur spontaneously leading to formation of polyplexes through electrostatic interactions. In this study, the ability of PAAs to form nanostructures through condensation of pDNA was assessed by gel retardation. The ability of cationic polymers to condense DNA is considered necessary for protection of DNA against degradation by enzymes and thus for efficient gene expression (356). As shown in **Error! Reference source not found.** and **Error! Reference source not found.**, both individual (CP and HP) and crosslinked PAA polymers showed good pDNA binding ability as they completely inhibited pDNA migration at a low RU:Nt ratio of 2:1 which indicated that all of the pDNA was trapped inside the particles. It is important to note that the PEG side chain in CP and cross-linked system had no negative effect on complex formation with pDNA. Complexation behaviour of PAAs with different backbones had previously been studied and it was shown that PAA with p(MBA-DMEDA) backbone have efficient DNA condensing properties, probably because of the high cationic charge density compared to the others (211).

The correlation between size of pDNA polyplexes and successful gene delivery has already been reported where a particle size of less than 200 nm in diameter is required for efficient cellular uptake via endocytosis and subsequent transfection (357, 358). To investigate the ability of PAAs to condense DNA efficiently, particle size was evaluated as a function of RU:Nt ratios through DLS analysis. It was found that the particle sizes of the cross-linked polyplexes decreased with increasing RU: Nt ratios which could be due to the presence of the appropriate proportion of PEG that provides a steric barrier around the complexes (359, 360). However, both PEGylated and non-

PEGylated polymers formed polyplexes with a diameter around or below 200 nm that are suitable for endocytosis (357).

Zeta potential is considered as an indicator of the surface charge of the pDNA polyplexes, which plays a pivotal role in cellular uptake following electrostatic interactions between the positively charged polyplexes and the anionic cell membranes (361). The zeta potentials measured for PAA complexes did not show significant differences at the corresponding RU: Nt ratios. However, polyplexes formed with HP had a zeta potential of > 30 mV at the highest ratios, which iscomparable to that produced by BPEI. In contrast, PEG terminated PAAs produced less positively charged complexes than BPEI, possibly due to the shielding effect of neutral PEG on the polyplex surface (362) which shifts the shear plane away from the particle core resulting in reduced surface charge (223). Therefore, given the low zeta potential, CP and crosslinked polyplexes would be expected to exhibit lower transfection efficiency and cytotoxicity as compared to HP and BPEI (223).

Polyplexes require an efficient mechanism to escape from the endosome into the cytosol (243). The buffering capacity of gene delivery vectors between the physiological pH (pH 7.4) and late-endosomal pH (pH 5.1) is associated with their ability to escape from the endosome (357). It has been shown that there is a correlation between the buffering ability of the polymers in the endosome and the amount of secondary and tertiary amine groups they contain, as these amines have a tendency for protonation over the relevant pH range (243). It is noted that both PEGylated copolymers (CP) and unPEGylated homopolymer (HP) showed a similar trend but with a lower buffering capacity in comparison to BPEI within the pH range of 7.4 to 5.1. The high buffering capacity of BPEI is related to the presence of many secondary and tertiary amine groups in its structure, so it can buffer endosomal pH by taking up

protons via the proton-sponge effect (355, 363), and in turn mediate its efficient escape from endosome to cytosol by disrupting the endosomal membrane. However, the buffer capacities of both PEGylated and unPEGylated PAAs were about the same which confirmed that the existence of the PEG molecules in the copolymer have no effect on the protonation of these polymers (362).

3.4 Conclusion

Both individual (HP, CP) and HP:CP blend PAA polymers showed efficient pDNA binding and they were able to condense pDNA into nanosized complexes of <200 nm in diameter with a positive surface charge. However, PEGylated PAA polyplexes exhibited lower zeta potential compared to BPEI polyplexes at the same RU:Nt ratio. All PAAs showed a similar buffering capacity, but this was lower than that of BPEI. Based on the physico-chemical evaluation, only polyplexes prepared at 2:1, 5:1 or 10:1 RU:Nt ratio and 2:1 HP:CP ratio were further used for biological evaluation.

Chapter four

4 *In vitro* cytotoxicity of PAA based pDNA polyplexes

4.1 Introduction

The assessment of *in vitro* cytotoxicity of any biomaterial is an early step to estimate its biocompatibility. Biocompatibility is a descriptive term which indicates the ability of a material to perform with an appropriate host response, in a specific application (364). In simple terms it implies compatibility or harmony of the biomaterials with the living systems. In general, polycations such as linear polyamidoamines (PAA) are considered promising polymers for biomedical application due to their good biodegradability and biocompatibility (365). They have lower cytotoxicity than other usual polycationic vectors (366) (**Table 4.1**). Previous studies showed that PAAs based on MBA DMEDA monomers have a lower cytotoxicity compared to other cationic polymers (205). However, the cytotoxicity of PAA polymers and corresponding polyplexes has been frequently evaluated by using the MTT assay without providing insight into the underlying mechanisms. There are many limitations undermining the accuracy of the MTT assay which include reduction of the MTT tetrazolium salt occurring not only in the mitochondria but also within the cytoplasm, on the plasma membrane, endosomal or lysosomal membrane, or even in the extracellular environment. Moreover, the reduction of the MTT tetrazolium salt is decreased by compounds that cause elevation of the ROS due to disruption of mitochondrial enzyme activity (367). Cationic polymers and their polyplexes can cause cytotoxicity by compromising the cell membrane integrity as well as through interactions with other intracellular processes (368). Mitochondria are membrane-enclosed organelles located in the cytoplasm of eukaryotic cells and play a pivotal role

in the generation of ATP and reactive oxygen species (ROS) (369). Cell viability is indicated by the mitochondrial membrane potential (MMP) which is an important physiological mitochondrial parameter (370). MMP is generated by the proton pump of the electron transport chain or mitochondrial respiratory chain (371). The MMP controls ATP synthesis, generation of ROS and calcium uptake. The optimum MMP is critical for preserving important cellular functions and mitochondrial processes as well as keeping the physiological function of the respiratory chain (372). Loss of MMP leads to cellular stress and cell death, and dissipation of MMP can result in apoptosis (373). Moghimi *et al* reported that the release of LDH and the translocation of phosphatidylserine from the inner plasma membrane to the outer cell surface that occur within the first hour of incubation with BPEI are hallmarks of Phase I toxicity as a result of cell membrane disruption (374), whereas Phase II toxicity results from intracellular effects of the same polymer through the formation of channels in the outer mitochondrial membrane which leads to the release of proapoptotic cytochrome c that activates caspase 3 (375, 376). Translocation of caspase 3 into the mitochondria causes a loss of mitochondrial transmembrane potential (MMP) and eventually leads to apoptosis (374, 377, 378).

Nanoparticles can also cause oxidative stress as a result of intracellular reactive oxygen species (ROS) formation (379). Reactive oxygen species (ROS) are reactive chemical species that contain oxygen molecules. ROS can be categorized into free radicals ROS and non-radical ROS. Free radical ROS are radicals possessing one or more unpaired electrons in their outer molecular orbital making the radical high reactive. Unlike free-radical ROS, non-radical ROS do not contain any unpaired electrons. However, non-radical ROS remain chemically reactive and can be converted into free-radical ROS through chemical interactions. Superoxide anion

($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals are free radicals and non-radical species includes hydrogen peroxide (H_2O_2) (380). ROS are highly reactive in nature and have a very short half-life in cells. Conversion of superoxide into H_2O_2 and other reactive products is a very quick process, so there are difficulties in distinguishing one particular type from another by using ROS assays. Superoxide anions ($O_2^{\bullet-}$), nitric oxide (NO) and hydroxyl radicals are the commonly found ROS in biological systems and serve a wide array of important physiological functions in cells. They have the ability to cause biological damage and are involved in aging and the pathology of many conditions, including cancer and cardiovascular, inflammatory (381) and degenerative diseases (382).

Biological systems continuously encounter exogenous and endogenous sources of reactive oxidants. These reactive species are generated as inevitable product of oxygen metabolism, especially in the reduction of oxygen by the electron transfer chain (ETC) system in the mitochondria, mainly at complex I and complex III. Mitochondria is considered the major source of intracellular ROS and it is predicted that 1-2% of mitochondrial O_2 consumed goes on to form ROS (383). Other organelles also contribute to levels of ROS, including peroxisomes and the endoplasmic reticulum, as well as external sources such as UV and ionizing radiation, toxic chemicals and drugs (384-387). Various enzymes, for example- Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs), xanthine oxidase, nitric oxide synthase and peroxisomal constituents also generate ROS (388). The formation of superoxide anion radical creates a cascade leading to the formation of another ROS. Superoxide anion is converted to hydrogen peroxide and oxygen by the enzyme manganese superoxide dismutase and transformed furthermore to the highly reactive hydroxyl ion via Fenton reactions (389) . The hydroxyl radical can react immediately with any biomolecule from which it can abstract a hydrogen atom leading to a free radical that is more stable and longer-lived than the hydroxyl

radical. ROS and in particular the hydroxyl radical are able to interact with all biological macromolecules (lipids, proteins and nucleic acids) and cause damage (390).

ROS play an important role in the series of cellular processes, including cell adhesion, immune response, apoptosis, cell growth and differentiation, and act as second messengers in intracellular signalling (391-393). Production of ROS at low levels is considered important for performing of these cellular processes, whereas high levels of ROS are involved in the mechanism of numerous diseases, including various neurological disorders (394) and cancer (395). ROS can cause oxidative modification of DNA, proteins and lipids. Severe increase of ROS in cells could cause irreversible oxidative damage that leads to cell death (396, 397).

Production of ROS can cause damage to the cellular components such as lipids, proteins and DNA and finally lead to apoptotic cell death (398). Moreover, apoptosis could also be induced by lysosomal proteases that are released into the cytoplasm as a result of endo/lysosomal escape of the polycations (399, 400).

Various factors can influence the toxicity of cationic polymers such as molecular weight, molecular architecture, and charge density. In general, high molecular weight and high charge density cationic polymers show high cytotoxicity (399, 401). Moreover, unmodified polymers may cause potential membrane damage and have already been reported to cause apoptosis in different human cell lines (374), whereas changes in the degree of branching and chemical structure or the introduction of hydrophilic moieties (e.g. PEG) into a polymer can reduce the cytotoxicity to an acceptable level (402). As a consequence, assessment of the underlying mechanisms behind PAA toxicity is considered necessary for the development of efficient gene delivery system.

This chapter aims to advance the knowledge of interactions between PAA polyplexes and cells by examining their effects on cell membrane, mitochondrial function and production of ROS. To determine the effects of the PAA polymers on the metabolic activity of the cultured cells, the MTT assay was employed, whereas the Presto Blue assay was used for PAA polyplexes. The LDH assay was used to assess the effect of PAA polymers and polyplexes on membrane integrity. To probe whether PAA polyplexes have the ability to induce ROS generation, the ROS sensitive H2DCFDA dye was employed and the effect of PAA polyplexes on MMP was examined by using the JC-1 assay.

Table 4.1 Cytotoxicity of PAAs

Polymer	Cell line (Cytotoxicity is expressed as an IC_{50} (µg/ml) mean ± SD)			
	B16F10	Mew	HepG2	HL60
NG30 (MBA-DMEDA)			400 ± 22.5	>10
ISA1	3100 ± 700	2200 ± 400		
ISA4	3500 ± 1200	1900 ± 500		
ISA22	4000 ± 1400	4600 ± 500		
ISA23	>5000	4200 ± 1100		
DMEDA-BAC	>5000			
DEEDA-BAC	>5000			
DMEPDA-BAC	3550 ± 310			
DMEXA-BAC	230 ± 60			
Poly- L-lysine (PLL)	100	10		
PLL (22 kDa)			300 ± 8.4	
PLL (243 kDa)			70 ± 8.9	
PLL (56.5 kDa)	50 ± 10			
Polyethylenimine (PEI)	10 ± 10			

4.2 Results

4.2.1 Effect of free polymers on cell metabolic activity

The appropriate cell density for conducting the MTT assay was first determined. An increase in the absorbance was observed with increasing cell number until this reaches a plateau (**Figure 4.1 Represents the absorbance of untreated cells at different densities.**). This can be explained as the ability of cells to reduce the reagent increases as their number increases, reflected in a high absorbance, until a state of little or no change is reached because the wells became overconfluent and led to saturation of the assay. According to these results, we considered 10000 cells/well as the appropriate cell density to be used in the next experiments.

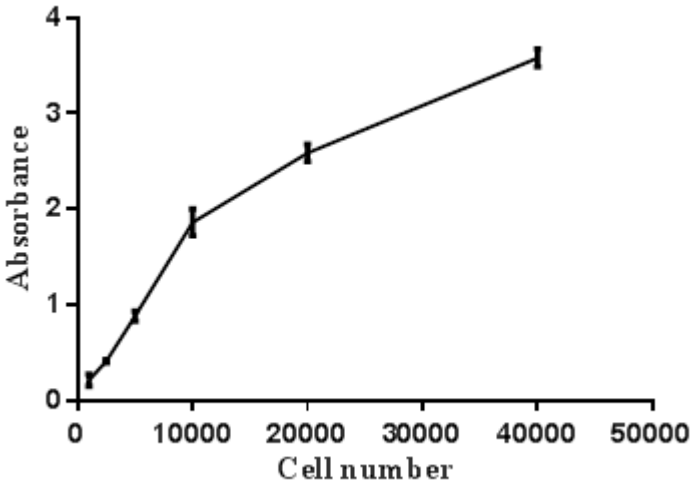


Figure 4.1 Represents the absorbance of untreated cells at different densities. A549 cells were seeded for 48 hrs and incubated with MTT (0.5 mg/ml) for 2hrs. MTT was prepared with serum-free medium (DMEM). Each point represent mean of 6 wells ± SD.

In this section the cytotoxicity of the free polymers, PAAs and BPEI, was investigated on A549 cells in the absence of serum, under conditions matching those during *in vitro* transfection. The metabolic activity of cells was assessed by MTT assay after incubation with a range of polymer concentrations from 0.1µg/ml to 1000 µg/ml. The

results show that BPEI causes a great reduction in the metabolic activity of A549 cells at low concentrations in comparison to the PAA polymers and also demonstrate that the metabolic activity decreased with increasing polymer concentration (**Figure 4.2 Dose-response curve showing the metabolic activity (%) of A549 cells treated with BPEI and individual PAAs for 4 hours.**). It is noteworthy that no alteration in the trend of the dose response curve was observed under the concentration of 100 µg/ml for both PAA polymers tested. Cytotoxic effects of polymers were compared by using IC_{50} values **Table 4.2**. The IC_{50} values for HP->10, CP and BPEI are 279 ± 13 µg/ml, 314 ± 6 µg/ml and 8 ± 1 µg/ml respectively.

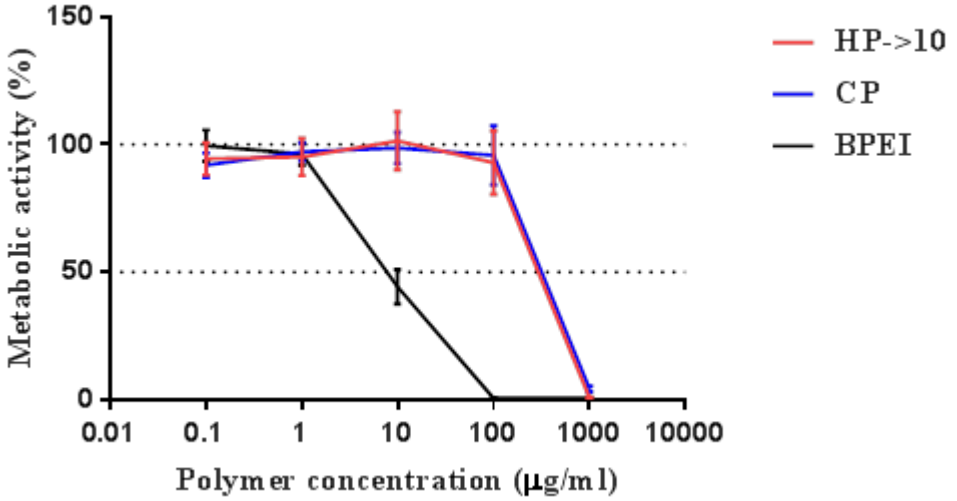


Figure 4.2 Dose-response curve showing the metabolic activity (%) of A549 cells treated with BPEI and individual PAAs for 4 hours.
Sample of polymers were prepared in cell culture medium (OptiMEM) at increasing concentrations of (0.1, 1, 10, 100, 1000) µg/ml. Triton X-100 (4% v/v) and OptiMEM were used as positive and negative controls respectively. The metabolic activity was measured by MTT assay which was performed 24 hours after polymer exposure, mimicking the transfection assay conditions. Data represents mean ± SD (6 replicates, n = 2).

Table 4.2 Cytotoxicity of polymers

Type of polymer	IC_{50} ((μg/ml)
BPEI	8 ± 1
HP->10	279 ± 13
CP	314 ± 6

4.2.2 Effect of pDNA polyplexes on cell metabolic activity

The appropriate cell density for conducting the Presto Blue (PB) assay was first determined. The relationship between fluorescence and cell number was linear with a R^2 value = 0.99 as shown in **Figure 4.3 Linear regression between A549 cell number and their fluorescence.**. This can be explained as the ability of cells to reduce the reagent increases as their number increases. According to the results, we considered 50000 cells/well as an appropriate cell density to be used in the next experiments.

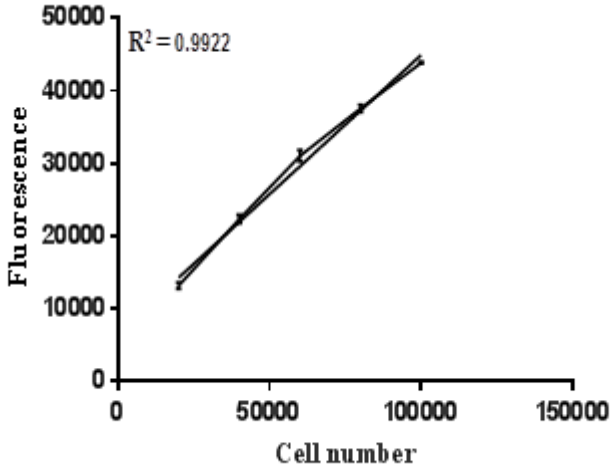


Figure 4.3 Linear regression between A549 cell number and their fluorescence. The cells were seeded for 48 hrs and incubated with Presto Blue for 30 min. PB was prepared at a 1:9 ratio with HBSS solution. Each point represent mean of 3 wells ± SD.

PAA polyplexes could maintain a high metabolic activity (86 -94%) at RU:Nt ratios of 2 and 5. However, after exposure to 10:1 RU:Nt ratio the cell metabolic activity was reduced significantly to (61-76%). Moreover, cells incubated with the cross-linked polyplexes showed a significantly lower metabolic activity in comparison to those exposed to the other PAA polyplexes at the same 10:1 RU:Nt ratio. In contrast, BPEI polyplexes caused a dramatic reduction in the metabolic activity, up to ~70%,

at the RU:Nt ratios of 5 and 10, (**Figure 4.4 Metabolic activity measured by Presto Blue assay 20h followingcell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.**).

