University of Strathclyde

Strathclyde Institute of Pharmacy and Biomedical Sciences

Preparation and Evaluation of Amino Acid-Bearing Polymers for Enhanced Gene Expression in Tumours

By

Hibah Aldawsari

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
Arg	Arginine
BBB	Blood brain barrier
CCD	Cooled, charge-coupled device
CLSM	Confocal Laser Scanning Microscopy
CPPs	Cell-penetrating peptides
Cs	Chitosan
D_2O	Deuterated water
DAB	Generation 3 diaminobutyric polypropyleneimine
DAPI	4',6-diamidino-2-phenylindole
DDAO	7-hydroxy-*9H*-(1,3-dichloro-9,9-dimethylacridin-2-one)
DDAO-G	*9H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-D galactopyranoside
DMEM	Dulbecco's Modified Eagle Medium
DMSI	Dimethylsuberimidate dihydrochloride
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPE	Dioleoylphosphatidylethanolamine
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetate
EPR	Enhanced permeation and retention effect
FBS	Foetal Bovine Serum
FR	Folate receptor
G	Generation
GAGs	Glycosaminoglycans
GFP	Green fluorescent protein
HA	Hyaluronic acid
HIV	Human Immunodeficiency Virus
HMP	Hexametaphosphate
HSA	Human serum albumin
IC_{50}	Growth inhibitory concentration
Leu	Leucine
L-PPI	Lactose-coated polypropyleneimine
Lys	Lysine
mcDNA	Minicircle DNA
M-PPI	Mannose-coated polypropyleneimine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear Magnetic Resonance
OEI	Oligoethylenimine
ONPG	O-nitrophenyl- β-D-galactosidase
PAMAM	Polyamidoamine
PBLG	Poly(γ-benzyl L-glutamate)
PBS	Phosphate buffered saline
pCMV β-Gal	Plasmid DNA encoding β-galactosidase
PCS	Photon correlation spectroscopy
pDNA	Plasmid DNA
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PIC	Protease Inhibitor Cocktail
PLB	Passive lysis buffer
PLL	Poly-L-lysine

PMSF	Phenylmethylsulfonyl fluoride
PPI	Polypropyleneimine
PTDs	Protein Transduction Domains
TBE	Tris-Borate-EDTA
TEM	Transmission electron microscopy
TNFα	Tumour necrosis factor
γ- PGA	Poly(γ-glutamic acid)

Summary

Non-viral gene delivery is emerging as potential safer alternative to the use of viral vectors for the treatment of various gene related diseases including cancer. Although non-viral vectors may not be as effective as the viral ones, the continuous research on rationally designing multifunctional non-viral polymeric gene delivery carriers resulted in improved delivery. In this study, our aim was to develop polyethyleneimine (PEI) and generation 3 diaminobutyric polypropyleneimine (DAB) polyplex formulations that could efficiently deliver the therapeutic genes to tumours after intravenous administration through the use of three different amino acids. Conjugation of amino acids such as arginine, lysine and leucine seems an attractive tool in virtue of their excellent cell penetration properties, which results in enhancing DNA transportation into cells and improving the transfection efficacy.

The conjugation of arginine, lysine and leucine with PEI led to an increase of the *in vitro* anti-proliferative activity in A431 cells, respectively by 34-fold, 51-fold and 40-fold compared to the unmodified polyplex. In T98G cells, the conjugation of the amino acids to PEI improved the anti-proliferative activity of the polyplex, by 2-fold for PEI-Arg, 89-fold for PEI-Lys and 269-fold for PEI-Leu. The intravenous administration of arginine-, lysine- and Leucine-bearing PEI polyplexes led to a significant increase of gene expression in the tumour, with a β-galactosidase expression at least 3-fold higher than that obtained after treatment with unmodified polyethyleneimine polyplex.

The grafting of arginine, lysine and leucine to DAB led to a significant increase of *in vitro* anti-proliferative activity in A431 cells, respectively by 30-fold, 22-fold and 20-fold compared to the unmodified DAB polyplex. In T98G cells, the conjugation of the amino acids to DAB improved the anti-proliferative activity of the polyplex, by 43-fold for DAB-Arg, by 47-fold for DAB-Lys and by 35-fold for DAB-Leu compared to the unmodified DAB polyplex. *In vivo*, the intravenous administration of amino acid-bearing DAB polyplexes resulted in an improved tumour gene expression, with the highest gene expression level observed after treatment with DAB-Lys polyplex.

This work corresponds to the first evaluation of gene expression of amino acid bearing non-viral delivery nanosystems in tumours following intravenous administration.

In conclusion, these results, together with the lack of toxicity, make arginine-, lysine- and leucine-bearing polyethyleneimine and generation 3 polypropyleneimine polymers highly promising gene delivery systems.

Chapter 1

Introduction

1.1 Challenges in Cancer therapy

Cancer is a major public health problem worldwide. It is considered a major cause of death around the world. The World Health Organization estimates that 84 million people will die of cancer between 2005 and 2015, and the incidence is expected to increase continuously as the world population ages (Danhier *et al.,* 2010).

The currently available conventional therapies include surgical intervention, radiation treatment and chemotherapy. Although chemotherapy is considered a major therapeutic approach in treating cancer, the clinical application of conventional anticancer drugs usually involves high risks of toxicity for the patients. Most patients will suffer from several undesirable side effects which affect their quality of life. Moreover, the outcome of conventional chemotherapy remains well below expectations, especially in the cases of breast, pancreatic, ovarian and oesophageal cancers (Wong *et al.,* 2007).

Generally, the aggressive growth profile of tumours made them resistant to conventional treatments (Morille *et al.,* 2008). Moreover, these therapies cannot be delivered specifically to the tumours, resulting in lack of efficiency of the treatments and development of side effects (Gu *et al.,* 2007). The delivery of therapeutic agents to solid tumours is a significant problem because of the obstacles facing drug transport through the unique structural properties of tumours. In addition to the transport barriers that limit the delivery of drugs to a tumour, another problem in cancer therapy is that chemotherapeutic agents are typically toxic to healthy cells as well as tumour cells, which leads to severe systemic life-threatening side effects such as cardiac and renal toxicity as multiple organs are involved (Chilkoti *et al.,* 2002; Wong *et al.,* 2007). Another obstacle that gives rise to chemotherapy resistance is the genomic instability and heterogeneity, either intrinsic or acquired, that need to be faced before a treatment can become effective (Gómez-Navarro *et al.,* 1999; Baird and Kaye, 2003). All those limitations accentuate the need for new strategies of more effective therapies for cancer. Among those strategies, nanotechnology was recognised as a promising approach in the biomedical community, owing to unique theoretical benefits from targeted delivery perspectives in imaging, gene therapy and drug delivery (Grobmyer *et al.,* 2010).