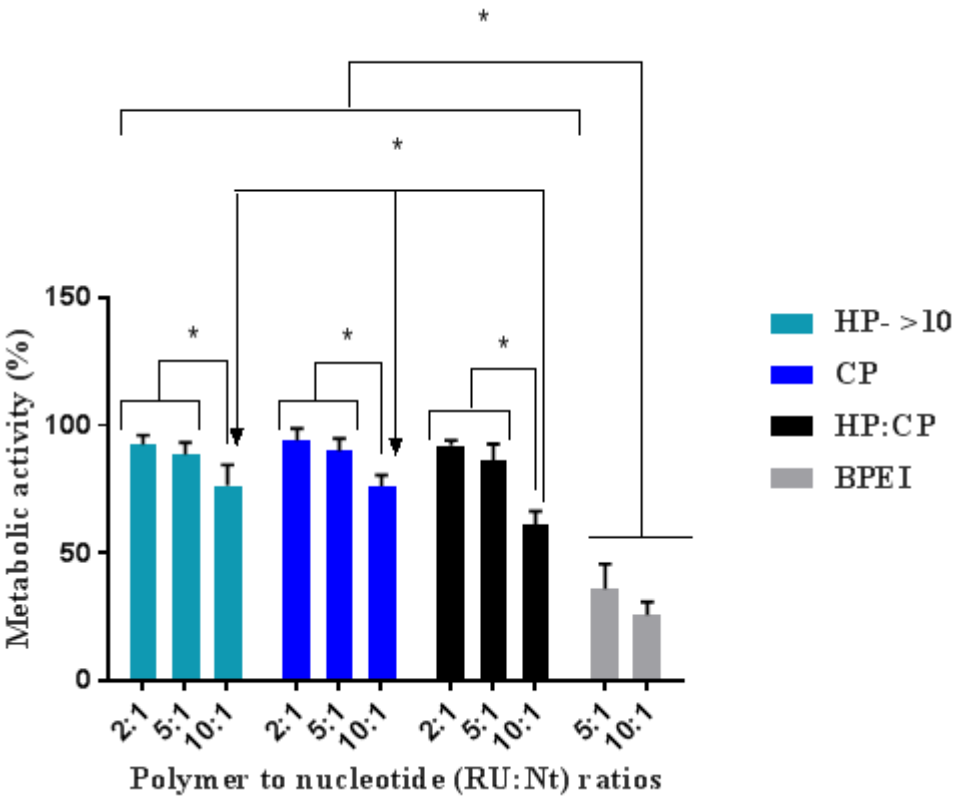


Figure 4.4 Metabolic activity measured by Presto Blue assay 20h followingcell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.
Cross-linked pDNA polyplexes were prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance ($p<0.05$) among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA followed by a Tukey's multiple comparison post-hoc test.

4.2.3 Effect of free polymers and pDNA 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.

The LDH released in response to the free HP->10 was negligible at the all tested concentrations, whereas free CP caused a significant LDH release, around 50% of the positive control, at the highest concentration (1000 μg/ml). In contrast, 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 (**Error! Reference source not found.**).

Again, cell exposure to PAA polyplexes did not induce any significant LDH release at any RU:Nt studied (**Figure 4.6 LDH released from A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.**). In comparison, BPEI based vectors caused an extracellular leakage of the protein at the highest RU:Nt of 10:1 (**Figure 4.6 LDH released from A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.**).

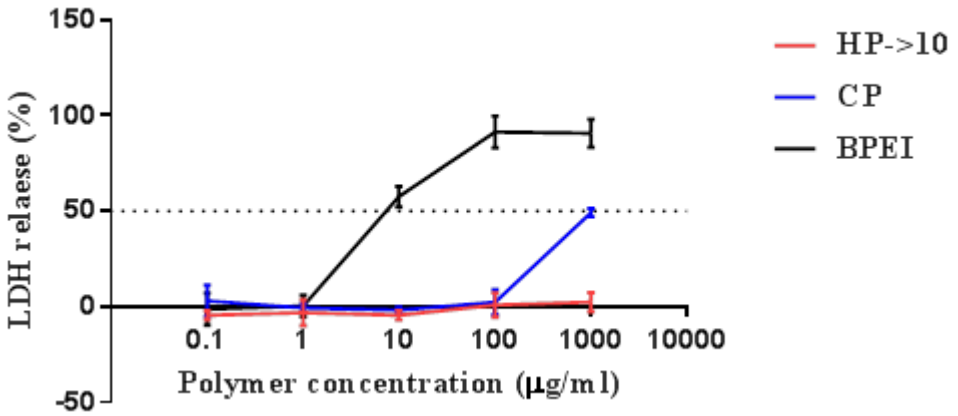


Figure 4.5 Dose-response curve showing LDH release (%) from A-549 cells treated with BPEI and individual PAAs for 4 hours.
Sample of polymers were prepared in cell culture medium (OptiMEM) at increasing concentrations of (0.1, 1, 10, 100, 1000) µg/ml. Triton X-100 (4% v/v) and OptiMEM were used as positive and negative controls respectively. LDH assay was performed 4 hours after polymer exposure. Data represents mean ± SD (6 replicates, n = 2).

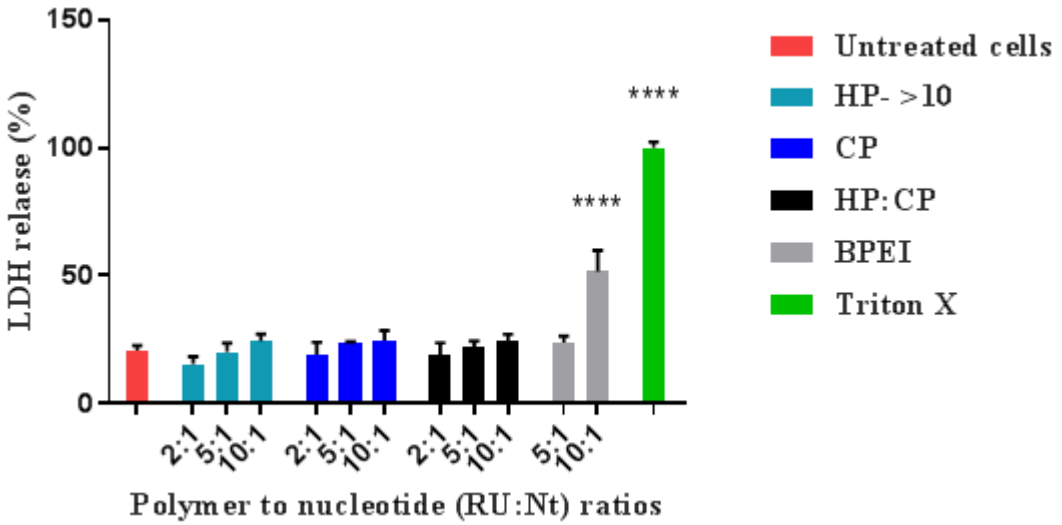


Figure 4.6 LDH released from A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.
Cross-linked pDNA polyplexes were prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. The data are expressed as mean ± SD (3 replicates, n=2). * indicates a statistically significant release compared to untreated cells, as determined by one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test. (****P < 0.0001).

4.2.4 Mitochondrial membrane potential measurement

The ability of the polyplexes to trigger apoptosis was determined through measurement of the MMP in a JC-1 assay using the FCCP , a potent uncoupler of mitochondrial oxidative phosphorylation which acts by inhibiting the coupling between the electron transport and phosphorylation reactions leading to inhibition of ATP synthesis (403) and dissipation of MMP (404), 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 (405), the dye is converted into a monomeric form (green fluorescence).

As shown in **Figure 4.7 Evaluation of MMP by JC-1 dye 24 h following A549cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.**, 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.

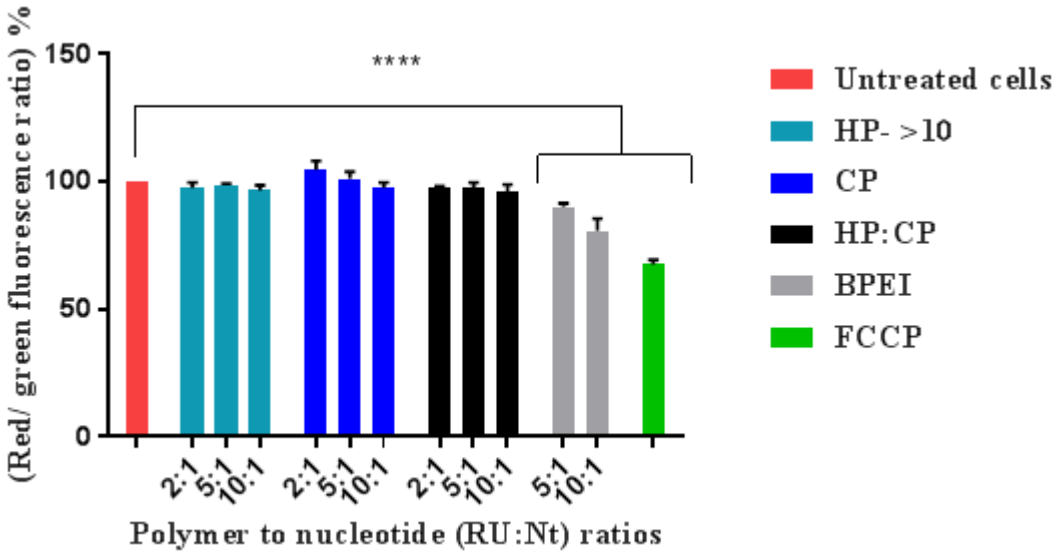


Figure 4.7 Evaluation of MMP by JC-1 dye 24 h following A549cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.
Cross-linked pDNA polyplexes were prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. As a positive control, cells were treated with 10 µM FCCP. The data are expressed as mean ± SD (3 replicates, n=2). * indicates a statistically significant increase in ROS compared to untreated cells, as determined by one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test (****P < 0.0001).

4.2.5 Intracellular formation of reactive oxygen species

The generation of intracellular reactive oxygen species (ROS) after cell exposure to the polyplexes was probed using a cell-permeable non-fluorescent H2DCF-DA dye which is de-esterified by cellular esterases into a non-fluorescent deacetylated intermediate (H2DCF) and then turns to highly fluorescent dichlorofluorescein (DCF) upon oxidation by ROS (406). The fluorescence measured in the DCF assay is directly related to the ROS levels generated (407). Quantitative measurement of the intracellular ROS generation in A549 cells was performed at different time points (2h

and 4h) and with different RU:Nt ratios of each pDNA polyplexes. Intracellular ROS generation after 2 h exposure to the different polyplexes at RU:Nt ratios of 2, 5, 10:1 was first measured by using flow cytometry, as shown in **Figure 4.8 Evaluation of intracellular ROS production by A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 2 hours.**; treatment of H_2O_2 (800 Mm) for 2 h was used as a positive control.

Transfection with the cross-linked polyplexes caused a significant increase in ROS production at RU:Nt ratios of 5 and 10 compared to the control cells, whereas cells treated with HP-10, CP and BPEI polyplexes showed a significant generation of ROS only at a 10:1 RU:Nt ratio (**Figure 4.8 Evaluation of intracellular ROS production by A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 2 hours.**). Quantitative analysis by TECAN multimode microplate reader has been carried out to confirm ROS generation by these polyplexes after 2 and 4 h incubation. There was significant difference in the intracellular ROS level between the untreated and polyplexes treated cells similar to those obtained by flow cytometry analyses apart from BPEI polyplexes at 5:1 RU:Nt ratio which showed significantly higher ROS production than the untreated group only after 4 h transfection (Figure 4.9).

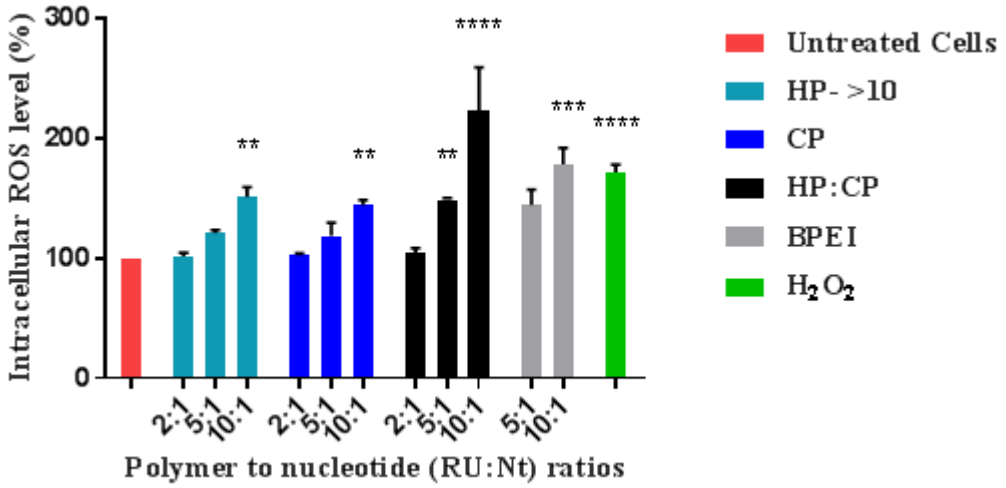


Figure 4.8 Evaluation of intracellular ROS production by A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 2 hours.
The ROS level was determined by measuring fluorescent DCF production using flow cytometry and is expressed as the percent ROS increase (Untreated cells = 100%). Cross-linked pDNA polyplexes were prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. As a positive control, cells were treated with 100 µM hydrogen peroxide (H_2O_2). The data are expressed as mean ± SD (2 replicates, n=2). * indicates a statistically significant increase in ROS compared to untreated cells, as determined by one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test (**P< 0.01; ***P < 0.001; ****P < 0.0001).

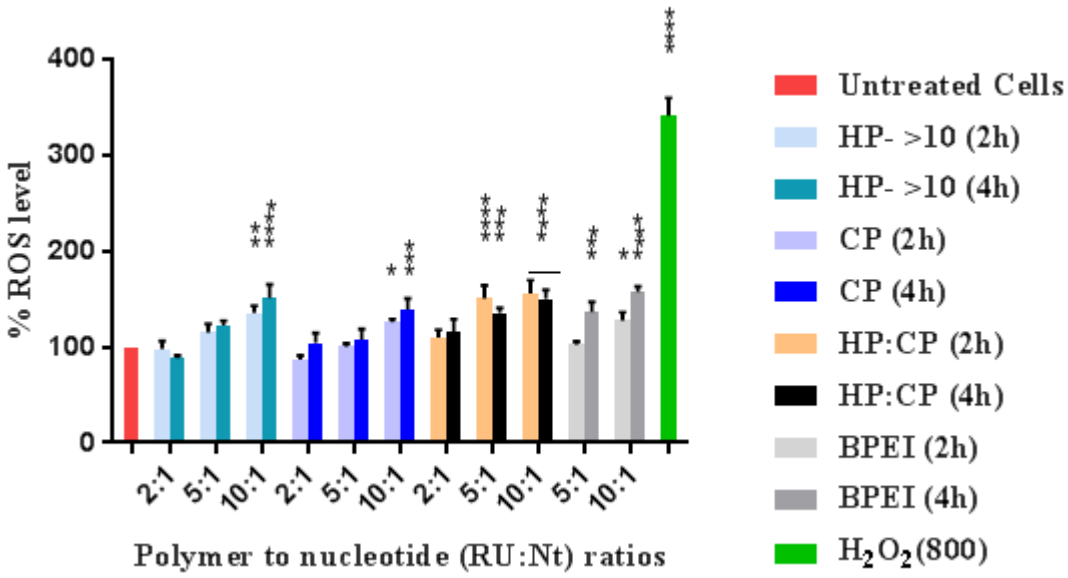


Figure 4.9 Evaluation of intracellular ROS production by A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 2 and 4 hours.
The ROS level was determined by measuring fluorescent DCF production using TECAN multimode microplate reader and is expressed as the percent ROS increase (Untreated cells = 100%). Cross-linked pDNA polyplexes were prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. As a positive control, cells were treated with 800 µM hydrogen peroxide (H_2O_2). The data are expressed as mean ± SD (3 replicates, n=2). * indicates a statistically significant increase in ROS compared to untreated cells, as determined by one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test (*P < 0.05; **P< 0.01; ***P < 0.001; ****P < 0.0001).

4.3 Discussion

Cytotoxicity is one of the most critical features of the synthetic polycationic carriers used in gene delivery. The MTT assay was utilized to determine the effect of PAAs and BPEI polymers on A549 cell metabolism in relation to their structures and concentrations. All PAAs used in the current project were based on the MBA-DMEDA co-monomers as detailed in Chapter 2 (Figure 2.1). BPEI exhibited a more pronounced dose dependent cytotoxicity compared to PAAs as the cell metabolic activity dropped to around zero at a concentration of 100 μg/ml, whereas the reduction in metabolic activity caused by PAAs at the same concentration was less than 10%. The cytotoxicity of the experimental polymers was compared through calculation of their IC_{50}. According to IC_{50} values, CP showed a significantly lower cytotoxicity than the HP->10 polymer. The low cytotoxicity of this polymer could be due to the presence of PEG in its structure which may sterically shield charges on the backbone. Results presented here are in agreement with those obtained by Rackstraw *et al*., where the parent PAA homopolymer showed a significantly higher toxicity (IC_{50}: 160 μg/ml) on Human HepG2 cells in comparison to the corresponding PEGylated PAA with an IC_{50} of 275 μg/ml (223). However, both PAA polymers (HP->10 and CP) were found significantly less cytotoxic compared to BPEI (35-40 fold). The high level of cytotoxicity showed by BPEI may be due to its non-degradability and accumulation (408), as well as its high positive charge density and the degree of branching (409), whereas the low cytotoxicity of PAA polymers could be related to their ability for degradation into oligomeric products (199, 220) in addition to the low amino density in their core (357).

In term of BPEI cytotoxicity, our findings were in agreement with Kim *et al*. who examined the effect of BPEI25k on C2C12 and HeLa cells. BPEI reduced the cell

viability to ≤ 20% even at a low concentration (20 µg/ml) (410). On the other hand, a study conducted by Hill *et al.* showed that a homopolymer [PAA of 21500Da] with a similar backbone to that used in the current project [MBA-DMEDA] had an IC_{50} of 400 µg/ml following 48 hrs incubation with human liver cancer (HepG2) cell line in the presence of serum (205).