1.2 Nanotechnology in cancer therapy

In 1959, Feynman identified nanotechnology as the future approach to the development of substances atom by atom. Nanotechnology is a term which refers to the design, production and employment of functional structures of substances on the nanometer scale. The prefix “nano” derives from the Greek word for “dwarf”. At the nanoscale, the behaviour and the fundamental characteristics of a given substance is different from its corresponding bulk substances, and can be accurately controlled by nanotechnology without changing its chemical composition. The main aim of nanotechnology is to enhance the therapeutic index of drugs either by improving their administration or by increasing the exposure of the diseased tissues to therapeutics. This is achieved by the ability to apply the nanoscale rules at the atomic, molecular and supramolecular levels to explore and create material systems by gathering molecules into objects. Nanotechnology provides answers for the transformation of biosystems by gathering all aspects of technologies, techniques and processes for real life purposes. Nowadays, nanotechnology has many potential applications in medicinal fields such as diagnostic devices, drug and gene delivery vectors, nanoscale surgery, biosensors and cancer therapy (Kelsall *et al.,* 2005; Park *et al.,* 2008; Oliveira *et al.,* 2010).

As mentioned earlier, most conventional cancer chemotherapeutics have no tumour selectivity and are distributed randomly in the body, which result in lowering the therapeutic index. As cancer has become a multidisciplinary challenge, a better knowledge of cancer pathophysiology combined with developing novel technologies that deliver anticancer materials to its site of action, is the ultimate goal which will lead to more effective therapies for cancer. This may be achieved by taking advantage of the distinct pathophysiological features of the diseased tissues (Vasir and Labhasetwar, 2005). The potential of nanocarriers in delivering therapeutics lies in their ability to carry therapeutic or diagnostic agents to the diseased tissues by the virtue of their small size. Nanomedicine is a term used to describe materials that are formulated in the nanometer size range (Moghimi *et al.,* 2005; Peer *et al.,* 2007). There are many advantages of nanomedicines over the free form of drugs such as protection from premature degradation, improved drug absorption into a selected

tissue, controlled pharmacokinetics and biodistribution, and enhanced intracellular penetration (Peer *et al.,* 2007). Furthermore, the physicochemical properties of these nanocarriers can be customized by surface modification to improve their performance (Moghimi *et al.,* 2005). Examples of commercially available nanoscale systems are liposomal formulations of the anticancer drugs Doxorubicin (Doxil®) and Daunorubicin (DaunoXome®) (Koo *et al.,* 2005). More recently, Paclitaxel albumin-stabilized nanoparticle formulation (Abraxane™) was approved by the Food and Drug Administration (FDA) to treat metastatic and recurrent breast cancer (http://www.cancer.gov/cancertopics/druginfo/fda-nanoparticle-paclitaxel).

Polymeric nanocarriers bearing physically entrapped or chemically conjugated drugs are an attractive strategy for improving the efficiency of targeted cancer therapy. These nanoscale delivery systems have shown promising pharmacokinetics at both the whole body and at cellular levels. Initially, receptor-mediated targeting was thought to be the only available technique to improve tumour selectivity. Accordingly, many investigators worked on developing conjugates bearing tumour-specific antibodies or peptides. However, several studies have shown recently that polymer-conjugated drugs and nano-particulates exhibit prolonged circulation time in the blood and accumulate passively in tumours even in the absence of targeting moieties, suggesting the existence of passive retention mechanism which is achieved by the help of nanotechnology (Duncan, 2003; Park *et al.,* 2008).

In the context of nanotechnology, new concepts in cancer therapy have been explored over the years in the hope to turn molecular discoveries in laboratories into more realistic benefits for the public. From this point, researchers have sought to develop novel cancer therapeutics by a combination of nanotechnology and gene therapy.

1.3 Gene therapy

Gene therapy has emerged as a promising approach due to its potential to overcome consequences of genetic mutations that are characteristics of many

diseases, including cancer. The discovery of the complementarity of deoxyribonucleic acid (DNA) in 1953 (Watson and Crick, 1953) led to investigating the hereditary mechanism and consequently exploring new developments in the area of genetics. More recently, the completion of the Human Genome Project in 2001 has provided huge amounts of knowledge of the molecular mechanisms of inherited genetic diseases (Venter *et al.,* 2001). This has paved the way for gene therapy as alternative curative method for both inherited and acquired genetic diseases (Park *et al.,* 2008). Thanks to advances in genomic research and molecular biology, several diseases such as Human Immunodeficiency Virus (HIV), Parkinson's disease, sickle cell anaemia, Huntington's disease and Alzheimer's disease, have their genetic identity explored for which gene therapy may be used as a possible cure (Pawliuk *et al.,* 2001; Burton *et al.,* 2003; von Laer *et al.,* 2006; Wong *et al.,* 2006).

Gene therapy involves transferring genetic materials (DNA or RNA) into specific cells or tissues of patients with therapeutic intent, either by replacing defective or missing genes, silencing undesired gene expression or introducing new cellular biofunctions (Dalgleish, 1997). Recently, investigators have realised that gene transfer technology can provide novel approaches to a large variety of illness not traditionally thought of as genetic diseases. The fact that cancer can be a result of a progression of genetic mutations, where one cell proliferates in abnormal way to a malignant cell, has encouraged scientists to apply gene therapy techniques to treat it (Kasai *et al.,* 2002). A better understanding of the genes associated with the development and growth of cancer and other acquired diseases such as acquired immunodeficiency syndrome (AIDS) is guiding the scientists to new approaches to eradicate this disease by treating it at its source (Dutta *et al.,* 2010). It is worth mentioning that a relatively short expression of therapeutically active proteins may be sufficient to eradicate this disease, in contrast to other genetic diseases, such as cystic fibrosis, which requires long-term expression and regulation of gene expression (Ogris and Wagner, 2002). Among the currently available gene therapy clinical trials, cancer addresses almost 66.5% of these trials (Edelstein, *et al.,* 2007).

There are two main potential strategies for gene therapy: the *ex vivo* and the *in vivo* approaches. In the *ex vivo* approach, target cells are extracted from the

patient, modified by transfection with the therapeutic gene, then re-implanted in the patient. In the *in vivo* strategy, therapeutic genes are directly administered to the patient and the cells are transfected *in vivo* (Dutta *et al.,* 2010).

To administer a therapeutic gene into the body of the patient, a delivery system is required. Up to date, specific and efficient delivery of genetic material to diseased sites and to particular cell populations is the challenge that is being addressed in gene therapy using a variety of viral and non-viral delivery systems. Each system has diverse advantages and disadvantages. The main requirements of a suitable vector for clinical applications are low cytotoxicity/immunogenicity, high transfection efficiency, tissue specificity and cost effectiveness. Unfortunately, to date there is no single vector synthesized with all of these essential features. As a result, the need for multidisciplinary research focusing on innovative and novel delivery vehicles remains unchanged (Canine and Hatefi, 2010).

1.3.1 Method of gene transfer

Theoretically, there are three different approaches in the application of gene delivery. The first approach consists of direct injection of naked DNA to the tumour site. The resultant high level of gene expression together with the ease of this application led to its use in several experimental protocols (Shi *et al.,* 2002; Walther *et al.,* 2003). However, this method is only limited to the tissues that are readily accessible by direct injection such as the skin and muscles, and not suitable for systemic administration due to the presence of serum nucleases (Mansouri *et al.,* 2004).

The other approaches involve using viral or non-viral vectors that are capable of transferring the genetic materials into the host cells to bring out the maximum therapeutic effects.

1.3.1.1 Viral vectors for gene delivery

Engineered viruses were the first delivery vectors used in gene therapy. Viral vectors are biological systems derived from viruses capable of transferring their genetic materials into host cells. The most commonly used viruses in gene therapy clinical trials to date are adenoviruses (24.7%) and retroviruses (22.8%). This is due to their high transduction efficiency in cells *in vivo*. Other viruses as adeno-associated viruses, herpes simplex and lentivirus have been employed as well (Edelstein, *et al.,* 2007). However, the limitations associated with viral vectors, in term of lack of specificity towards target cells, high cost of production and safety concerns such as risk of potential immunogenicity and oncogenicity responses has led researchers to focus on safer alternatives (Jeong *et al.,* 2007).

1.3.1.2 Non-viral vectors for gene delivery

While viral vectors remain the dominant vehicles for gene delivery due to its high transfection efficiency, future gene delivery will utilize synthetic non-viral vectors for their favourable safety profile (Gabrielson and Cheng, 2010). Moreover, the application of nanotechnological tools in human gene therapy resulted in the emerging of an enhanced version of non-viral vectors (usually 50-500 nm in size) as potential safer alternatives (Davis, 1997).

A variety of materials are being investigated for non-viral gene delivery applications, which are mainly of a cationic nature: cationic polymers, dendrimers and cationic lipids (Figure 1.1) (Dufès *et al.,* 2005). The employment of cationic polymers and lipids for gene transfer was first introduced by Felgner *et al.* (1987) and Wu and Wu (1987), respectively. Since then, their use has been rapidly progressing in the field of non-viral gene therapy.

Despite the rapid revolution in nanotechnology and the enhanced knowledge of cellular pathway, there are various challenges to overcome before a safe and effective nano-size gene delivery vectors can be clinically available for cancer therapy.

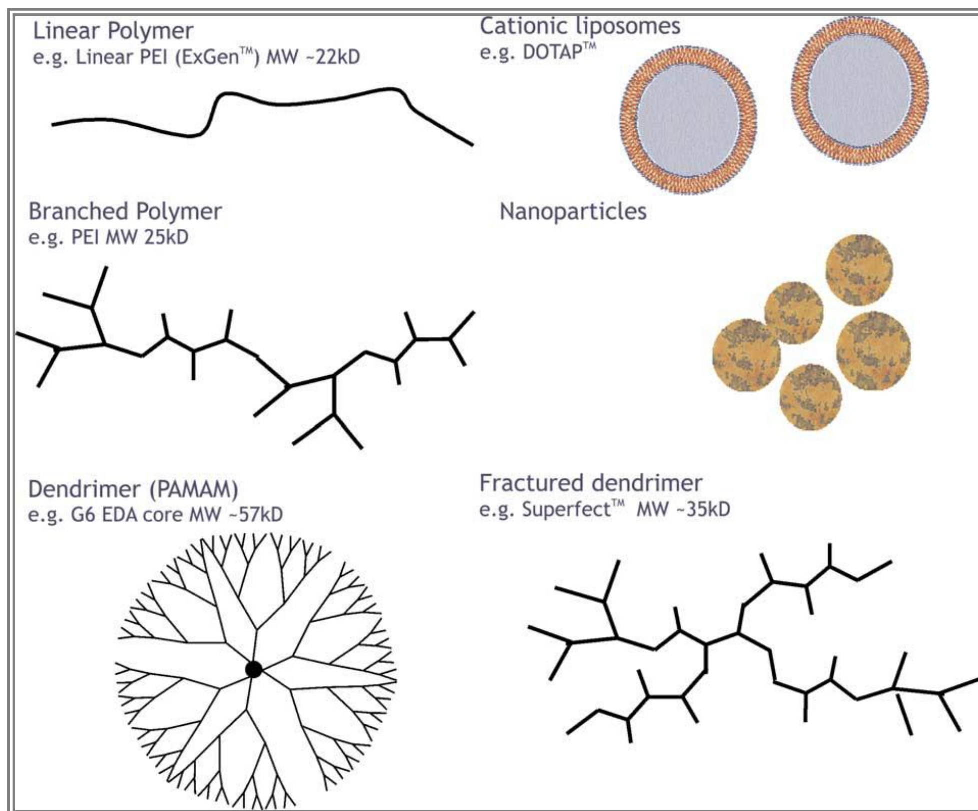

Figure 1.1: Non-viral delivery vectors *(After Dufès et al., 2005).*

In recent years, more information has become available about the mechanism and the cellular pathway involved in the use of cationic polymer/DNA complexes (polyplexes) and cationic lipid/DNA complexes (lipoplexes) in gene transfection. The general mechanism of delivery is based on the hypothesis that nano-sized polyplexes or lipoplexes are formed when cationic polymers or liposomes are mixed with DNA in buffered aqueous solution (Elouahabi and Ruysschaert, 2005).

These positively charged particles bind to the anionic plasma membrane of mammalian cells via non-specific electrostatic interaction (Boussif *et al.,* 1995; Piers *et al.,* 1999) and then enter cells by endocytosis or endocytosis-like mechanism. Once inside, the pH of the endosome compartments drops from pH 7 to 5.5 and part of the bound nucleic acids escape into the cytosol. The process of cytoplasmic transport of endosomes plays an essential role in carrying the transfecting substances near the nucleus. This is followed by the dissociation of polyplexes or lipoplexes which released the plasmid DNA (Figure 1.2) to enable its entry to the nucleus by

either passive or active transport. Both mechanisms have inspired scientists to rationally design an efficient safe gene carrier with a targeted cell-specific delivery. Nowadays, targeting strategy is considered one of the most important issues in regards to the present gene delivery methodologies.