In addition, the cytotoxicity of PAA based polyplexes was investigated on A549 cells as a function of polymer/pDNA ratios by using PrestoBlue® (PB) assay. PB assay is based on measuring the metabolic activity of mitochondrial enzymes. PB is the fastest live assay with high sensitivity to assess cell viability. It can measure cell viability after a short incubation period of 10 min, and can detect a low number of cells (12 cells per well) (411). Moreover, PB is water soluble and non-toxic to cells, therefore normal metabolism is less likely affected and cells can be used in subsequent tests (412). In contrast, 3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyl tetrazoniumbromide (MTT) is insoluble in cell culture media, less sensitive and considered an end-point assay because cells are killed by the reagent forming crystals (413). PB was shown to be faster than alamarBlue as a viability indicator for *Streptococcus mutans*, *Prevotella intermedia*, and *Mycobacterium tuberculosis* treated with antimicrobial (414). Again, the use of the fluorometric PrestoBlue assay was advised for assessing cytotoxicity towards human umbilical vein endothelial cells (HUVECs) in comparison to MTT (415). In a more recent investigation, PB was found to be more reliable than MTT, but similar to AlamarBlue® in determining the viability of human corneal epithelial cells (411). All these investigations strongly confirmed that PB assay can be applied to obtain more consistent/reliable data than other assays. Based on our findings, PAA polyplexes showed a significantly lower cytotoxicity than BPEI polyplexes at all the RU:Nt ratios tested. This finding may again be attributed to the biodegradable nature

of PAAs (416) and low charge density (357). These results are consistent with previous studies that have shown both PEGylated and unPEGylated PAA polyplexes based on N, N′-cystaminebisacrylamide- 4-amino-1-butanol p(CBA-ABOL) have a lower cytotoxicity on MCF-7 and NIH 3T3 cell lines in comparison to PEI (362). With respect to the toxicity of PAA polyplexes, higher levels of cellular toxicity occurred at the RU:Nt ratio of 10 compared to the other ratios which may be attributed to the presence of excess free polymers in the nucleic acid/polymer polyplexes as previously reported (417). A possible explanation for the high cytotoxicity of BPEI polyplexes is the presence of primary amines in BPEI structure that are responsible for its high charge density (418) which could cause activation of some unknown deleterious cellular response (416) compared to that of PAA which has secondary and tertiary amines. Different authors discussed the role of the type of amines for toxicity. Ferruti *et al*. found that the partial or total substitution of primary amino groups in PLL with dimethylacrylamide leads to derivatives that were less toxic than the parent compound (419). Another study conducted by Dekie *et al*. showed that the presence of primary amines in poly L-glutamic acid derivatives led to a significant toxic effect on red blood cells (117). However, cytotoxicity is not only influenced by the type of amino function, but the charge density resulting from the number of cationic residues has also an important effect (161).

Interaction of polymers and polyplexes with cell membranes was reflected by the release of the cytosolic enzyme LDH into the extracellular region which is considered as a marker for cell membrane damage (420). In this study, the PAA polymers caused much lower membrane damage than PEI. However, cells exposed to CP exhibited more LDH release around 50% at 1000 µg/ml compared to HP->10 which showed negligible LDH release over a broad concentration range tested. This increase in LDH

release occurring at high concentrations of CP was likely due to destabilization of the lipid monolayer (421). Moreover, it has been found that polyethylene glycol (PEG) acts as pore former; its capacity to form pores increasing with its molecular weight (422). The lower membrane activity of PAAs could be related to their repeating units behaving independently of one other and not depending on the degree of protonation of neighbouring repeating units unlike many other polyamines (e.g., polyethyleneimine and poly- L-lysine) (202, 203).

Cell exposure to different PAA polyplexes did not induce significant LDH release over the entire range of ratios studied. In contrast, a significant extracellular leaking of LDH was observed following treatment with BPEI at the highest RU:Nt ratio of 10, suggesting that BPEI had a much higher membrane damaging effect on A549 cells than PAA. These results are consistent with another study which revealed that different concentrations of PAA/ lysozyme nanocomplexes were non-cytotoxic to human umbilical vein endothelial cells (423). This is not surprising because BPEI at the highest ratio possesses a greater charge density compared to PAA, in agreement with its zeta potential results, as a result of its primary amine groups which leads to strong electrostatic interaction with the plasma membrane and in turn results in destabilisation and ultimately cell membrane dysfunctions (424). Moreover, no differences in LDH release were detected comparing free polymers to PAA/pDNA complexes. Therefore, direct membrane toxicity was ruled out as a major cause of the decreased cell viability after treating A549 cells with PAA polyplexes.

Mitochondrial membrane potential is a key indicator of mitochondrial function and cell viability, which reflects the pumping of hydrogen ions across the inner membrane during the process of electron transport and oxidative phosphorylation, the driving force behind ATP production (425). The loss of MMP occurs in the early stages of

apoptosis (405) and plays a central role in mitochondrial-mediated cell death (426). JC-1 dye was mainly used to examine the effect of different polyplexes on mitochondrial membrane potential (MMP). This probe was shown to be more reliable in determining MMP changes compared to rhodamine 123 (R123) and 3,3'-dihexiloxocarbocyanine iodide [$DiOC_6(3)$]. DiOC6(3) and R123 are less useful for analysing MMP changes due to their inconsistent behaviour (427). R123 probe showed lower sensitivity to changes in MMP because of quenching some of the probe to several energy-independent binding sites, so their movement is independent on the energy status of mitochondria (428), whereas $DiOC_6(3)$ dye is more sensitive to changes in plasma membrane potential than to MMP. Moreover, addition of MMP dissipating agents such as carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) or valinomycin led to changes in the fluorescence of cell stained with JC-1 probe compared to DiOC6(3) which was only affected by FCCP, and R123 did not respond to either agents (427). Furthermore, JC-1 does not reveal any concentration-dependent quenching effect (429). The use JC-1 has another advantage over the other probes that it can be both qualitative and quantitative due to dual-emission characteristics according to the MMP condition (430). This probe has been found to be sensitive and efficient in numerous cell types such as Jurkat T lymphoma cells (431); U937, HL60, and K562 cell lines (430, 432). All these characteristics make the use of JC-1 as a fluorescent probe more suitable than other potential membrane dyes for determination of MMP. MMP has been evaluated 24 h after cell exposure to different polyplexes to see if MMP would be affected permanently due to localization of the polymers in the mitochondria. PAA polyplexes did not induce any loss in MMP at any of the tested RU:Nt ratios indicating that PAA polyplexes did not localize in the mitochondria. Mitochondrial depolarization can occur by one of the potential

mechanisms which include direct mitochondrial membrane permeabilization, activation of the mitochondrial permeability transition pore, and interference with mitochondrial membrane proton pumps, specifically Complex I of the electron transport chain and F0F1-ATPase (273, 306, 433, 434). The results obtained are in line with the cytotoxicity results of LDH and Presto Blue assays. In contrast, BPEI polyplexes at the tested ratios caused a significant reduction in MMP compared to untreated control, which are in agreement with previous studies on Jurkat T and HeLa cells (374, 435). It has been found that BPEI induce dissipation of the MMP because of its high escape efficiency from the endosomes to the cytosol, followed by interaction with and then localization in the mitochondria, which in turn disrupts the mitochondrial membrane leading to release of pro-apoptotic factors and eventual cell death (435).

One of the major mechanisms of cytotoxicity that can be induced by many types of nanoparticles such as gold, silica, titanium oxide, iron oxide, lipoplexes, and polyplexes (436-438) is oxidative stress through ROS generation. Intracellular ROS can cause oxidation to the lipids, proteins and the genomic DNA (439) which could eventually lead to cell death due to breakage of DNA strands and accumulation of damaged proteins in the endoplasmic reticulum lumen (440). Therefore, the ability of the selected polyplexes to induce oxidative stress was investigated in A549 by evaluating intracellular ROS. The formation of intracellular ROS can be detected by a fluorometric assay using 2', 7'-dichlorofluorescin diacetate (H_2DCFH-DA) probe. The advantages of using DCFH-DA as a fluorescence probe are its ease of use, sensitivity to changes in redox state, low cost and ability to measure ROS production in cells over time (407). Compared to untreated cells, we have indeed detected a higher level of ROS in polyplex treated cells after 2 and 4 h incubation. There is no significant

increase in the generation of cellular ROS by PAA polyplexes as incubation time increased compared to BPEI polyplexes. Although BPEI polyplexes are more toxic than PAA counterparts, no significant differences were found among the highest RU:Nt ratio (10:1). This is most likely because cells produce ROS normally through metabolic processes; as cells die by BPEI polyplexes, there would be a decrease in metabolism leading to a decrease in ROS. ROS production by BPEI polyplexes were in agreement with the results obtained by Lee *et al*. who observed that transfection with BPEI 25/pDNA polyplexes induced a significant production of ROS in Human hepatoma cancer cells after 4 and 24 h incubations (379). Induction of ROS by BPEI polyplexes is possibly associated with their localisation in the mitochondria after release into the cytoplasm leading to additional production of free radicals (441, 442), oxidative stress and further reduction in MMP, which eventually can culminate in apoptosis (443). These findings were in agreement with MMP results where BPEI polyplexes caused a significant decrease compared to the control group after 24 hr. Moreover, the generation of ROS has been found to be dependent on the number of surface primary amino groups of the cationic polymers (185) so that BPEI showed elevated intracellular ROS level at both 5 and 10 RU:Nt ratio due to the presence of primary amino groups in its structure compared to the PAA polymers which have only secondary and tertiary amines. Dey *et al*. showed that a peptide linked polyethylenimine polymer hybrid produced lower levels of ROS compared to BPEI alone due to the introduction of L-Carnosine as a side chain which might reduce the toxic effect of the large number of the available primary amine groups in BPEI. In addition, they revealed that introduction of Boc groups caused a reduction in the toxicity of BPEI by blocking some amine groups and making the other primary amine groups inaccessible for reaction with intracellular components (444). Surprisingly,

although PAA polyplexes did not lead to significant loss of MMP compared to BPEI complexes as shown above, HP->10 and CP polyplexes showed an increase in ROS level at the highest RU:Nt ratio of 10 whereas cross-linked polyplexes exhibited significant ROS production at 5 and 10 RU:Nt ratios. The potential reason behind ROS generation by these polyplexes is their trafficking with the endosomal organelles after endocytosis. Mukherjee and Byrne observed that there are two phases of ROS generation after cell exposure to polyamidoamine dendrimer (PAMAM). Early-stage ROS generation occur during the first 6 h and result from encapsulation of the dendrimers in in early-stage subcellular vesicles, most likely endosomes, while the later, secondary response has been associated with release of the nanoparticles into the cytoplasm by endosomolysis and subsequent incorporation into the mitochondria (190, 445). ROS generation in the previous study is related to the reactive NH2 surface groups in PAMAM structures (190). Generation of ROS by PAA polyplexes at the highest RU:Nt ratios may be related to the high amount of free polymer compared to the lower ratios. Moreover, the presence of PEG in cross-linked polyplexes resulted in higher production of ROS so that appeared significantly at 5:1 RU:Nt ratio compared to HP->10. Our findings were in agreement with a study conducted by Beyerle *et al*. who showed that PEGylated BPEI caused higher upregulation of oxidative stress genes than non-PEGylated BPEI which may be due to the higher molecular weight of the PEGylated PEI-based polymers (446).

4.4 Conclusion

Polyamidoamine (PAA) polymers showed a significantly lower cytotoxicity compared to BPEI. However, at the concentration of 1mg/ml, HP->10 caused cell death by reducing cell metabolic activity without affecting cell membrane integrity compared to CP and BPEI which affect both. PAA/DNA complexes demonstrated favourable effects towards the metabolic activity and membrane integrity of A549 cells. Although PAA polyplexes can induce cellular ROS production at the highest RU:Nt ratios, no MMP depolarization was observed by PAA polyplexes at any ratio, suggesting their cytocompatibility is good. BPEI polyplexes were not associated with free polymer at the tested RU:Nt ratios, but nevertheless resulted in higher cytotoxicity compared to their PAA counterparts, for which an excess of free polymer was observed.

Chapter five

5 Mechanisms behind the transfection efficiency of PAA based pDNA Polyplexes

5.1 Introduction

Several studies have shown a relationship between transfection efficiency and cytotoxicity for cationic polymers. Grandinetti *et al*. revealed that nuclear membrane permeability is induced by cationic polymers such as linear PEI and hydroxyl-containing poly (L- tartaramidoamine) (T4) that are capable of inducing a high level of protein expression but also cytotoxicity (447). A recent study reported that intracellular reactive oxygen species (ROS) such as H_2O_2, hydroxyl radical, and superoxide generated by cancer cells due to oncogenic transformation can be utilized as stimulus for mediating efficient intracellular gene delivery (448). A study conducted by Hsu *et al*. showed that the transfection efficiency of the hybrid vector of recombinant adeno-associated virus and PEI decreased after pre-treatment of cells with antioxidants such as vitamin E or N-acetylcysteine (NAC) suggesting a role of ROS in gene delivery through actin polymerization induction which in turn facilitates cellular internalisation of the hybrid vectors (449).

Endocytosis is the generally accepted pathway for polyplexes internalization into cells (120). Once DNA polyplexes enter a cell via endocytosis, endosomal and then lysosomal trafficking will occur, therefore, their escape from the endosome into the cytosol is considered necessary to avoid metabolism in the endolysosome (450), which is a major bottleneck for successful delivery and transfection (451). It has been suggested that cationic polymers such as BPEI facilitate endosomal escape of the polyplexes by the alleged mechanism of proton sponge effect as shown in Figure

1.2(104). However, endosomal escape following the proton sponge effect is still controversial (452). Benjaminsen *et al* showed that PEI functions as a "proton sponge" but this doesn't lead to a change of pH (because of the activity of the ATPase pump) and the bursting of the endolysosomes (453). Moreover, recent studies showed contribution of the highly cationic charge in the endosomal escape via direct phospholipid membrane destabilization (158, 225, 363, 454). Unlike many other polyamines, PAAs have been found to mediate endosomolytic effects by different mechanisms. A study conducted by Barbucci *et al*. showed that PAA could escape form the endosome by changing its conformation at low pH from a coil to rod-like structure due to electrostatic repulsion between protonated nitrogens (203). However, Griffiths *et al*. showed that there are no changes in the morphology of the polymer or the vesicle after addition of amphoteric PAA to vesicles, prepared using either hydrogenous or tail deuterated lipids, mimicking the intracellular membranes and concluded that no interaction occurred between them at different pH (455). Therefore, the precise mechanism of endosomal escape of PAA polyplexes is still ambiguous and further experiments are required to elucidate the mechanisms underlying their ability to mediate gene transfer. The aim of this study is to determine the roles of the ROS in PAAs mediated transfection efficiency and to investigate the mechanism of endosomal escape of PAAs and its effect on transfection efficiency and cytotoxicity.

5.2 Results

5.2.1 *In vitro* transfection efficiency of pDNA polyplexes

The transfection efficiency of various polyplexes was evaluated in A549 cell line in serum-free medium by using the luciferase activity assay. Transfection efficiency of HP->10 polyplexes was increased significantly with increasing RU:Nt ratio to reach the maximum level at 5:1 ratio and then decreased significantly to the lowest level at that of 10:1. In contrast, no significant differences were found among RU:Nt ratios of CP and cross-linked polyplexes (**Figure 5.1 Transfection efficiency measured by luciferase assay 20hfollowing a 4 h cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).**). BPEI achieved a peak transfection activity at 5:1 RU:Nt ratio which was significantly greater than that at 10:1. Although all polyplexes showed superior transfection efficacy compared to the DNA only control, the level seen with BPEI polyplexes was significantly higher than those obtained with the PAA counterparts. However, no significant differences in transfection efficiency were found between the 5:1 ratio of HP-10 polyplexes and the 10:1 ratio of BPEI complexes. CP polyplexes have been excluded from further studies due to their low transfection efficiency.

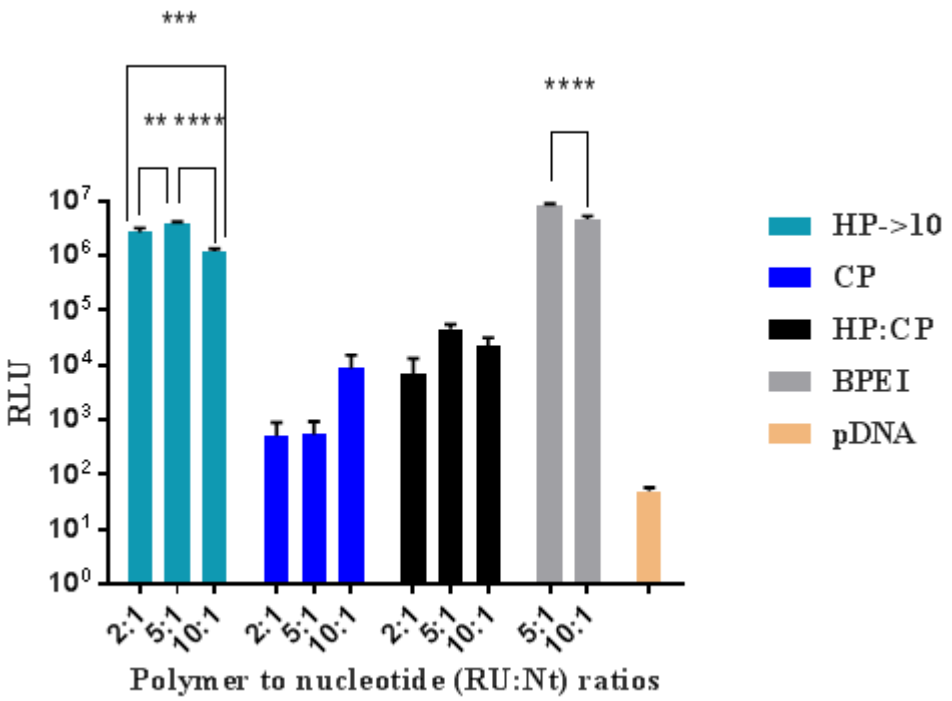


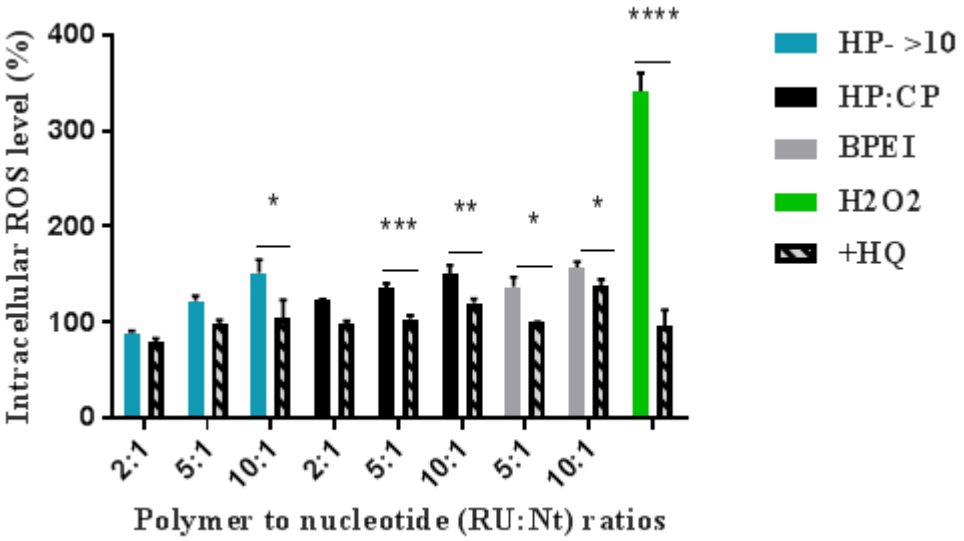
Figure 5.1 Transfection efficiency measured by luciferase assay 20hfollowing a 4 h cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).
Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (**p< 0.01; ***p< 0.001; ****p< 0.0001).