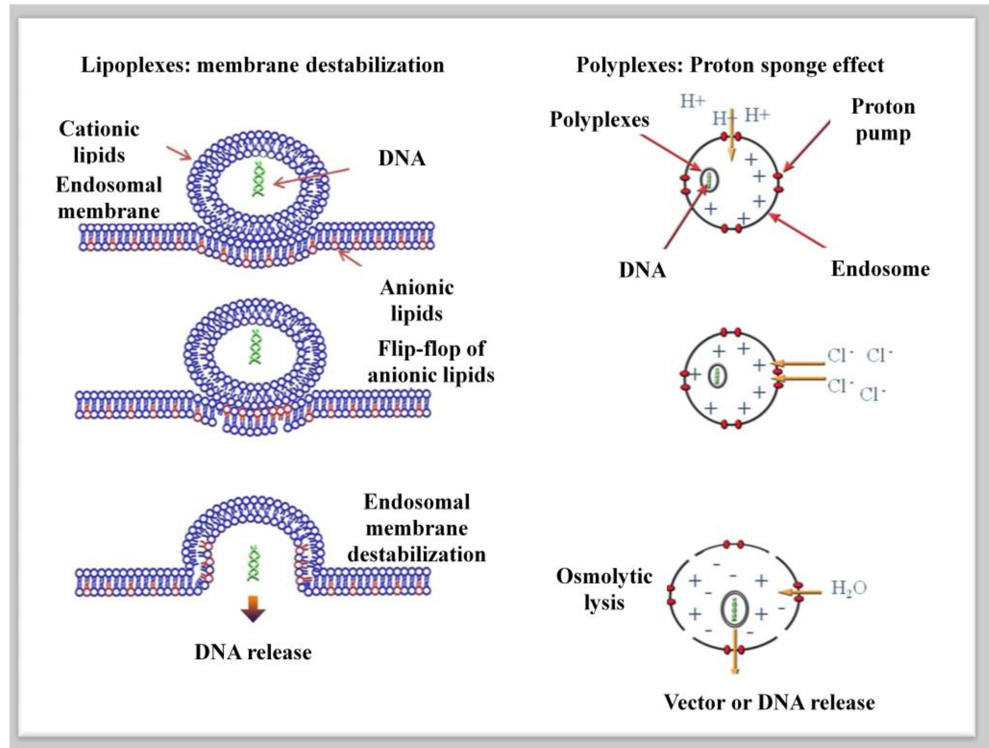

Figure 1.2: Hypothesis of endosomal escape of lipoplexes and polyplexes gene delivery systems (*Adapted from Morille et al., 2008*).

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1.3.2 Targeting

On the cellular level, numerous polyplexes or lipoplexes have been carefully designed to gain entry into the cell by either passive or active tumour targeting strategies.

1.3.2.1 Passive tumour targeting method

Passive tumour targeting refers to the entry of DNA into the nucleus during cell division when the nuclear membrane disintegrates temporarily (Brunner *et al.,* 2000; Tros de Ilarduya *et al.,* 2010).

Unlike normal blood vessels, which are represented in well organized and functional structure, tumour blood vessels show leaky, heterogeneous and tortuous structures (Folkman 2007; Kang *et al.,* 2010). Other abnormal morphology includes large openings and fenestrations in the vasculature network, lack of constant blood flow or direction, lack of pericytes and dilated capillaries (Cassidy and Schätzlein, 2004; Alexis *et al.,* 2008; Maeda *et al.,* 2009). These defective vascular structures are the result of the rapid vascularisation required to supply oxygen and nutrients for cancer cells, leading to cancer growth with decrease lymphatic drainage. As a result, antitumour drugs can pass through tumour blood vessels without being removed easily, and tend to accumulate more in tumours than in normal tissues (Figure 1.3) (Park *et al.,* 2008; Kang *et al.,* 2010).

This phenomenon was first identified by Matsumura and Maeda (1986), and has been named the "enhanced permeation and retention (EPR) effect". Since its identification, several studies have shown that the EPR effect results in passive accumulation of macromolecules and nano-sized particulates (as polymeric conjugates, polymeric micelles, liposomes and dendrimers) in tumour tissues with the resulting increase of their therapeutic indexes and decreasing side effects, subsequently (Park *et al.,* 2008).

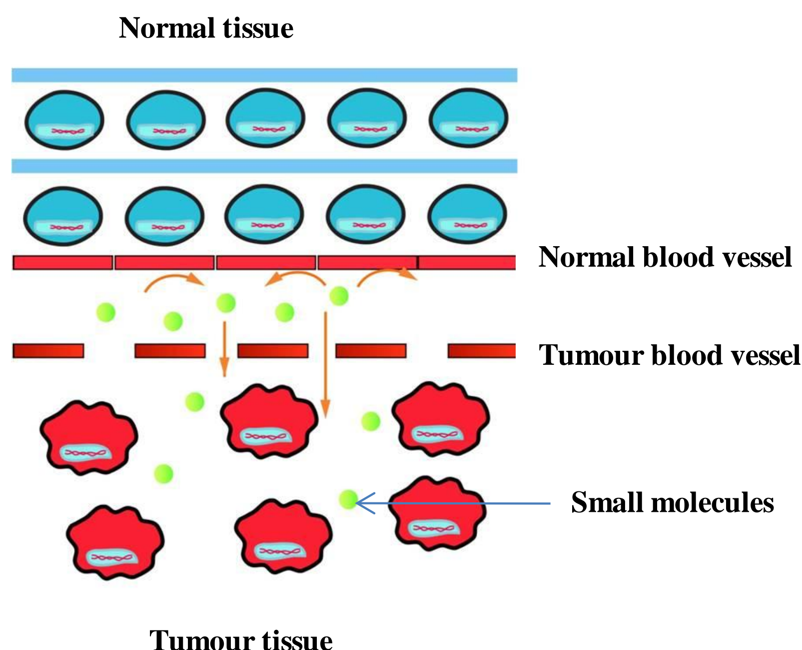

Figure 1.3: Passive tumour targeting method.

As passive targeting is influenced by the intrinsic properties of the system, the physicochemical properties are of great impact on the EPR effect. The molecular weight of the nano-size systems should be higher than 40 kDa, as low molecular weight nanoparticles will diffuse into the circulating blood and removed rapidly from the body (Yuan *et al.,* 1995; Kang *et al.,* 2010). Furthermore, nanoparticles should possess prolonged circulation time in the blood to allow for adequate accumulation in solid tumours (Matsumura and Maeda, 1986). Vascular permeation is higher for positively charged nanoparticles, but their plasma clearance will be faster compared with negatively charged or neutral nanoparticles (Dellian *et al.,* 2000).

The diameter of nanoparticles is a critical factor in delivering cancer therapies using the EPR effect. Tumour blood vessels may be permeable to nanoparticles of up to 400 nm in diameter (Yuan *et al.,* 1995). However, studies using nano-sized systems have shown that the cut-off size of the tumour vessel's pores range from 200 nm-1.2 µm (Yokoyama *et al.,* 1991; Yuan *et al.,* 1995). As the particle size and extended circulation time are of special importance in delivering nanoparticles via EPR effect, researchers have focused on the development of techniques that produce

small particle size delivery systems with prolonged blood half-life. Self-assembling nanoparticles, micellar nanoparticles with hydrophobic lipid core and a hydrophilic shell and a resultant particle size range below 200 nm are examples of systems used to deliver genes in tumour targeted delivery based on the EPR effect (Torchilin 2007; Ko *et al.,* 2009; Kang *et al.,* 2010). The employment of hydrophilic fragments such as poly(ethylene glycol) (PEG) and poly(N-vinylpyrrolidone) (PVP) onto these nanoparticles promoted prolonged circulation time, and subsequent accumulation in a tumour. Furthermore, water-soluble conjugates, such as polymer-protein (mostly PEGylated enzymes and cytokines) or polymer-drug conjugates also show extended circulation time and tumour targeting via the EPR effect compared to non-PEGylated enzymes (Kang *et al.,* 2010; Duncan *et al.,* 2005).

A number of polyplexes have gained access into cells via charge mediated interactions with proteoglycans that are present on the cell surface (Mislick and Baldeschwieler, 1996). Proteoglycans are composed of a membrane-associated core protein from which a chain of sulphated or carboxylated glycosaminoglycans (GAGs) extend into the extra cellular space (Hardingham and Fosang, 1992). These highly anionic GAGs units are responsible for the level of interaction between the cell surface and the extracellular macromolecules, in addition to the overall negative charge of cell membrane (Yanagishita and Hascall, 1992). Although the exact mechanisms by which GAGs facilitate cellular internalization remain unclear, they are believed to play a critical role in the endocytic uptake of many non-targeted cationic gene delivery systems (Mislick and Baldeschwieler, 1996; Kichler *et al.,* 2006). Another opportunity for cellular uptake relies on the interaction between lipophilic residues conjugated to the cationic vector and the phospholipid layers that comprise the cell membrane (Tagikawa and Tirrel, 1985).

More recently, with the aim of enhancing cationic gene delivery vectors, cell-penetrating peptides (CPPs) have been extensively investigated for their ability to facilitate membrane translocation. These peptides, which derived from viral proteins originally, are 5-40 amino acids in length, amphipathic in nature and positively charged. Some CPPs served as DNA-binding and cell-penetrating components by the virtue of their overall positive charge (Ignatovich *et al.,* 2003; Rittner *et al.,* 2002;

Rudolph *et al.,* 2003). Although the exact mechanism by which CPPs enable cellular uptake remains unclear, the basic hypothesis include (1) formation of pores by peptide within the membrane, followed by (2) direct penetration via the membrane into the cytoplasm, (3) transient uptake into a membrane-bound micellar structure that inverts to release the CPPs and its genetic load inside the cytosol and finally endocytosis induction (El-Andaloussi *et al.,* 2005). Those several non specific targeting strategies are illustrated in Figure 1.4.

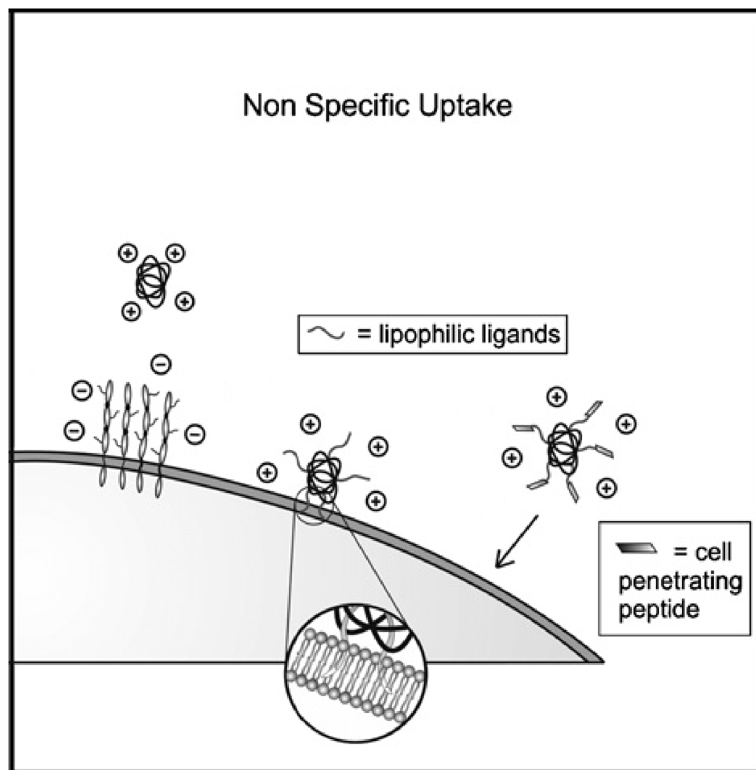

Figure 1.4: Cellular entry-non specific uptake strategies (*After Wong et al., 2007*).

1.3.2.2 Active tumour targeting method

Cancer cells are often characterized by an overexpression of cell surface proteins which are found on normal cells at low levels (tumour-associated antigens), as well as proteins which are found only on cancer cell surface (tumour-specific antigens). It is well known that the rapidly expanding necrotic mass of tumour needs an increased supply of nutrients. Increased rate of transcription, proliferation of tumour cells and altered morphology of blood vessels interpenetrating the tumour

mass, leads to expression of various receptors on the surface of these cells (Horsman and Siemann, 2006).

These hyperactivated receptors and intracellular signals can become the target for tumour-specific gene delivery. Active targeting is often achieved by chemically coupling a targeting moiety which interacts specifically with the receptors or antigens displayed on the target tissue. This process led to the accumulation of the therapeutics in the targeted cells, tissues and organs (Park *et al.,* 2008).

Upon administration, when a carrier containing a ligand binds to each ligand specific receptor, both the carrier and receptor are transferred into the cytosol by receptor-mediated endocytosis, mainly in the form of endocytic vesicles. This endocytic vesicle has the ability to recognize endosomes by receptor-mediated fusion with the endosome. The carrier-ligand complex and the receptor are then separated under the influence of the low pH environment of the endosome. The carrier may then be degraded in, or escape from, the endosome and the receptor is recycled back to the cell surface allowing the delivery of additional conjugates (Figure 1.5) (Park *et al.,* 2008).

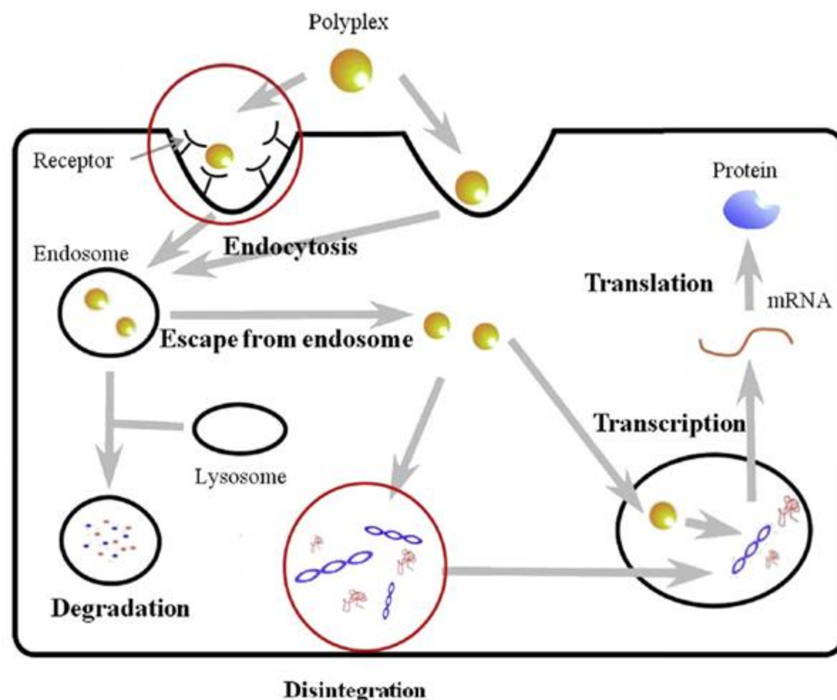

Figure 1.5: Active tumour targeting method (*After Kang et al., 2010*).