5.2.2 Effect of hydroquinone on reactive oxygen species (ROS) formation, metabolic activity and transfection efficiency

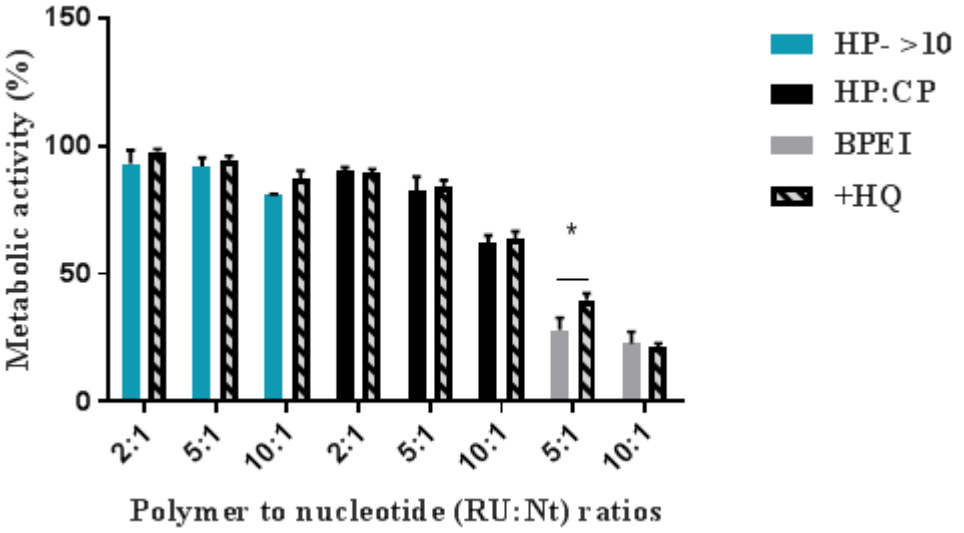
In order to probe the correlation of ROS generation with transfection efficiency and cytotoxicity, A549 cells were pre-treated with HQ. It has been reported that HQ has antioxidant effect with radical scavenging activities (456, 457). When HQ was added to the culture medium, the ROS levels mediated by different RU:Nt ratios of various polyplexes decreased significantly (**Figure 5.2 Effect of HQ on A) Intracellular ROS production B) Metabolic activity and C) Transfection efficiency.A**), indicating that HQ worked as an effective free radical scavenger. Interestingly, the

results in **Figure 5.2 Effect of HQ on A) Intracellular ROS production B) Metabolic activity and C) Transfection efficiency.B** showed that HQ improved the metabolic activity of A549 cells treated with the polyplexes. This improvement appeared significantly at a 5:1 ratio of BPEI, but was marginal for the other polyplexes. Moreover, pre-treatment of cells with HQ caused a significant increase in the transfection efficiency mediated by BPEI at 5:1 and 10:1 RU:Nt ratios, whereas no significant changes were seen in cells transfected with PAA polyplexes (**Figure 5.2 Effect of HQ on A) Intracellular ROS production B) Metabolic activity and C) Transfection efficiency.C**). Cross-linked polyplexes were excluded from further studies due to their lower reproducibility and transfection efficiency.

A)


Intracellular ROS level (%)
400
300
200
100
0
*
***
**
*
*
****
2:1
5:1
10:1
2:1
5:1
10:1
5:1
10:1
Polymer to nucleotide (RU:Nt) ratios
HP- >10
HP:CP
BPEI
H2O2
+HQ

B)


Metabolic activity (%)
150
100
50
0
*
2:1
5:1
10:1
2:1
5:1
10:1
5:1
10:1
Polymer to nucleotide (RU:Nt) ratios
HP- >10
HP:CP
BPEI
+HQ

C)

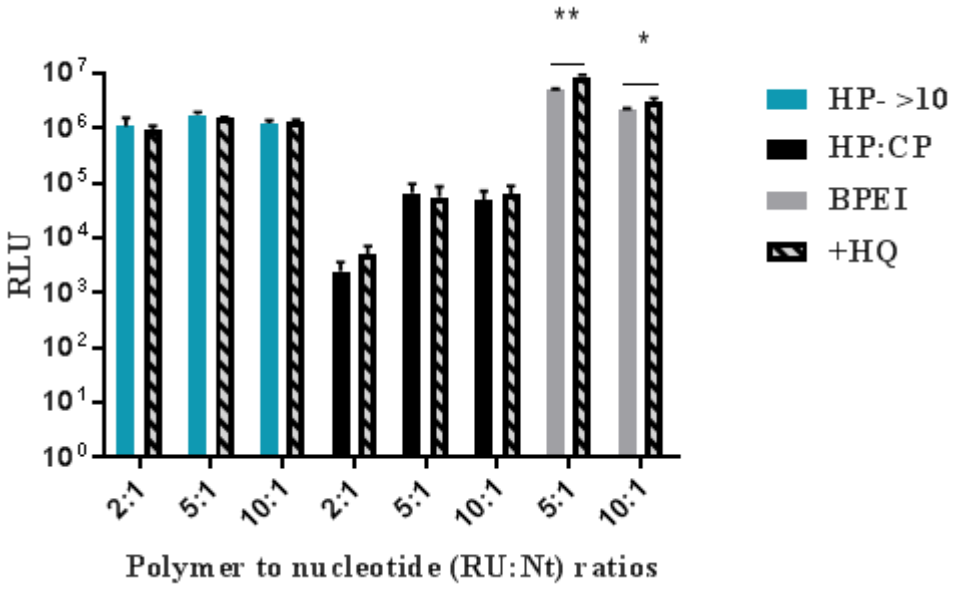


Figure 5.2 Effect of HQ on A) Intracellular ROS production B) Metabolic activity and C) Transfection efficiency.
A549 cells were pre-treated with HQ (0.5 µM) for 30 minutes and then exposed to HP->10, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for an additional 4 hours. Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-test (*P < 0.05; **P< 0.01; ***P < 0.001; ****P < 0.0001).

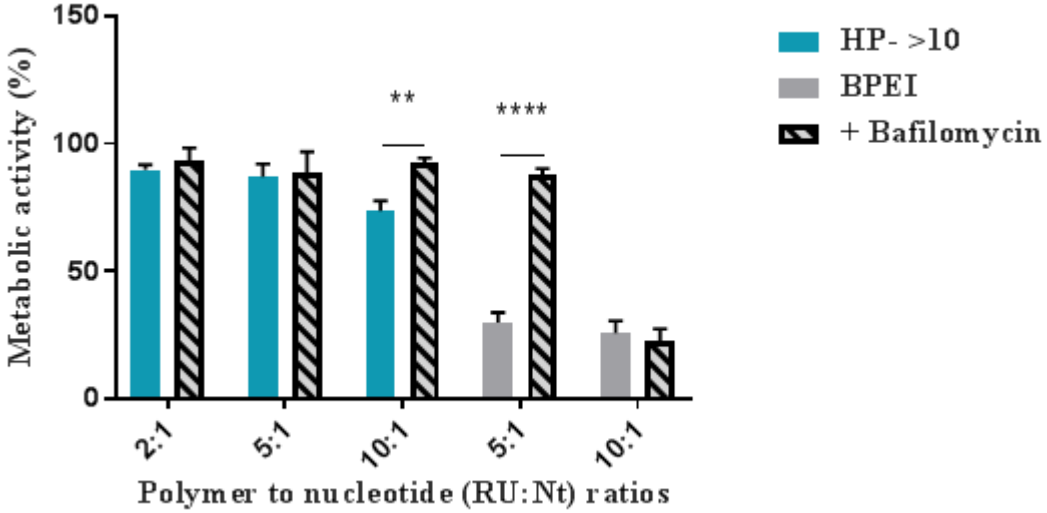
5.2.3 Effects of bafilomycin A1 on transfection efficiency and metabolic activity

To investigate if the endosomal buffering ability of polymers contributed to their endosomal escape and in turn to their cytotoxicity and transfection efficiency, bafilomycin A1 was coincubated with HP->10 and BPEI polyplexes during transfection. Bafilomycin A1 is a specific inhibitor of the Vacuolar-type H^+-ATPase (V-ATPase) and therefore, prevents endosomal acidification (458-460).

Bafilomycin A1 caused a dramatic reduction (> 75%) in the luciferase activity measured following transfection with the PAA polyplexes at all RU:Nt (**Figure 5.3 Effect of bafilomycin A1 (500 nM) on (A) A549 metabolic activityand (B) the**

transfection efficiencyB). Similarly, the transfection efficiency of BPEI polyplexes was significantly decreased by ~50% and ~90 % at a 5:1 or 10:1 ratio, respectively (**Figure 5.3 Effect of bafilomycin A1 (500 nM) on (A) A549 metabolic activityand (B) the transfection efficiencyB**). On the other hand, the metabolic activity of cells transfected with a 10:1 RU:Nt ratio of HP-10 polyplexes and a 5:1 ratio of BPEI counterparts was increased after drug treatment (**Figure 5.3 Effect of bafilomycin A1 (500 nM) on (A) A549 metabolic activityand (B) the transfection efficiencyA**), whereas no significant change observed at the highest ratio of BPEI polyplexes.

A)



B)

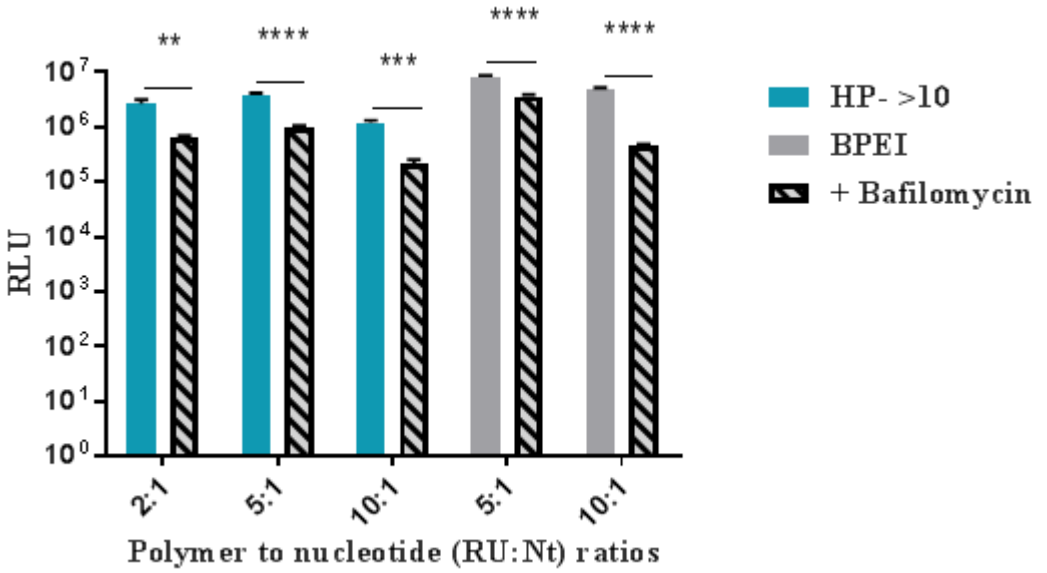


Figure 5.3 Effect of bafilomycin A1 (500 nM) on (A) A549 metabolic activityand (B) the transfection efficiency measured by Presto Blue and luciferase assays, respectively 20h following cell exposure to HP-10 and BPEI pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.
The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance from the control without inhibitor, as determined by two-tailed Student's t-test (**P< 0.01; ***P < 0.001; ****P < 0.0001).

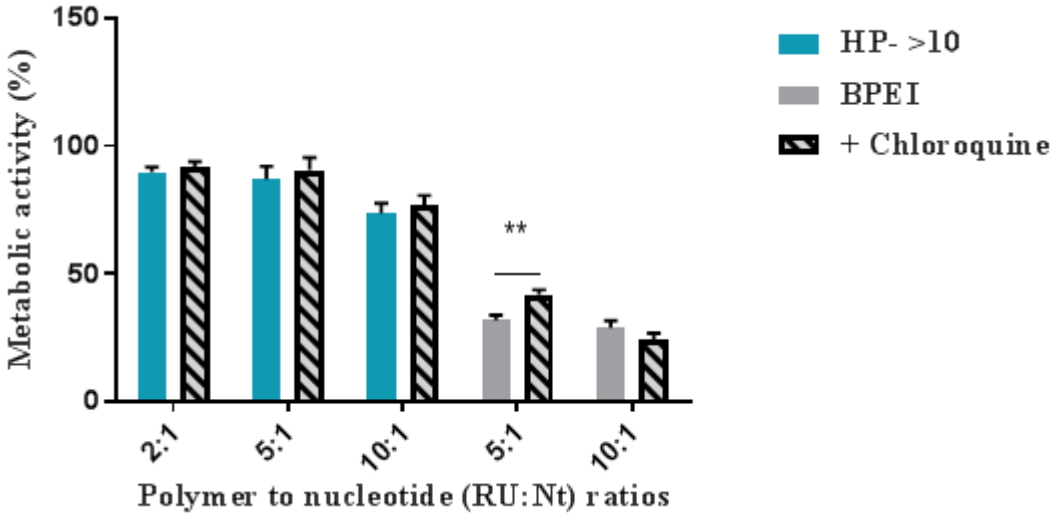
5.2.4 Effects of chloroquine on transfection efficiency and metabolic activity

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 (123-126). As a consequence, it raises their pH (127, 128), impairs the fusion of endosomes and lysosomes, and inhibits the degradative effects of lysosomal enzymes (129).

Co-incubation of the cells with HP->10 polyplexes and chloroquine resulted in a significant increase in the transfection efficiency by 3-fold at 2:1 RU:Nt ratio, whereas the transfection level at 5:1 and 10:1 ratios decreased significantly by 2- or 3.5-fold, respectively (**Figure 5.4 Effect of chloroquine (100 µM) on (A) the metabolic and activity and (B) the transfection efficiencyB**). Again, addition of chloroquine led to a 2.5-fold significant increase in the transfection efficiency of BPEI polyplexes at a 5:1 ratio, whereas no change was observed at the 10:1 ratio (**Figure 5.4 Effect of chloroquine (100 µM) on (A) the metabolic and activity and (B) the transfection efficiencyB**).

As shown in **Figure 5.4 Effect of chloroquine (100 µM) on (A) the metabolic and activity and (B) the transfection efficiencyA**, 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.

A)



B)

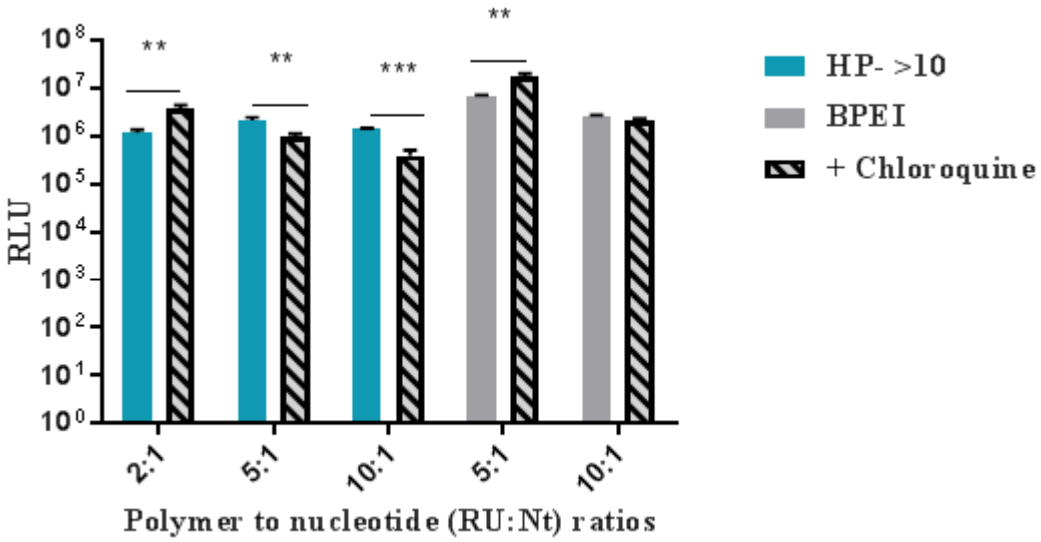


Figure 5.4 Effect of chloroquine (100 µM) on (A) the metabolic and activity and (B) the transfection efficiency measured by Presto Blue and luciferase assays, respectively 20h following cell exposure to HP-10 and BPEI pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.
The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance from the control without inhibitor, as determined by two-tailed Student's t-test (**P< 0.01; ***P < 0.001).

5.3 Discussion

The transfection efficiency is considered another essential factor for assessment of the quality of gene delivery vectors. On the other hand, plasmid DNA (pDNA) quality has an impact on the efficiency of pDNA biopharmaceuticals (461). The process used to produce pDNA can influence its delivery, biological activity, and stability (462). For example, one may hypothesize that (1) the presence of residual amounts of nucleases can impinge on pDNA stability; (2) anionic impurities such as genomic DNA fragments and lipopolysaccharide can change the size, charge, and morphology of complexes formed when packing pDNA with cationic vectors; (3) the ability of pDNA to adsorb to microparticles used for delivery can be compromised by the presence of impurities; and (4) proteins that remain tightly attached to pDNA can hinder the binding of transcription factors. Furthermore, the process steps and materials (e.g., salts, detergents, alcohols) used throughout purification may have an effect on the proportion of the biologically active supercoiled pDNA in the final product and/or irreversibly alter the overall conformation and degree of compactness acquired by the supercoiled isoforms. The latter could subsequently have an impact on the formation of delivery complexes and particles and hence in transfection efficiency (463). The transfection efficiency of pDNA polyplexes was examined by using a luciferase reporter assay. This assay relies on luciferase enzymes, which catalyse the conversion of luciferins to oxyluciferin and lead to emission of light (464, 465). The reporter genes mainly used in transfection experiments are based on either fluorescence such as the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, or luminescence including firefly luciferase (FLuc) from *Photinus pyralis* (466, 467). GFP is autofluorescent and nonenzymatic compared to luciferase, so can be easily visualized, without a substrate being required. (468, 469). On the other hand, FLuc is widely used

in bioassays because excitation light energy is not required for luminescence compared to fluorescence. This leads to a lower background signal and a more sensitive assay. Another advantage from the lack of excitation light source is the absence of interference from compound fluorescence and fluorophore photobleaching (470). Furthermore, FLuc can be utilized for studying dynamic changes in gene expression due to shorter half-lives (3 hr) of their intracellular protein compared to GFP (26 hr) (471). However, the signal intensity in luminescence assays is weaker than that in fluorescence based assays (472). As a result of these properties, FLuc has been widely employed to report gene delivery (473), and gene silencing (474).