Delivery systems recognizing hyperactivated receptors show reduced cytotoxicity toward normal cells with high tumour affinity and subsequent increase in the therapeutic effect. However, their efficiency may depend on several factors such as the activated receptors concentration in the tumour cells, the affinity of the attached ligands to each receptor and the pH (Khalil *et al.,* 2006; Bareford and Swan, 2007; Kang *et al.,* 2010).

In cancer therapy, several ligands have been utilized and examined for active targeting to tumours such as transferrin (Kircheis *et al.,* 2001; Dufès *et al.,* 2004b; Yang *et al.,* 2009, Koppu *et al.,* 2010), glucose transporter (GLUT-1) (Dufès *et al.,* 2004a), epidermal growth factor (Park *et al.,* 2008), integrin receptors (Aina *et al.,* 2007; Merkel *et al.,* 2009) and folate receptors (Kim *et al.,* 2007; Low *et al.,* 2008).

In general, the role of the gene delivery vectors is to effectively deliver the DNA into the nucleus of a cell. This process is mainly limited by barriers that exist between the administration site and the nucleus of the target cells.

1.3.3 Delivery barriers for non-viral vectors

The body defence mechanisms at the humoral and cellular level have evolved to effectively prevent the intrusion of external entities to the system. Viruses avoid the host defence systems by evolution-driven acquisition of efficient machineries for delivering their genetic substances to the target cells. As synthetic cationic non-viral carriers do not exhibit such efficient gene transfer machineries as viruses, they generally show far lower gene transfer efficacy especially in *in vivo* conditions. However, synthetic cationic non-viral carriers have well defined physico-chemical properties and are highly flexible to allow chemical amendment by which their features can be changed to overcome the barriers they face (Jeong *et al.,* 2007).

1.3.3.1 Physicochemical properties

Upon systemic administration, DNA is rapidly degraded by nucleases in a few minutes (Houk *et al.,* 2001). Most cationic polymers and lipids protect DNA by forming polyelectrolyte complexes with nano-scale dimensions via electrostatic

interaction. The formed DNA complex has its unique properties that depend on several factors such as the vector used, the mixing ratio, ionic strength of the solution and the diluents used during the mixing stage (Nishikawa and Huang, 2001; Dutta *et al.,* 2010). Upon DNA condensation, DNA will be folded as a series of similar sized adjacent loops, forming short rod-like structures (Golan *et al.,* 1999; Jeong *et al.,* 2007). The complexes formed usually have diameter size of (50-300 nm) and contain a few thousand copies of DNA (Lai and van Zanten, 2001). Among the various properties, particle size is an important factor which determines the tissue distribution, such as passage through the capillaries (around 5 µm) and via the *fenestrae* between discontinuous endothelial cells (30-500 nm). Moreover, the endocytosis uptake is also a size-limiting process (Nishikawa and Huang, 2001; Dutta *et al.,* 2010).

The overall electrical surface charge of the complex which greatly affects the biodistribution process depends on the mixing molar stoichiometry of DNA to polycation. Complexes with a positive surface charge are generally utilized for transfection. A positively charged surface increases the solubility of the complexes in aqueous medium and facilitates its interaction with the cells (Jeong *et al.,* 2007). As cell membranes are negatively charged due to presence of glycoproteins and glycolipids, they represent a good target for the cationic complexes to induce cellular uptake (Pouton *et al.,* 1998).

1.3.3.2 Stability in biological fluids

Despite the fact that cationic non-viral vectors are favoured in cell culture studies, when administered systemically they readily interact with serum components, such as negatively charged serum albumin which exists in abundance. This results in neutralising its charge to form large aggregates that are rapidly cleared by reticuloendothelial system (RES) or phagocytes, thereby decreasing their transfection efficiency (Dash *et al.,* 1999; Jeong *et al.,* 2007). Surface modification with hydrophilic compounds, such as polyethylene glycol (PEG), has been shown to prevent the aggregation of the cationic delivery systems by masking their positive

charge. PEGylation of polycations increases the blood circulation time of the complexes following intravenous administration, which may be attributed to the highly flexible PEG chains that sterically interfere in the non-specific interaction process which occurs between the serum components and the polycations (Sagara and Kim, 2002; Jeong *et al.,* 2007). In addition, surface modification of polyplexes with PEG provides more stable (Lee *et al.,* 2002) and soluble polyplexes in aqueous solutions (Katayose and Kataoka, 1997). One drawback to PEGylation is that it will hinder the interaction of the complex with the cell membrane, thus decreasing the overall transfection efficiency of the delivery system (Sung *et al.,* 2003). To overcome such a problem, attachments of targeting ligands to the surface of the complexes have been proposed (Choi *et al.,* 1998; Dutta *et al.,* 2010).

Another obstacle of the cationic complexes is their recognition by the immune system upon administration. Plasmid DNA will be recognized as a foreign substance by the mononuclear phagocytic system (Kupffer cells in the liver and splenic macrophages). This immune reaction is part of the body's defence mechanism which identifies foreign materials and removes them from the body (Dutta *et al.,* 2010).

1.3.3.3 Cellular uptake

When the cationic DNA complexes reach target cells, the positively charged particles will interact in a non-specific way with the anionic plasma membrane. Due to the lack of cell specificity of the cationic carriers, cellular uptake mostly takes place via endocytosis. Following endocytosis, the cationic DNA complexes are largely retained within the endosomes / lysosomes and are finally degraded. This is considered one of the major barriers to successful transfection as it limits their transport into the cytoplasm. To improve cellular uptake specificity towards the target cells, cationic carriers are often modified by conjugation to cell-specific ligands, which particularly identify membrane-bound proteins of the target cells and binds to them (Jeong *et al.,* 2007). Moreover, due to the leaky vasculatures of highly

proliferating solid tumours, nano-carriers can passively target tumour cells via the enhanced permeation and retention effect (Maeda *et al.,* 2000).

1.3.3.4 Endosomal escape

Once the cellular uptake process occurs either via adsorptive or receptor-mediated endocytosis, cationic complexes are localized within the endosomal compartments. The pH rapidly drops from 6 to 5 and the late endosomes then mature into lysosomes, where the complexes will release the DNA to be degraded by various degradative enzymes (Figure 1.6) (Wong *et al.,* 2007).