The transfection efficiency of HP->10 polyplexes was higher than that of CP08 and the cross-linked polyplexes for the same RU:Nt ratios. On the other hand, BPEI polyplexes achieved higher transfection efficiency than PAA polyplexes. However, BPEI mediated highly efficient transfection when cell metabolic activity was around 30% of the control (**Figure 4.4 Metabolic activity measured by Presto Blue assay 20h followingcell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.**) indicating its ability to hijacks resources of the cell for expression of the transgene at the expense of native expression levels, whereas HP->10 polyplexes showed maximum transfection activity when cells maintained around 90% of their metabolic activity (**Figure 4.4 Metabolic activity measured by Presto Blue assay 20h followingcell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.**). Moreover, transfection efficiency of HP->10 polyplexes at the optimal ratio was not accompanied by apoptosis initiated by reduction of MMP (**Figure 4.7 Evaluation of MMP by JC-1 dye 24 h following A549cell exposure to HP->10, CP, BPEI and cross-linked**

pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.) and generation of ROS (**Figure 4.8 Evaluation of intracellular ROS production by A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 2 hours.**) compared to BPEI counterparts. The lower transfection efficiency of PEGylated polyplexes may be related to the presence of PEG which reduced the surface charge of the polyplexes and in turn leads to poor cell association and internalization in addition to reduced endosomal escape (362). Although, HP->10 and BPEI polymers have comparable particle size and zeta potential at the optimal ratio (5:1), they exhibited differences in transfection efficiency which indicates that these variations are not related to the physicochemical properties of both complexes but can be attributed to the structure of the polymer. At the highest RU:Nt ratio (10:1), luciferase gene expression after transfection with HP->10 and BPEI polyplexes decreased. This reduction could be due to the condensation of pDNA into more compact complexes which may limit unpacking or the higher cytotoxicity observed at that ratio, consequently reducing the number of cells expressing the transgene (475).

One of the potential cause of cell death is oxidative stress which can occur across different cell types and treatments (476). In order to probe the correlation of ROS generation with transfection efficiency and cytotoxicity, A549 cells were pre-treated with HQ. It has been reported that HQ has antioxidant effect with radical scavenging activities (456, 457). Co-treatment with HQ counteracted the rise in ROS levels mediated by both PAA and BPEI polyplexes. However, the metabolic activity and transfection efficiency were only increased significantly in cells transfected by the BPEI polyplexes, reflecting an increase in the number of viable cells that expressed the luciferase reporter gene as a result of an improvement in antioxidant defence after

addition of HQ. These results reveal the role of ROS generated by BPEI in cell damage which in turn decreased the transfection efficiency. This is indicative of an impairment of redox homeostasis mediated by BPEI polyplexes compared to their PAA counterparts which probably induce a ROS generation that is transient and managed by the intracellular glutathione. In terms of the effect of antioxidant on the transfection efficiency of BPEI, our results were similar to those previously showed by Hall *et al*. (420).

The mechanism behind the transfection efficiency of HP->10 polyplexes could be elucidated by using bafilomycin A1. Bafilomycin A1 is a specific inhibitor of the Vacuolar-type H+-ATPase (V-ATPase) found in early endosomes (458, 459) and therefore prevents their acidification and the formation of late endosomes (460). It has been shown to prevent the endosomal escape of cationic vectors by inhibiting their protonation (130). Bafilomycin A1 caused a significant reduction in the transfection efficiency of HP-10 and BPEI polyplexes at all RU:Nt ratios. This reduction was not related to cytotoxic effects of bafilomycin A1 as revealed by metabolic activity results. It indicates instead the crucial role of endosomal acidification in the transfection efficiency of both PAA and BPEI polyplexes. One possible interpretation is that the proton sponge effect mediates the endosomal escape of the vectors. However, bafilomycin A1 caused a lower reduction in the transfection efficiency of BPEI at 5:1 RU:Nt ratio compared to that obtained with the PAA polyplexes. These variations could be related to the differences in the pKa values of PAA and BPEI. The pKa values for PAA with p(MBA-DMEDA) backbone has been previously calculated as pKa1= 8.1 and pKa2 = 4.5 (205), whereas the pKa values of BPEI are 4.5 for primary, 6.7 for secondary, and 11.6 for tertiary amine groups (477). Protonation of amine occurs when the pH value of the environment is below the pKa value of the specific amines present

in the polymer. Hence at physiological pH (or in the presence of bafilomycin A1) and acidic pH, higher percentage of amines in BPEI was protonated in comparison to PAA and therefore more BPEI polyplexes escaped from the endosomes. Moreover, the BPEI repeating unit has a much lower molecular weight than that of PAAs (~43 vs ~326 Da) and features four independent protonable groups, whose protonation is affected by the protonation state of neighbouring sites (478). 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. On the other hand, bafilomycin A1 led to a significant reduction in the cytotoxicity of PAA and BPEI polyplexes at 10:1 and 5:1 RU:Nt ratio, respectively. This increase in the metabolic activity at these RU:Nt ratios indicates that their cytotoxic effects mainly originate from their endosomal escape. However, the reduction in metabolic activity caused by BPEI at 10:1 RU:Nt ratio was not reversed indicating that the cytotoxicity induced at this ratio is irreversible. 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 (203, 219). 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 (204, 206), although the overall positive charge of the polymers at the lower pH might also contribute to cell membrane destabilisation (478). PLL and PEI, on the other hand, showed haemolytic properties at both a neutral and acidic pH (204, 206). However, disruption of the lysosomal membrane by lysis or proton sponge effect will cause accidental cell death due to release of the lysosomal enzymes into the cytoplasm which in turn trigger apoptosis (479), whereas PAA polyplexes did not induce significant reduction in the mitochondrial membrane potential (MMP) compared to BPEI counterparts as previously shown in chapter three, indicating no initiation of

apoptosis by PAAs. Depending on the cytotoxicity data collected in this study we can suggest that HP->10 polyplexes escaped from the lysosomes through formation of pores in their membrane that might close shortly after release of polyplexes into the cytoplasm, therefore, they are less deleterious to the cell compared to BPEI counterparts. This explanation was in agreement with results obtained by Richardson *et al*. who revealed that addition of PAAs to isolated lysosomal vesicles in a suspension medium designed to minimise osmotic breakage did not increase their permeability in comparison to BPEI (480). This indicates that PAA might only interact with the cell membrane at a low pH, i.e., only when inside the endo/lysosomes while BPEI does it over a range of pH. Once released in the cytoplasm, BPEI interacts with other organelles, triggering apoptosis while PAA does not.

The effect of endolysosomal trafficking on the transfection efficiency of polyplexes was further investigated by using chloroquine. 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.8), which indicates that under control transfection conditions, a significant fraction of the vectors are trafficked from the endosomes to the lysosomes where pDNA is likely degraded by enzymes and acidic medium. 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 or due to inhibition of vesicle fusion by chloroquine and therefore the polymers weren't getting to the endolysosome. Surprisingly, chloroquine had a negative impact on the transfection efficiency of PAA polyplexes at higher RU:Nt without affecting their cytotoxicity (Fig.8). 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 permeabilization and/or DNA decomplexation.

5.4 Conclusion

Although PAA polyplexes achieved lower transfection than BPEI counterparts, their transfection efficiency was not associated with cytotoxicity. Generation of ROS did not contribute to the cytotoxicity and transfection efficiency of PAA in comparison to BPEI. Transfection efficiency of PAA polymers rests upon pH-dependent releases from both the early endosomes and late endosomes/lysosomes. Moreover, PAA polyplexes that have excess polymer require progression to a lower pH vesicle for DNA decomplexation.

6 General Discussion

6.1 Introduction and proposed hypothesis

The principal aim of this project was to increase understanding of linear polyamidoamine (PAA) promising behaviour in gene delivery by investigating cellular events occurring upon transfection with DNA polyplexes. PAA are particularly promising drug carriers due to their low cytotoxicity compared to other synthetic polymers such as poly-L-lysine (PLL) (481). However, a previous study conducted by Hill *et al*. showed that NG30, a PAA based on methylene bisacrylamide-dimethylethylene diamine (MBA-DMEDA), led to higher transfection efficiency and cytotoxicity towards A549 compared to other PAAs with different structures (205). While it has been suggested the ability of conventional cationic polymers to promote transgene expression is linked to their cytotoxicity (447, 482), certain members of the PAA class of polymers, such as the MBA-DMEDA structure (209) and the ISA series (204, 483), 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 polymers. We hypothesized that PAAs may be interfering with the cell membrane and affect mitochondrial membrane potential (MMP). Furthermore, PAAs could cause an increased production of reactive oxygen species (ROS).

For this purpose, pDNA polyplexes were prepared by using PAAs based on MBA-DMEDA including a PAA homopolymer (HP >10 kDa) and aPAA copolymer (CP) and a cross-linked system or BPEI 25 kDa. Comparative physicochemical properties

of pDNA polyplexes were investigated using simple techniques including gel electrophoresis, dynamic light scattering and zeta potential respectively (Chapter 3). A series of *in vitro* experiments were conducted in order to evaluate the biological effects of the PAA complexes in comparison to their BPEI counterparts. Cytotoxicity was evaluated in cultured cells both in terms of cell viability and membrane integrity, as well as effects on mitochondrial health and formation of reactive oxygen species (Chapter 4). To elucidate a link between endosomal escape and transfection efficiency, bafilomycin or chloroquine were incubated with pDNA polyplexes, whereas the effect of ROS generation on transfection efficiency was investigated by using hydroquinone as antioxidant (Chapter 5).

6.2 Synopsis of findings

All polymers showed good incorporation of pDNA during gel electrophoresis (**Error! Reference source not found.Error! Reference source not found.**) and produced polyplexes in a size range (Figure 3.4 Hydrodynamic diameter (nm) measurements for HP->10, CP, BPEIand cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).) suitable for cellular uptake. Moreover, zeta potential measurements show that pDNA polyplexes were positively charged (Figure 3.5 Zeta potential measurements for HP->10, CP, BPEIand cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).), but the surface charge potential of PEGylated PAA polyplexes was found to be less than that of non-PEGylated PAA and BPEI polyplexes.

PAA polyplexes did not induce cytotoxicity such as cell membrane disruption (**Figure 4.6 LDH released from A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.**) or mitochondrial depolarization (**Figure 4.7 Evaluation of MMP**

by JC-1 dye 24 h following A549cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.) in contrast to the BPEI counterparts*.* Furthermore, HP->10 and CP polyplexes only increased ROS levels at the highest RU:Nt ratio compared to the cross-linked and BPEI counterparts (**Figure 4.8 Evaluation of intracellular ROS production by A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 2 hours.**). At the optimal RU:Nt ratio of 5:1, a higher expression of the luciferase gene was observed when A549 cells were transfected with BPEI polyplexes than with their PAA counterparts (**Figure 5.1 Transfection efficiency measured by luciferase assay 20hfollowing a 4 h cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).**). However, PEGylated complexes showed significantly decreased transfection efficiency compared to complexes formed from HP->10. This reduction in the transfection efficiency of PEGylated polyplexes could be related to the presence of PEG. PEGylation offers a great advantage for bioactive molecules in pharmaceutical and biological applications by way of reducing protein immunogenicity and increased serum half-life of the drugs (484). However, PEGylation also brings the PEG dilemma, which is difficult for the cellular absorption and subsequent endosomal escape, therefore, results in significant loss of activity of the delivery system (485). BPEI mediated efficient transfection (**Figure 5.1 Transfection efficiency measured by luciferase assay 20hfollowing a 4 h cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).**) and high cytotoxicity (**Figure 4.4 Metabolic activity measured by Presto Blue assay 20h followingcell exposure to HP->10, CP, BPEI and cross-linked**

pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.) at the optimal ratio despite the absence of free polymer. On the other hand, HP->10 polyplexes exhibited maximal transfection activity and minimal cytotoxicity at the optimal ratio in spite of the presence of an excess of PAA polymer at that ratio (**Figure 4.4 Metabolic activity measured by Presto Blue assay 20h followingcell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.** and **Figure 5.1 Transfection efficiency measured by luciferase assay 20hfollowing a 4 h cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).**).

LDH assay (**Figure 4.6 LDH released from A549 cells following exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.**) revealed that HP->10 and BPEI polyplexes did not mediate transfection efficiency and/or cytotoxicity at the optimal (5:1) RU:Nt ratio by direct cell membrane permeabilization. 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 (366, 486, 487).

Transfection of A549 cells in presence of the Vacuolar-type H+-ATPase (V-ATPase) pump inhibitor bafilomycin A1 showed the important role for the acidification of the endosomes in the transfection of both BPEI and HP->10 polyplexes (**Figure 5.3 Effect of bafilomycin A1 (500 nM) on (A) A549 metabolic activityand (B) the transfection efficiencyB**). However, the reduction in the transfection efficiency of HP->10 complexes is more than that of BPEI (**Figure 5.3 Effect of bafilomycin A1 (500 nM) on (A) A549 metabolic activityand (B) the transfection efficiencyB**) indicating its lower charge density compared to BPEI.

HP->10 polyplexes at a 2:1 RU:Nt and BPEI polyplexes at a 5:1 ratio showed increased transfection in presence of chloroquine (**Figure 5.4 Effect of chloroquine (100 µM) on (A) the metabolic and activity and (B) the transfection efficiencyB**), indicate trafficking of these polymers to the lysosomes under normal conditions which has negative impact on their efficacy. On the other hand, chloroquine caused reduction in the transfection efficiency of HP->10 polyplexes at higher RU:Nt ratios (**Figure 5.4 Effect of chloroquine (100 µM) on (A) the metabolic and activity and (B) the transfection efficiencyB**) the necessity of movement of these vectors to the lysosomes where the lower pH allow them to accept more protons and as a result conformational changes and DNA unpacking occur.

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 (204, 206, 225, 228).

6.3 Contribution to the field of gene delivery

Understanding the mechanisms and pathways underlying toxicity associated with polyplex-mediated gene delivery could provide insight into the design of new polymers with a safer profile. We have successfully confirmed negligible cytotoxicity associated with PAA polyplex-mediated gene delivery using three different methods *in vitro*. It appeared that these polymers are safe and effective in *in vitro* A549 cell line and could open new and unexplored perspectives for the pharmaceutical industry

in areas such as gene delivery and, more importantly, provide a therapeutic approach for many untreatable diseases.

6.4 Limitations

One of the main limitations of the present research was being unable to establish an association between ROS generation and transfection efficiency of PAA polyplexes due to lacking cell line co-transfected with NF-Kb. Another limitation is that only one cell line was employed in this study and it is not known if similar observations could be made in other cell types. Furthermore, cross-linked polyplexes did not show reproducible data, therefore, this system cannot be used effectively in transfection *in vivo*.

6.5 Conclusion

In general, PAA polyplexes showed lower cytotoxicity than BPEI counterparts. The polymers with the highest transfection efficiency are the most toxic, as demonstrated herein by BPEI. However, there is no link between transfection efficiency and cytotoxicity of PAA polyplexes. It can be assumed that the presence of free polymer in PAA polyplexes has an essential role in the course of transfection. In addition, the presence of PEG led to a significant decrease in the transfection efficiency.

6.6 Future work

Although PAA polyplexes showed lower cytotoxicity during this study, further experiments on polyplex interaction with other organelles is necessary to make definite conclusions on this matter, since polyplexes are present throughout cells 4 h after transfection and could potentially interact with a myriad of intracellular processes. Moreover, the precise mechanisms by which PAAs are initially taken up by the cell and how exactly the release of the polyplexes from the endosome takes place need to be investigated. Similarly, more knowledge is required regarding the mechanisms by which the delivered DNA finally crosses the nuclear membranes, its nuclear entry. The lower transfection efficiency of cross-linked system is of considerable concern, and there would appear to be significant opportunities to increase their overall level of transfection by optimising these properties. This could be linked to the development of targeted systems, via covalent coupling of ligands to one of the polymer components. The targeting ligands that have been widely used are transferrin, which specifically targets transferrin receptors that are overexpressed in rapidly dividing tissues, such as tumours (224, 488), and folic acid that specifically target folate receptors, which are also overexpressed in proliferating tissues (489, 490). It is hoped that such modifications will compensate for the reduction in the cell uptake caused by PEG incorporation, which led to reduction in surface charge. Histidine residues could be added to the polymers to improve endosomal escape as a means to optimize transfection. The imidazole groups of histidine (pKa ~6) likely absorbed protons in the acidic environment (pH 5-6.5) of the endosome, leading to osmotic swelling, membrane disruption and gene escape (491). However, time restrictions and lack of financial resources limit these investigations which could not be completed during this PhD project.

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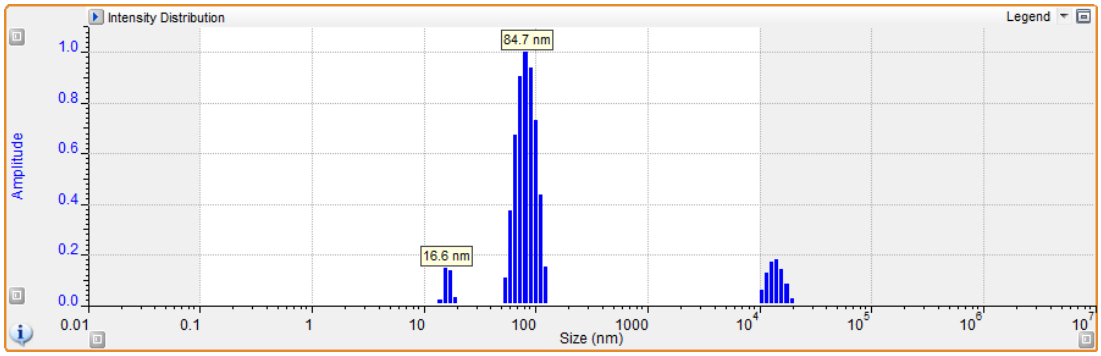
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Appendices

Appendix1: DLS analysis of pDNA polyplexes

a)



b)

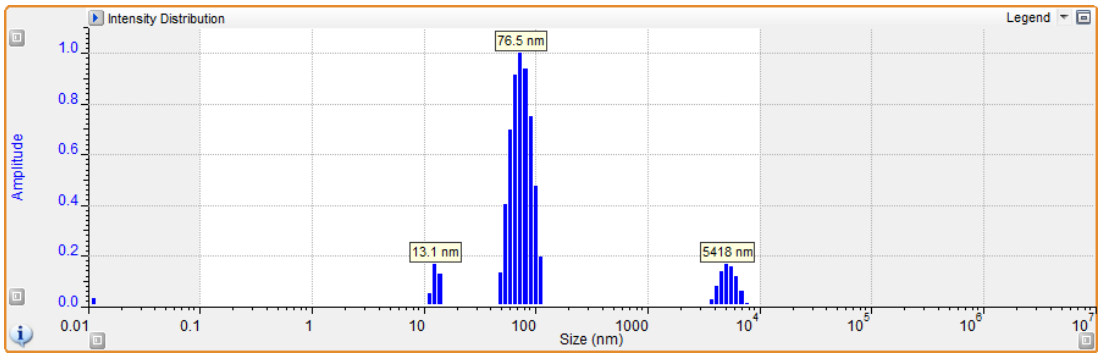
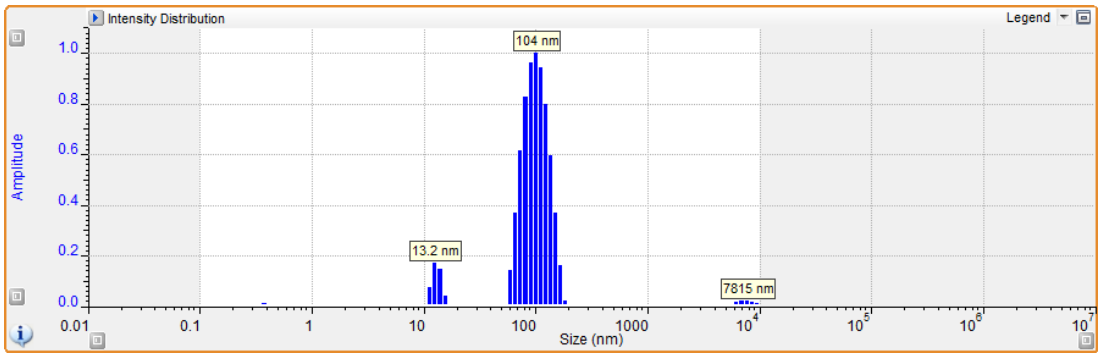
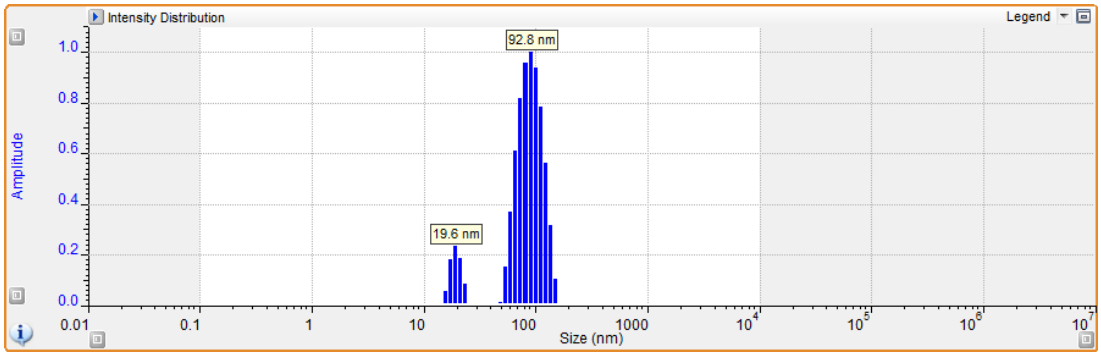


Figure 1.1 DLS analysis of BPEI polyplexes at RU:Nt ratios of a) 5:1 and b) 10:1

a)



b)



c)

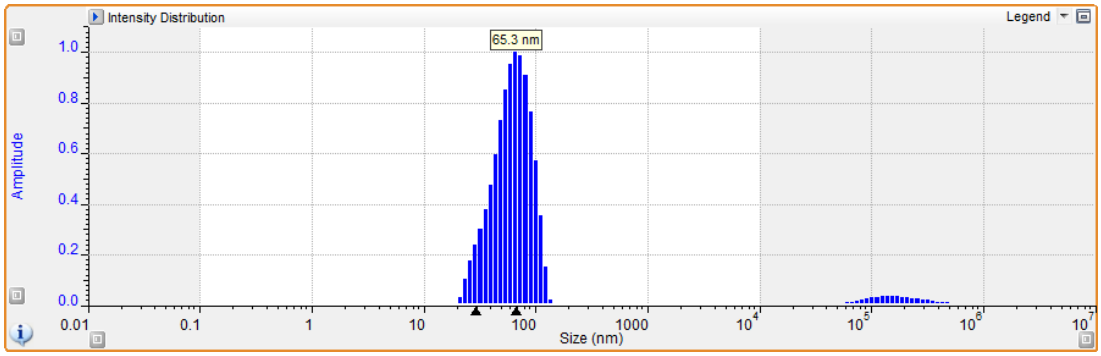
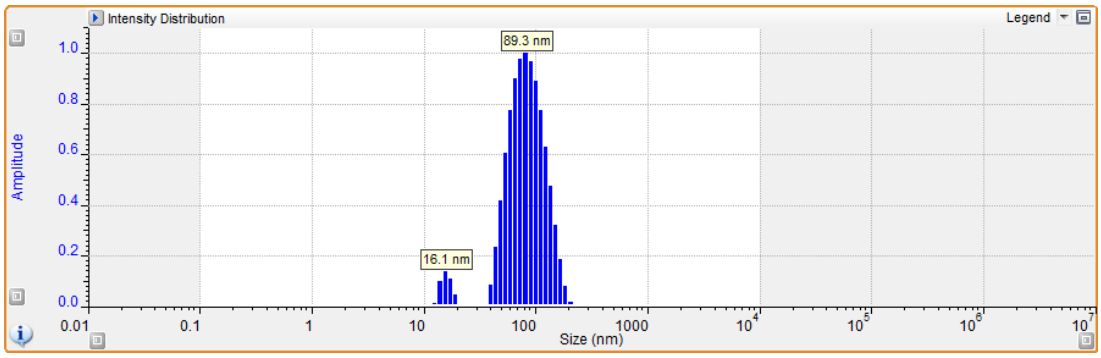
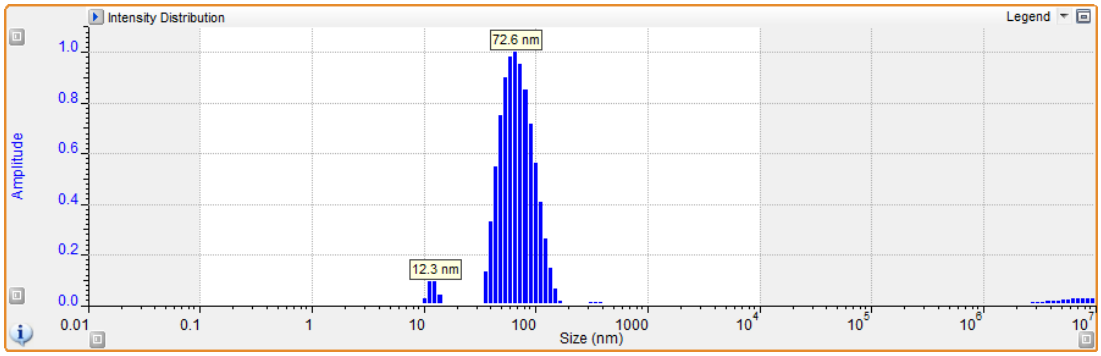


Figure 1.2 DLS analysis of HP->10 polyplexes at RU:Nt ratios of a) 2:1 b) 5:1 and c) 10:1.

a)



b)



c)

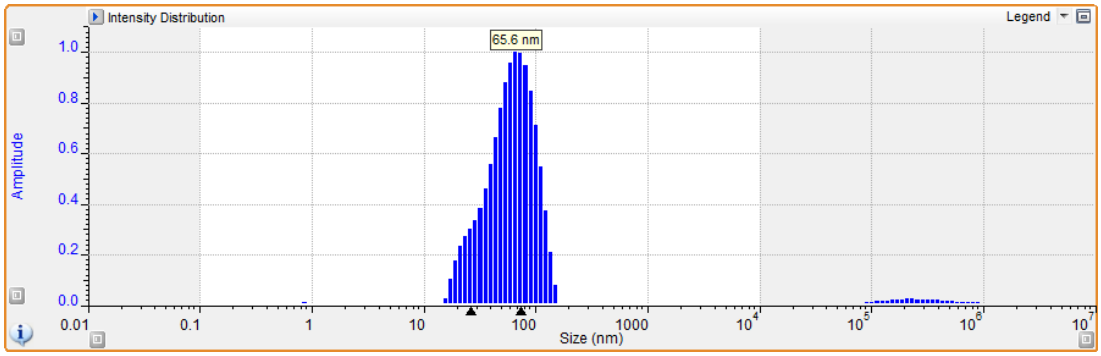
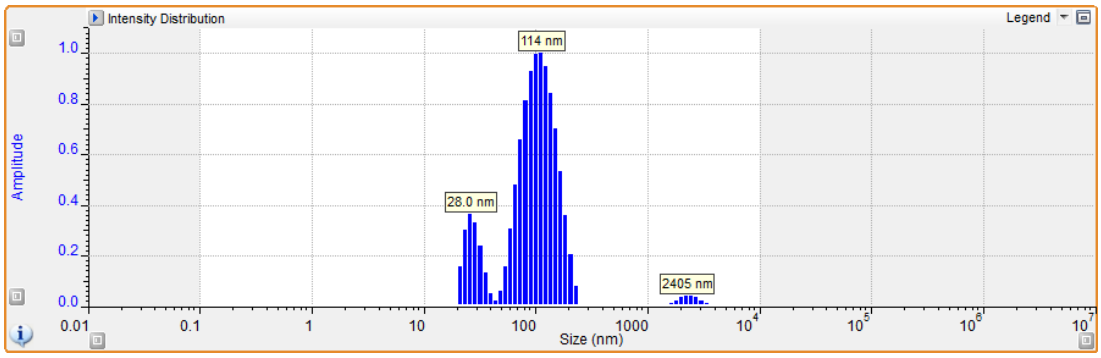
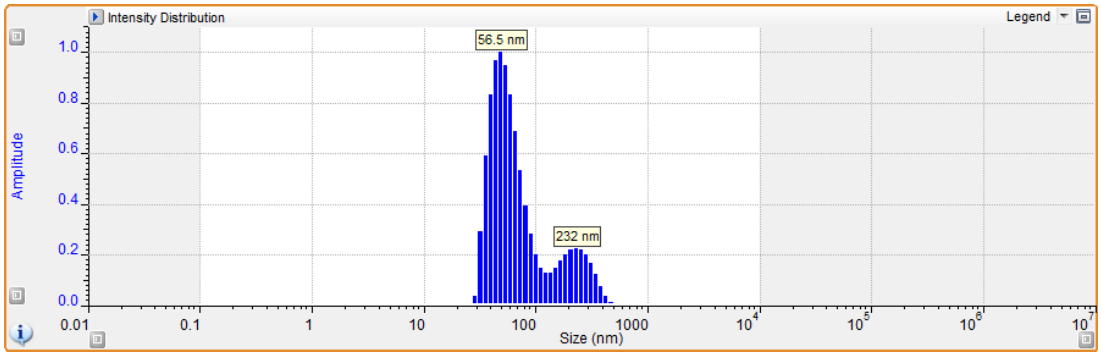


Figure 1.3 DLS analysis of CP08 polyplexes at RU:Nt ratios of a) 2:1 b) 5:1 and c) 10:1.

a)



b)



c)

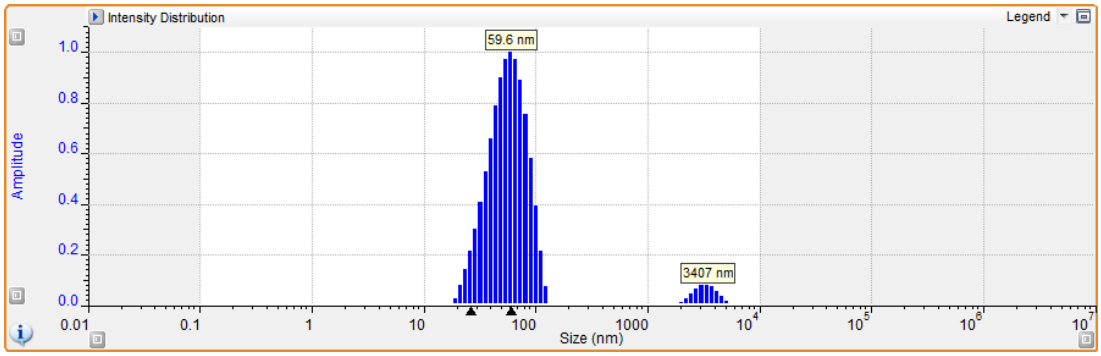
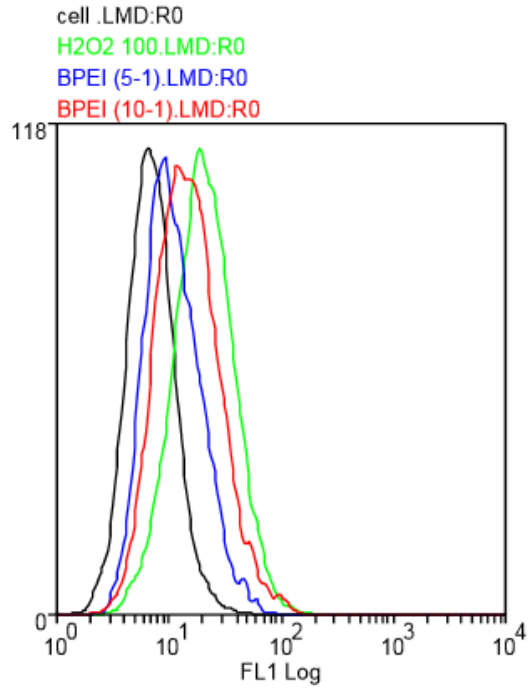


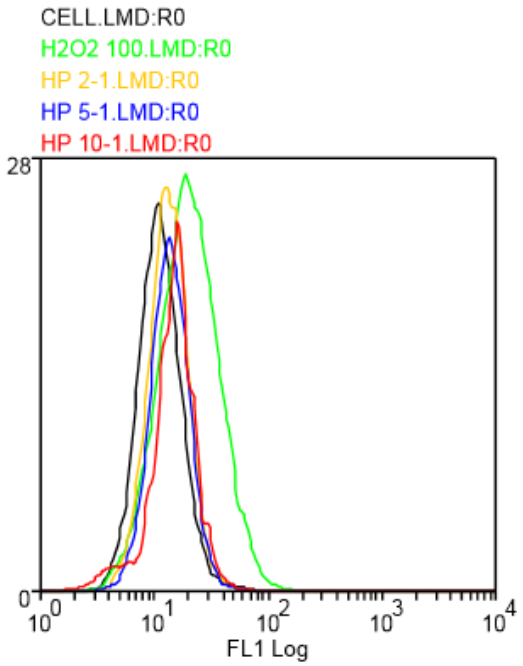
Figure 4 DLS analysis of HP->10: CP08 cross linked polyplexes at RU:Nt ratios of a) 2:1 b) 5:1 and c) 10:1 and HP: CP ratios of 2:1.

Appendix 2: Histograms of reactive oxygen species generation

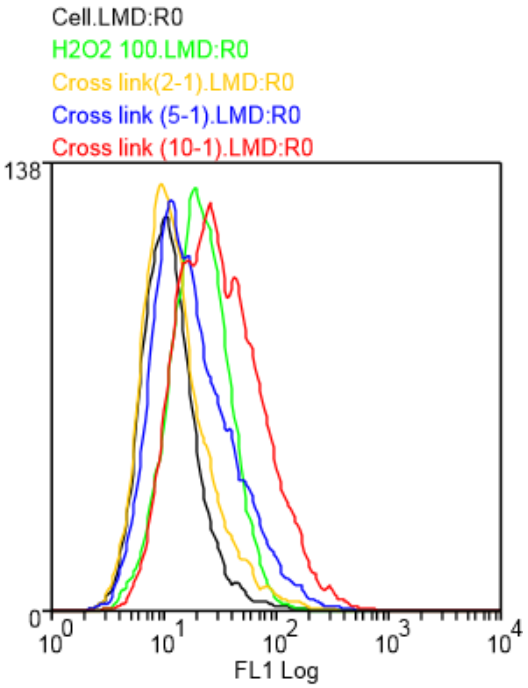
a)



b)



c)



d)

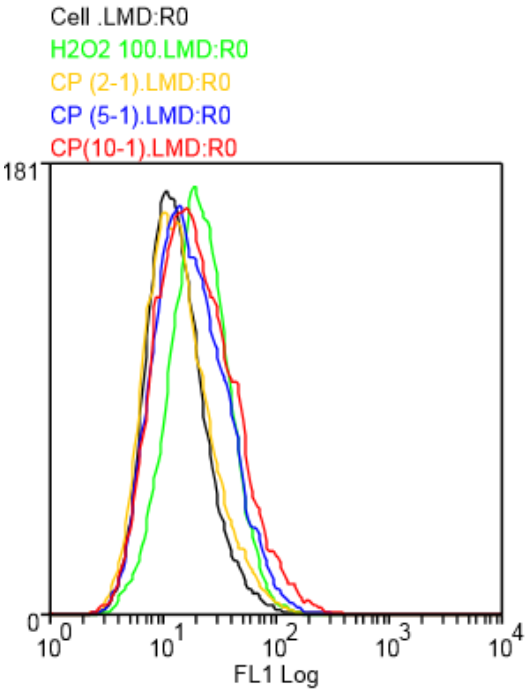
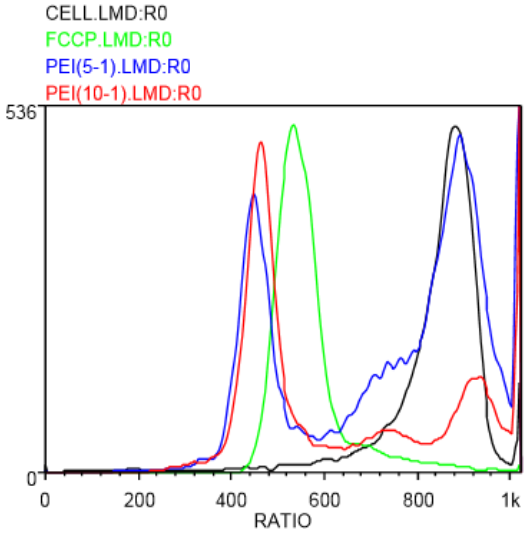


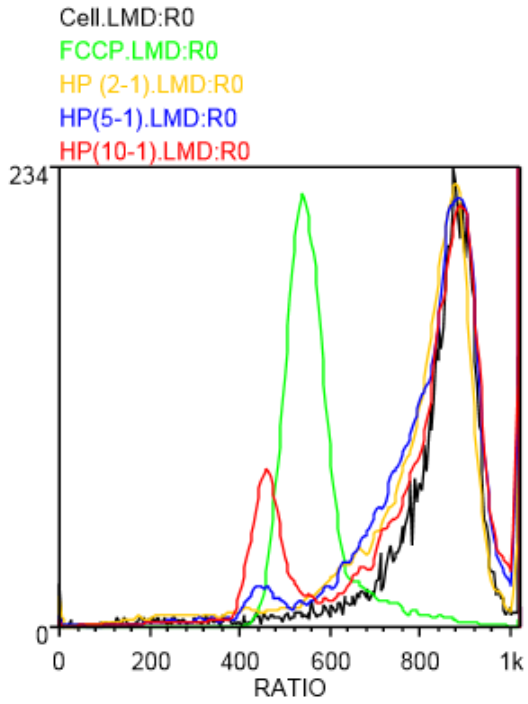
Figure 2.1 Flow cytometry analysis of ROS generation in A549 cells incubated with a) BPEI b) HP->10 C) HP->10: CP cross linked polyplexes d) CP polyplexes at RU:Nt ratios of a) 2:1 b) 5:1 and c) 10:1.
The fluorescence intensity of H2DCFDA was measured in the FL1 channel for green fluorescence using FITC emission.