For effective gene transfection, early escape of the cationic complexes from the endosome is considered to be a critical step to exert the desired therapeutic effect of the transfected genetic substances. Several strategies have been examined to overcome the endosomal barriers. In one approach, fusogenic peptides have been employed to disrupt the endosomal membrane thus releasing the plasmid (Mastrobattista *et al.,* 2002). Another technique is based on the use of high buffer capacity cationic polymers like polyethyleneimine (PEI), known as “proton sponge” effect. These polymers undergo protonation in the acidic pH of the maturing endosomes which result in rapid swelling of the polymer matrix and subsequent rupturing of the endosomes and thus releasing its contents (Cho *et al.,* 2003).

Following the endosomal release, the transgene will traffic toward the nucleus through the cytoplasm and may be exposed to cytoplasmic degradation. Only free DNA can reach the nucleus so the DNA must be dissociated from its vector by then. Following nuclear uptake, the transgene is expressed to messenger RNA which is exported to the cytoplasm and translated to the required protein (Haider *et al.,* 2005).

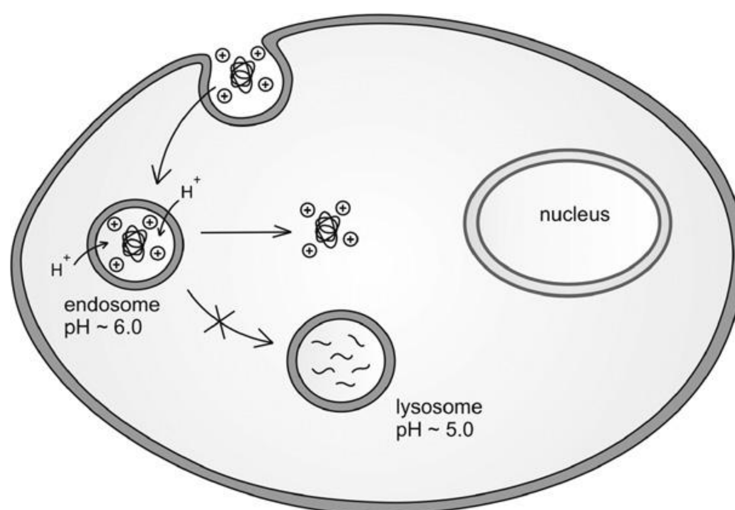

Figure 1.6: Endo-lysosomal escape (*After Wong et al., 2007).*

1.3.4 Types of non-viral nanocarriers

Current technologies in therapeutics tend to utilize tailored treatments that require high specificity and minimal side effects. Biological drugs such as proteins and nucleic acids are considered the next generation therapeutics are required to fulfil these requirements. Since most nucleic acid drugs are vulnerable to enzymatic degradation by nucleases, appropriate carriers are needed to efficiently deliver them to their target cells to bring out maximum therapeutic effects. Synthetic non-viral gene delivery carriers have recently attracted much attention due to the safety drawbacks issues of the viral carriers. The most common types of non-viral carriers mainly include cationic polymers, dendrimers, lipids and peptides. Every system has its unique physicochemical properties which lead to distinctive *in vitro* and *in vivo* behaviour, as well as individual advantages and disadvantages.

1.3.4.1 Cationic lipids

Cationic lipids are cationic amphiphilic molecules consisting of a cationic polar head group (amino group usually), a hydrophobic portion largely (alkyl chain or cholesterol usually), and a linker moiety that connects the polar head group with the non-polar tail (Lonez *et al.,* 2008). They are mainly used in the form of

liposomes (Figure 1.7). The positively charged head group is necessary for the binding of nucleic acid phosphate groups (Morille *et al.,* 2008). Since cationic lipids were first introduced as gene carriers by Felgner and his colleagues in 1987, they have become one of the most studied non-viral transfecting agents (Felgner *et al.,* 1987). A large number of cationic liposomes have been synthesized and used for delivery of nucleic acids into cells in culture, in animals and even in patients enrolled in phase I and II clinical trials (Lonez *et al.,* 2008).

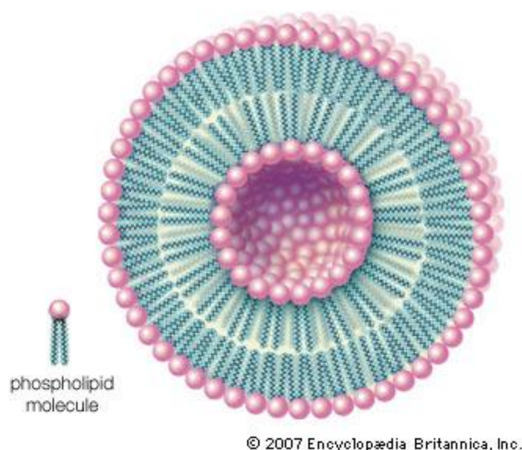

Figure 1.7: Schematic illustration of liposome *(After Encyclopedia Britannica, Inc. 2007).*

Several types of cationic lipids have been developed for DNA delivery, such as quaternary ammonium detergents, cationic derivatives of cholesterol and diacylglycerol, and polyamine lipid derivatives. The addition of cationic lipids to plasmid DNA lowers its negative charge and assists its interaction with the cell membrane. Helper lipids such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol are generally added to the cationic lipids to promote the DNA release from the endosome after the cellular uptake via endocytosis (Dutta *et al.,* 2010).

Some cationic polymers such as polylysine and protamine are incorporated into the cationic liposomes to facilitate DNA condensation and prevent aggregation and nuclear degradation (Liu *et al.,* 1997b). Cationic lipids are technically simple and quick to formulate, readily available commercially, and may be tailored for specific applications. One major issue was the degree of toxicity that cationic lipids exhibited

in cultured cells and that these effects were at times drastically pronounced in several animal studies (Dass, 2002).

1.3.4.2 Cationic polymers

Synthetic polymers can be divided into four main architectural groups: linear, cross-linked, branched and dendritic structures (Oliveira *et al.,* 2010). This group of gene vectors includes any synthetic cationic polymers which can be combined with DNA at the physiological pH to form a complex (polyplex) that is capable of transferring the genes to the target cells. They are more effective in condensing DNA than are cationic liposomes (Dutta *et al.,* 2010). The main difference between cationic polymers and cationic lipids is that the former lack a hydrophobic moiety and are completely soluble in water (Elouahabi and Ruysschaert, 2005). Being synthetic in nature, cationic polymers have greater flexibility which can be easily achieved by varying the mixture composition, the molecular weight, using linear, branched dendrimers or graft copolymer. The introduction of side chains or target-specific moieties to the cationic polymers is considered one of the most interesting areas for optimizing their performance (Nishikawa and Huang, 2001). Frequently studied cationic polymers include poly-L-lysine (PLL), chitosan, polyethyleneimine (PEI), and dendrimers.