Appendix 3: Flow cytometric analysis of mitochondrial membrane potential

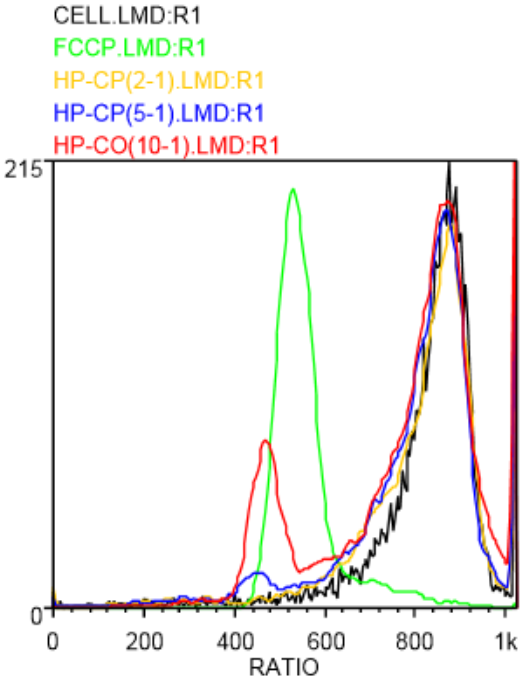
a)



b)



c)



d)

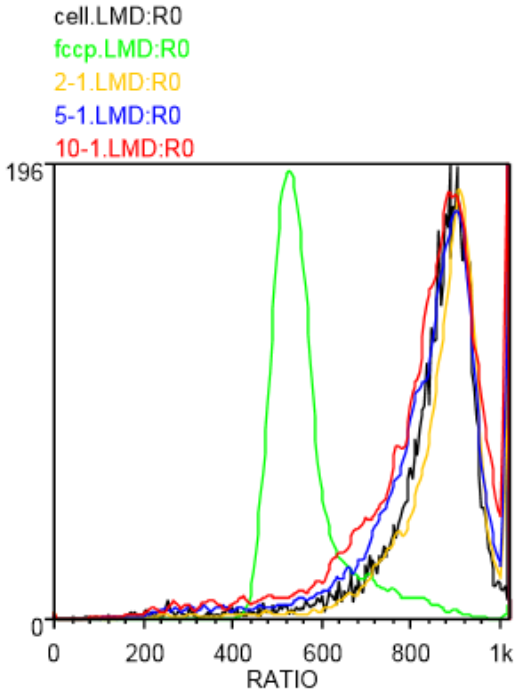


Figure 2.1 Flow cytometry analysis of MMP in A549 cells incubated with a) BPEI b) HP->10 C) HP->10: CP cross linked polyplexes d) CP polyplexes at RU:Nt ratios of a) 2:1 b) 5:1 and c) 10:1.

The fluorescence intensity of JC-1 aggregates was measured in the FL2 channel for red fluorescence and JC-1 monomers in the FL1 channel for green fluorescence.

Appendix 4

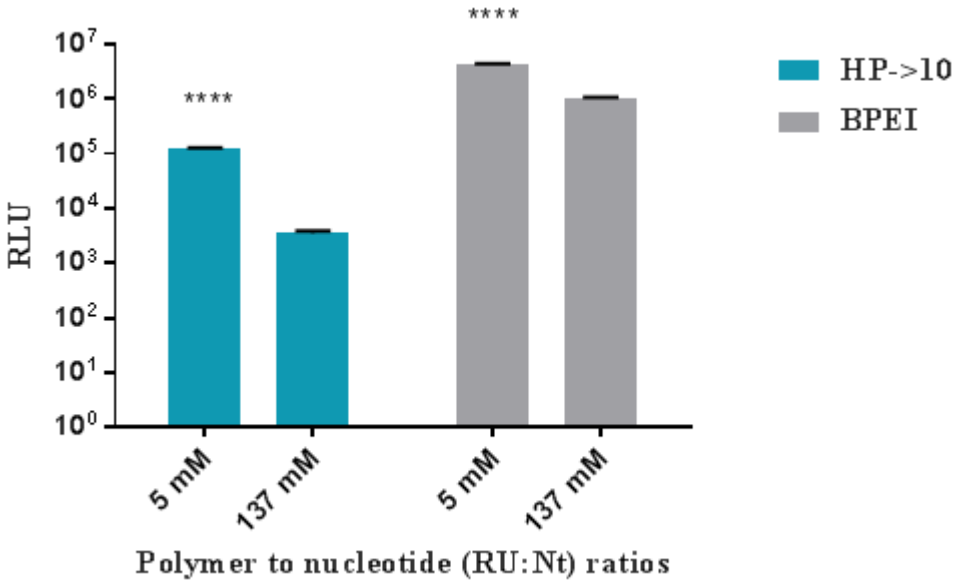


Figure 1 Transfection efficiency measured by luciferase assay 44 h following a 4 h cell exposure to HP->10 and BPEI pDNA polyplexes prepared at RU:Nt ratio 5:1 by using 5 mM and 137 mM PBS.
The data are expressed as mean ± SD (3 replicates, n=1). * indicates statistical significance from the control without inhibitor, as determined by two-tailed Student's t-test (****p< 0.0001).

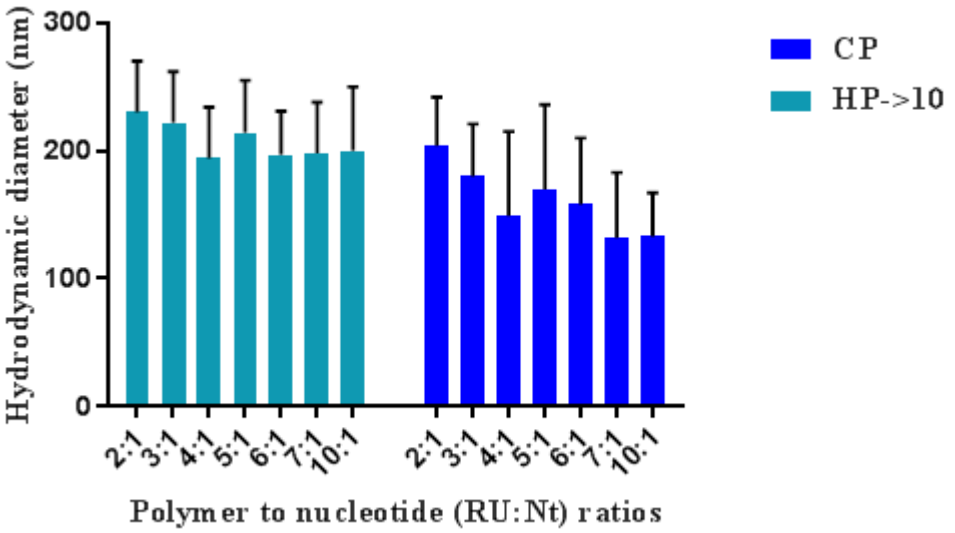


Figure 2 Hydrodynamic diameter (nm) measurements for pDNA polyplexes prepared by using HP->10 and CP at different RU:Nt ratios.
Each measurement represents the mean ± SD of 10 readings obtained from duplicate samples.

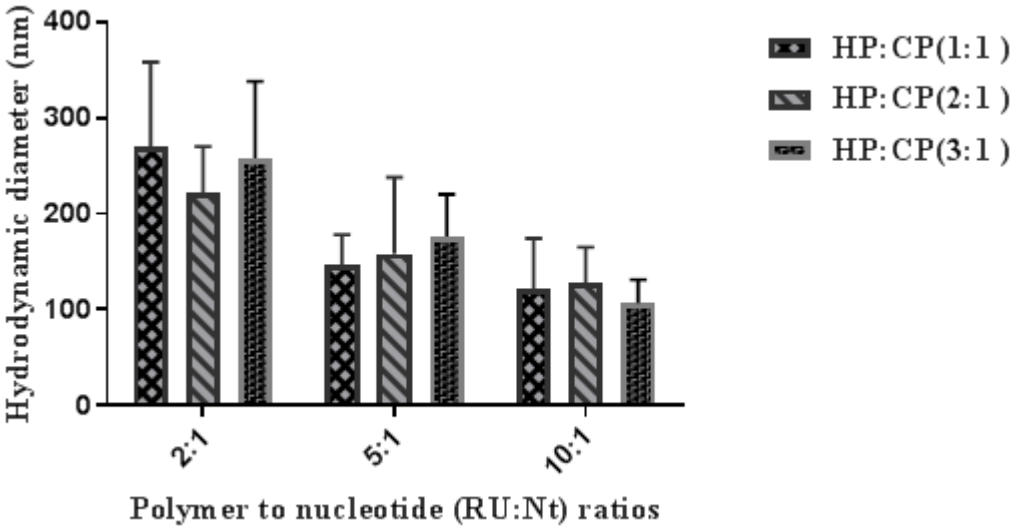


Figure 3 Hydrodynamic diameter (nm) measurements for cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at RU:Nt ratios of 2:1, 5:1 and 10:1 and HP:CP ratios of 1:1, 2:1 and 3:1.
Each measurement represents the mean ± SD of 10 readings obtained from duplicate samples.

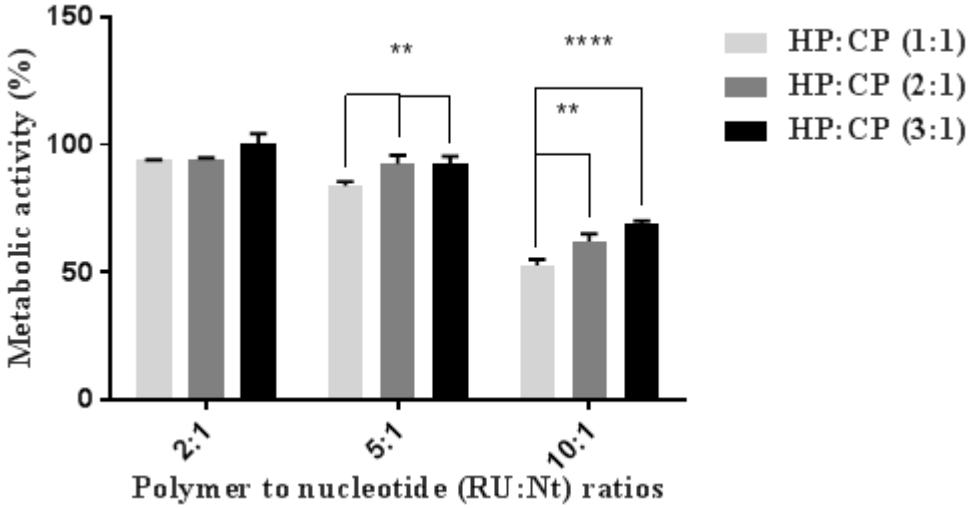


Figure 4 Metabolic activity measured by Presto Blue assay 20h following cell exposure to cross-linked pDNA polyplexes for 4 hours.
Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of (1:1, 2:1 and 3:1) and different RU:Nt ratios (2:1, 5:1 and 10:1). The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (**p< 0.01; ****p< 0.0001).

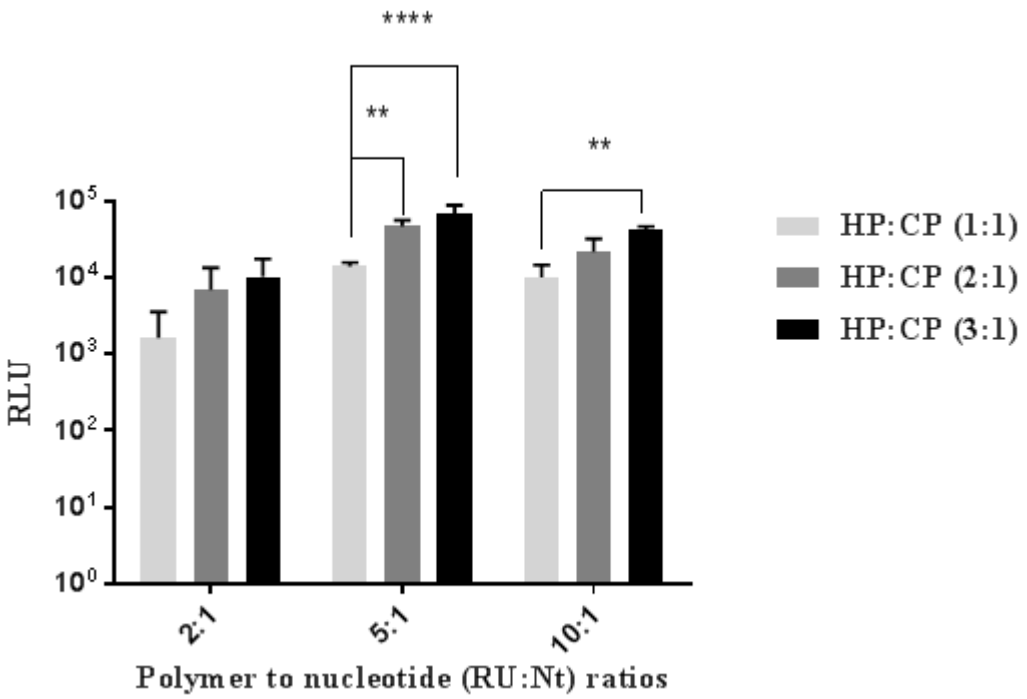


Figure 5 Transfection efficiency measured by luciferase 20h following cell exposure to cross-linked pDNA polyplexes for 4 hours.
Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of (1:1, 2:1 and 3:1) and different RU:Nt ratios (2:1, 5:1 and 10:1). The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (**p< 0.01; ****p< 0.0001).

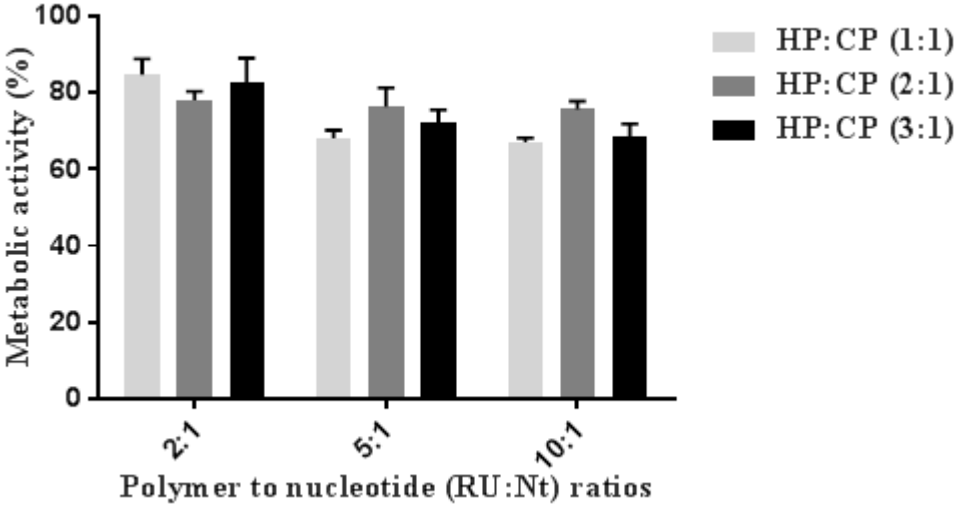


Figure 6 Metabolic activity measured by Presto Blue assay 44h following cell exposure to cross-linked pDNA polyplexes for 4 hours.
Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of (1:1, 2:1 and 3:1) and different RU:Nt ratios (2:1, 5:1 and 10:1). The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test.

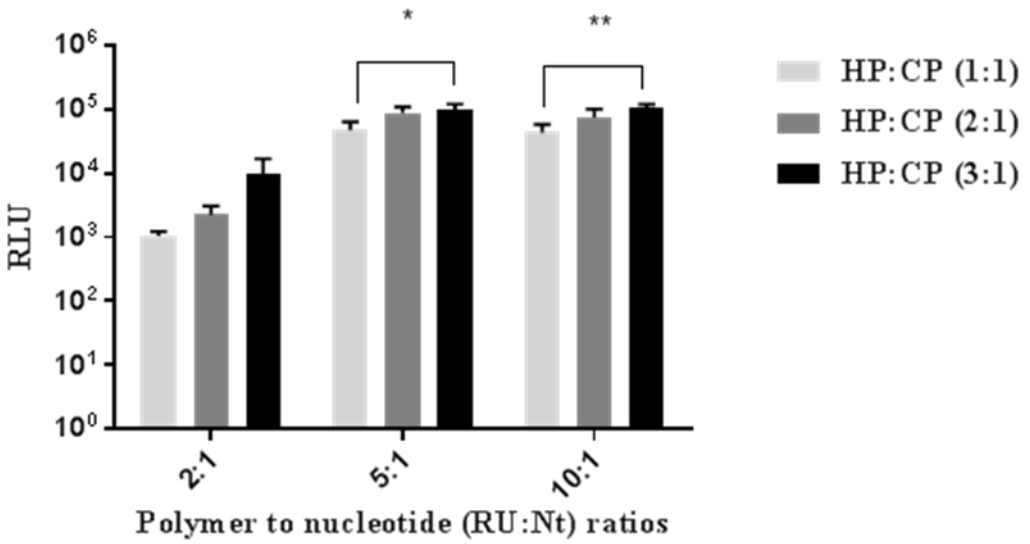


Figure 7 Transfection efficiency measured by luciferase 44h following cell exposure to cross-linked pDNA polyplexes for 4 hours.
Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of (1:1, 2:1 and 3:1) and different RU:Nt ratios (2:1, 5:1 and 10:1). The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (*P < 0.05; **p< 0.01).

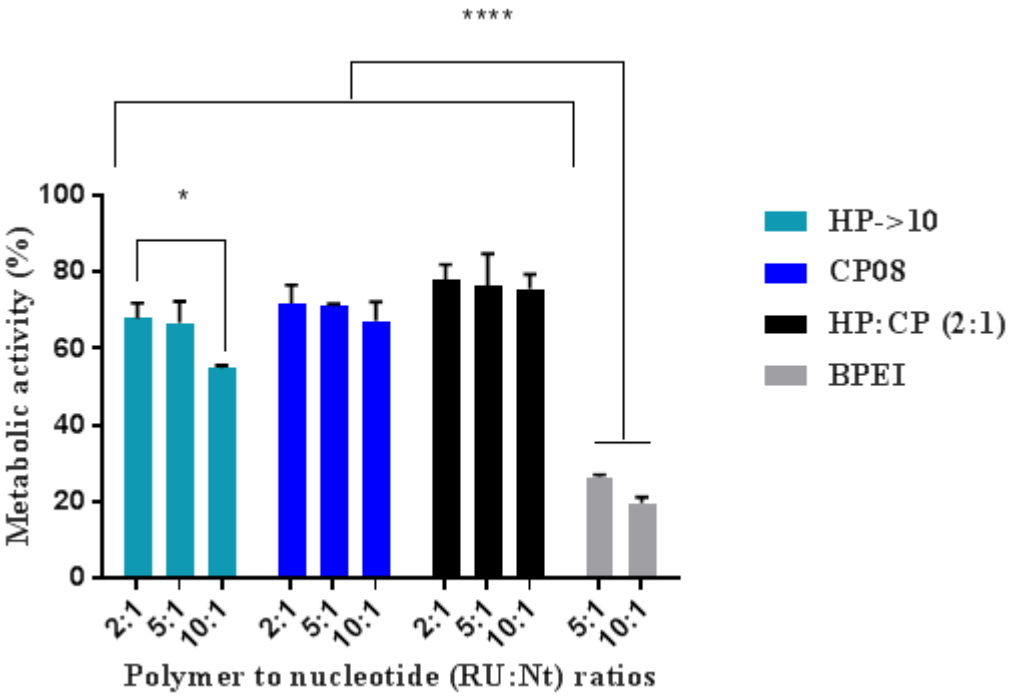


Figure 8 Metabolic activity measured by Presto Blue assay 44h following cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.
Cross-linked pDNA polyplexes were prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA followed by a Tukey's multiple comparison post-hoc test (*P < 0.05; ****p< 0.0001).

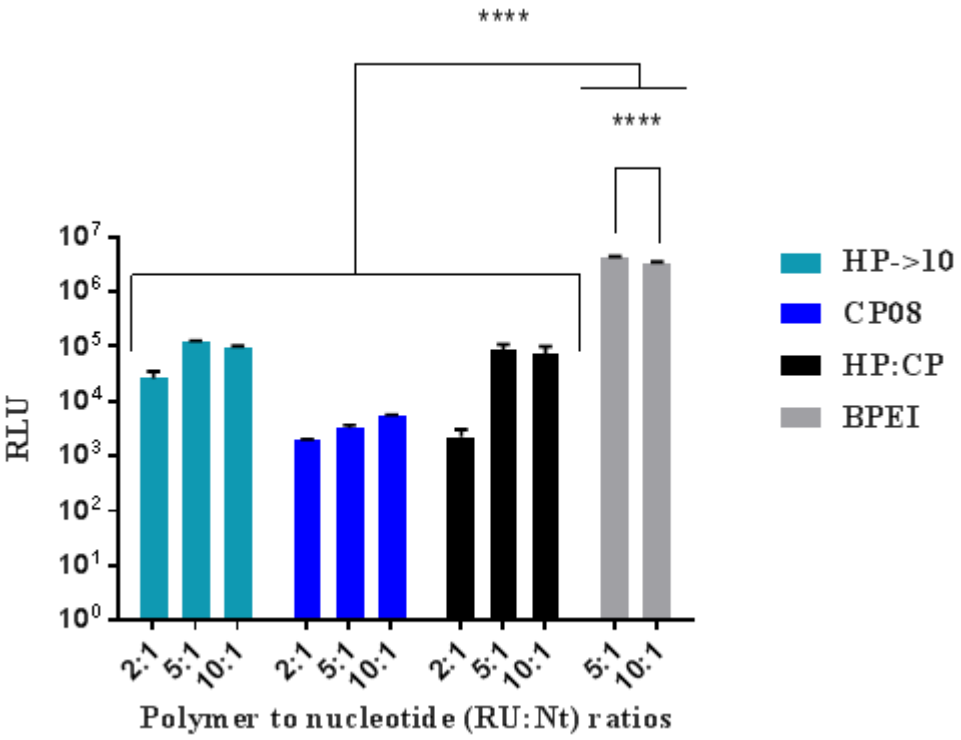


Figure 9 Transfection efficiency measured by luciferase assay 44h following a 4 h cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).
Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (****$p < 0.0001$).

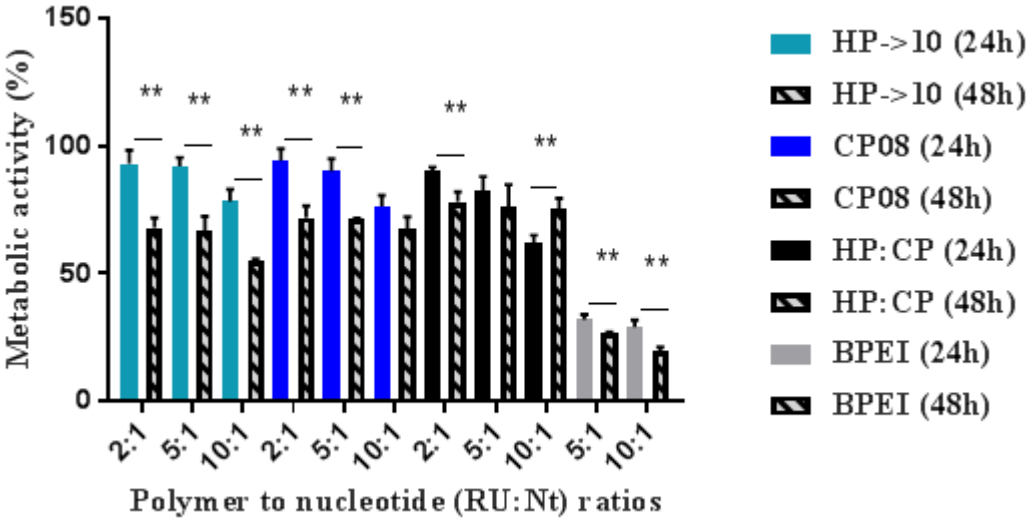


Figure 10 Metabolic activity measured by Presto Blue assay 20 and 44h following cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for 4 hours.
Cross-linked pDNA polyplexes were prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance from the control without inhibitor, as determined by two-tailed Student's t-test (**P< 0.01).

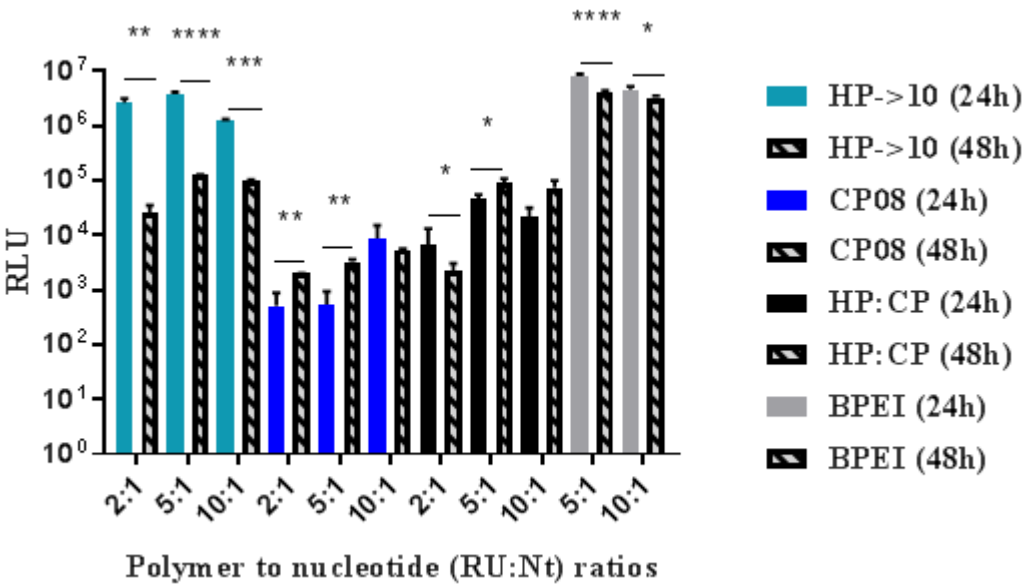
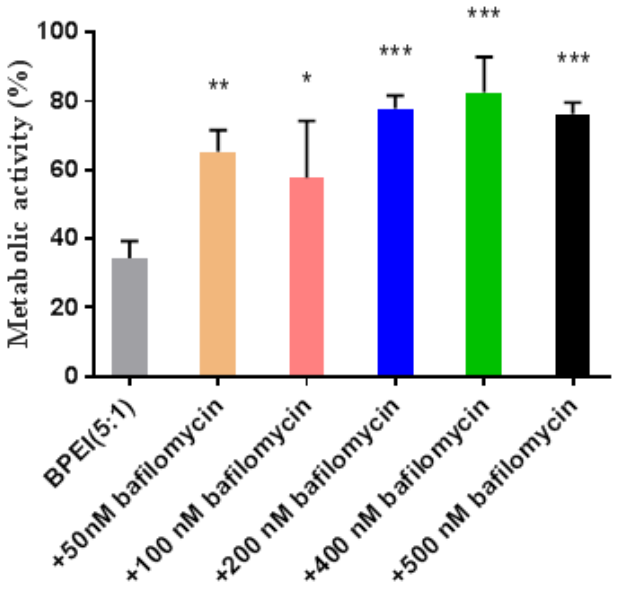


Figure 11 Transfection efficiency measured by luciferase assay 20 and 44h following a 4 h cell exposure to HP->10, CP, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1).
Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance from the control without inhibitor, as determined by two-tailed Student's t-test (*P < 0.05; **P< 0.01; ***P < 0.001; ****P < 0.0001).

A)



B)

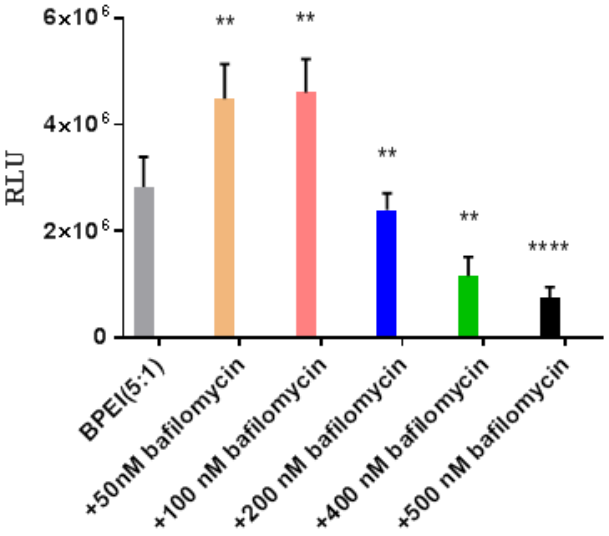


Figure 12 Effect of different concentrations of bafilomycin A1 (50, 100, 200, 400 and 500) nM on (A) A549 metabolic activity and (B) the transfection efficiency measured by Presto Blue and luciferase assays, respectively 20h following cell exposure to BPEI pDNA polyplexes prepared at different RU:Nt ratios 5:1 for 4 hours.

The data are expressed as mean ± SD (3 replicates, n=1). * indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test (*P < 0.05; **P< 0.01; ***P < 0.001; ****P < 0.0001).

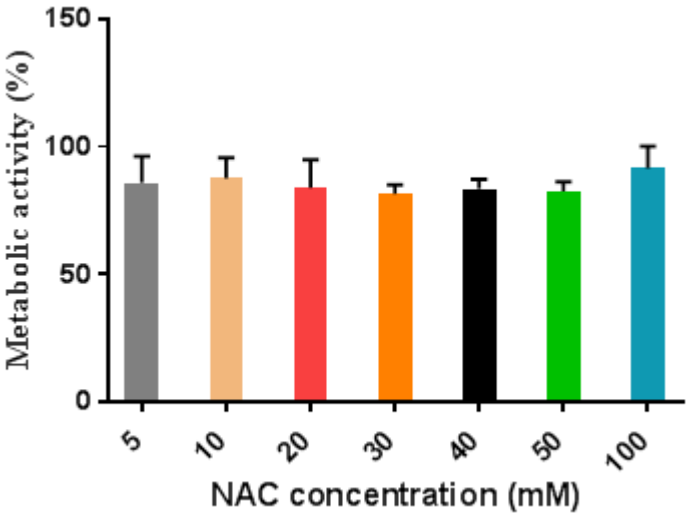
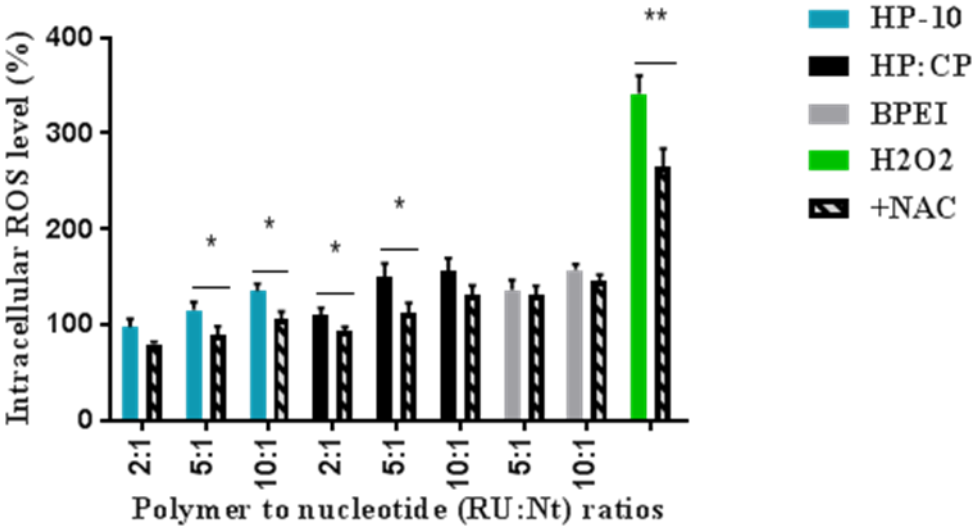


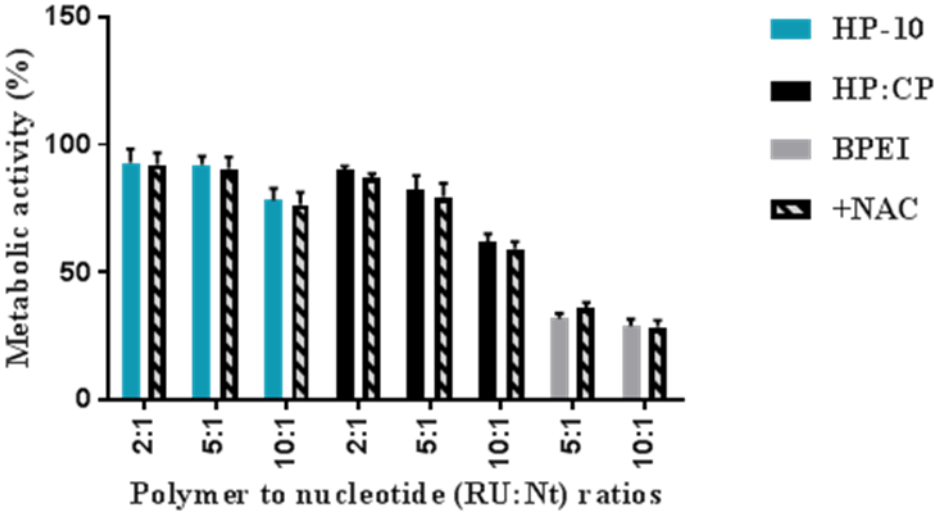
Figure 13 Effect of different concentrations of N-Acetyl-L-cysteine (NAC) (5, 10, 20, 30, 400, 500 and 100) mM on A549 metabolic activity measured by Presto Blue 2h following cell exposure to NAC.

The data are expressed as mean ± SD (3 replicates, n=1). * indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-hoc test.

A)


Intracellular ROS level (%)
400
300
200
100
0
*
*
*
*
**
2:1
5:1
10:1
2:1
5:1
10:1
5:1
10:1
Polymer to nucleotide (RU:Nt) ratios
HP-10
HP:CP
BPEI
H2O2
+NAC

B)


Metabolic activity (%)
150
100
50
0
2:1
5:1
10:1
2:1
5:1
10:1
5:1
10:1
Polymer to nucleotide (RU:Nt) ratios
HP-10
HP:CP
BPEI
+NAC

C)

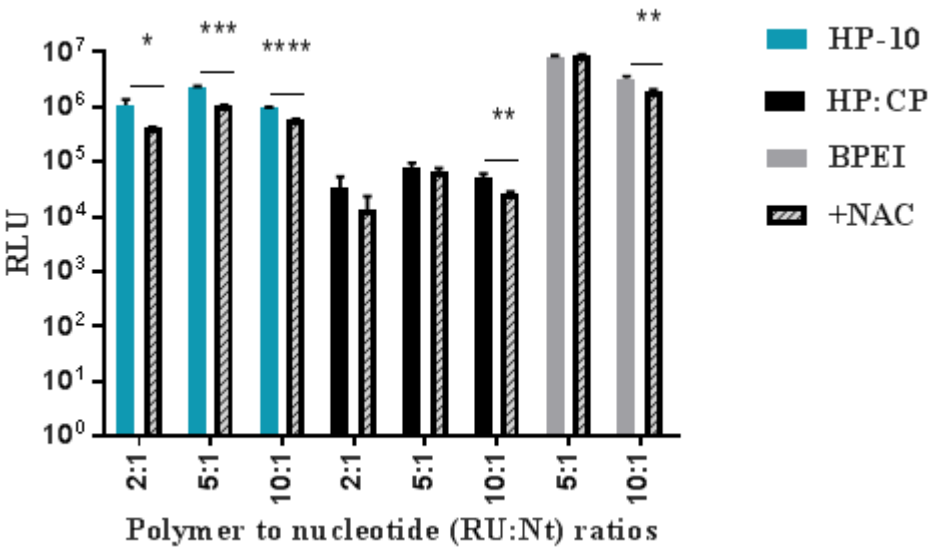


Figure 14 Effect of NAC on A) Intracellular ROS production B) Metabolic activity and C) Transfection efficiency. A549 cells were pre-treated with NAC (20 mM) for 2h and then exposed to HP->10, BPEI and cross-linked pDNA polyplexes prepared at different RU:Nt ratios (2:1, 5:1 and 10:1) for an additional 4 hours.

Cross-linked pDNA polyplexes prepared by using a polymer blend of HP->10 and CP at HP:CP ratios of 2:1. The data are expressed as mean ± SD (6 replicates, n=2). * indicates statistical significance from the control without inhibitor, as determined by two-tailed Student's t-test (*P < 0.05; **P< 0.01; ***P < 0.001; ****P < 0.0001).

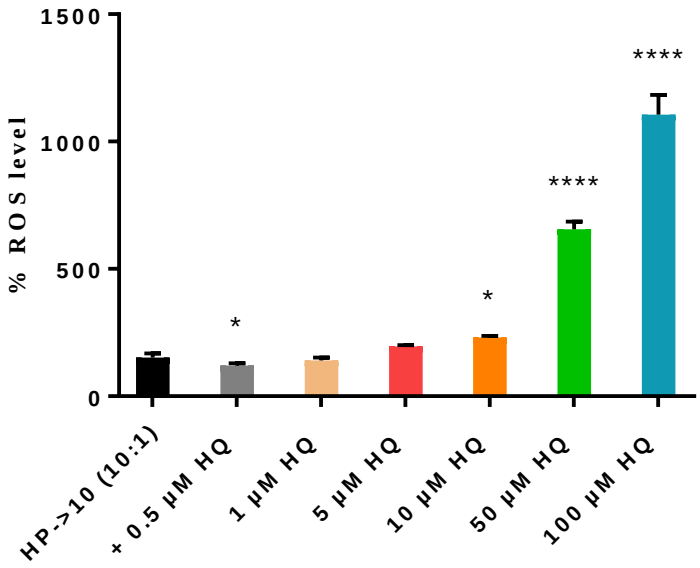


Figure 15 Effect of hydroquinone (HQ) on intracellular ROS. A549 cells were pre-treated with different concentration of HQ (0.5, 1, 5, 10, 50 and 100 µM) for 30 minutes and then exposed to HP->10 pDNA polyplexes prepared at different RU:Nt ratio 10:1 for an additional 4 hours.

* indicates statistical significance differences among RU:Nt ratios of pDNA polyplexes, as determined by one-way ANOVA and a Tukey's multiple comparison post-test (*P < 0.05; ****P < 0.0001).