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

PLL is a linear polypeptide with biodegradable nature (Figure 1.8) which is considered to be an advantageous characteristic for *in vivo* use. Until 2000, PLL has been popularly used as a cationic polymer for DNA delivery due to its high DNA condensation ability (Morille *et al.,* 2008). PLL also protects DNA from the extracellular enzymatic degradation by forming polyelectrolyte complexes (Jeong *et al.,* 2007). In general, low molecular weight PLLs are not suitable for gene delivery since they cannot form stable DNA complexes and are rapidly removed by the Kupffer cells of the liver (Kwoh *et al.,* 1999). PLL has lower transfection efficiency

when used alone without modifications (Farrell *et al.,* 2007). The relatively weak transfection ability of PLL is attributed to the fact that the entire primary Ɛ-amine groups of lysine in PLL are protonated at physiological pH. As PLL does not have any intrinsic proton buffering effect at endosomal pH, it will be difficult for the complex to escape from endosomes or for the DNA to be released from the complexes. To address this problem, several ligands have been introduced to the backbone of PLL to enhance its transfection efficiency by promoting the endosome escape property such as histidine (Midoux and Monsigny, 1999), chitosan (Yu *et al.,* 2007) and lipids (Abbasi *et al.,* 2008).

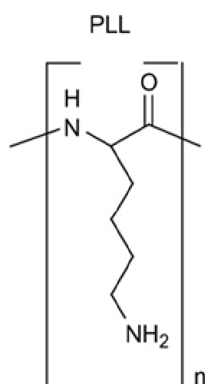

Figure 1.8: Structure of poly(L-lysine).

Cationic natural polysaccharides employed as gene delivery vectors have several advantages over the synthetic polymers. As polysaccharides are derived from natural sources, they are expected to show enhanced biocompatibility, biodegradability and safety profile. Chitosan is one of the popular cationic natural polymers for gene delivery which interact with DNA to form stable nano-size complexes. It consists of repeated D-glucosamine and N-acetyl-D-glucosamine units linked via (1-4) glycosidic bonds (Figure 1.9) (Okamoto *et al.,* 2002).

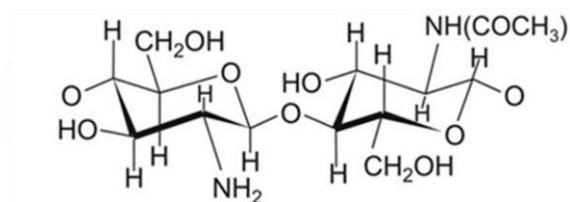

Figure 1.9: Structure of chitosan.

Gene transfection of chitosan is affected by several factors, such as the molecular weight, salt form, degree of acetylation and pH (Kim *et al.,* 2007). The use of chitosan as a gene carrier is limited compared to other cationic polymers due to its low water solubility and weak gene unpacking that compromise its gene transfection efficacy. Recently, various structural modifications of chitosan have been investigated to enhance its transfection efficiency. These include incorporation of hydrophilic and hydrophobic moieties (Kim *et al.,* 2007). Incorporation of anionic moieties such as hyaluronic acid (HA) or poly(γ-glutamic acid) (γ- PGA) to chitosan has been shown to significantly enhance its transfection ability. The improvement in transfection efficacy was attributed to the low charge density of the HA chain or the formation of γ- PGA/chitosan/DNA complexes that may disintegrate into smaller sub-particles after cellular internalization, both factors could facilitate DNA endosomal release (Duceppe and Tabrizian, 2009; Peng *et al.,* 2009). MacLaughlin *et al.* (1998) reported an enhanced *in vitro* transfection efficiency of chitosan-based polyplexes after modification with pH-sensitive endosomolytic peptide, suggesting that the chitosan lacks an intrinsic ability for endosomal escape. In their study, they demonstrated that the administration of chitosan/DNA complexes containing an endosomolytic peptide, to the upper small intestine and colon of rabbits led to reporter gene expression in the intestinal tissue and higher expression levels compared to the cationic lipid formulation Dioleoylphosphatidylethanolamine (DOPE) / N-[1-(2, 3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA/).

- ***Poly(ethyleneimine) PEI***

Over the past years, numerous efforts have been made to develop non-viral delivery vectors. Among the most investigated polymeric carriers is the polyethyleneimine (PEI), which is considered as a golden standard for non-viral gene transfection. PEI was first introduced in 1995 by Boussif and his co-workers (Boussif *et al.,* 1995), and since then several studies have been carried out to gain insights into the mechanism of action (Boeckle, *et al.,* 2004; Breunig *et al.,* 2006; Grosse *et al.,* 2007) and to further optimize this polymer (Rhaese *et al.,* 2003; Neu *et al.,* 2005; Nimesh *et al.,* 2007; Patnaik *et al.,* 2010). This efficient gene carrier comes in linear and branched forms (Figure 1.10), and in different molecular weights associated with different transfection efficiency (Boussif *et al.,* 1995).

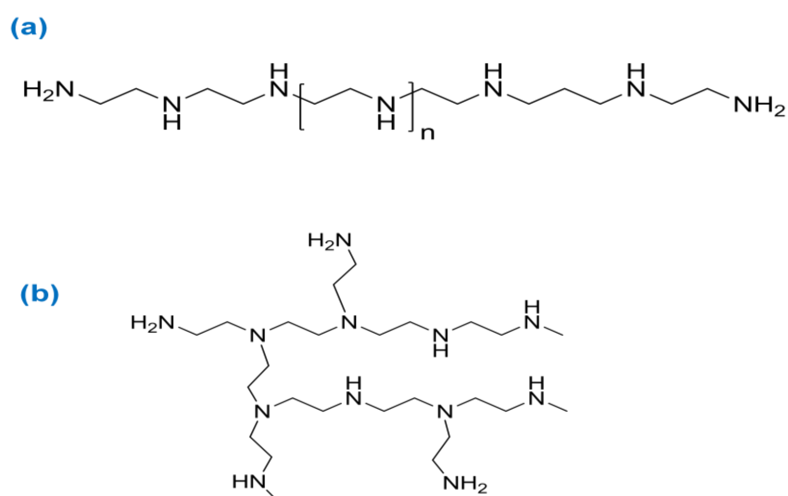

Figure 1.10: Structure of (a) linear and (b) branched PEI.

PEI is a water soluble polymer in which the basic unit of PEI consists of two carbons followed by one nitrogen atom. PEI is comprised of 25% primary amines, 50 % secondary amines, and 25% tertiary amines in which 20% of the nitrogens are protonated under physiological conditions (Park *et al.,* 2008).

Polyethyleneimine (PEI) contains primary amines for DNA binding, and secondary as well as tertiary amines for endolysosomal escape of DNA and its complexes. Therefore, it can induce efficient gene transfection in many cell lines,

and has been used as the gold standard vector for *in vitro* gene transfection. The positive charge of PEI allows its effective binding to the anionic plasmid DNA (pDNA), and the resulting condensation provides a protection for the pDNA from serum digestion (Zhang *et al.,* 2007). PEI has a unique characteristic known as “the proton sponge” effect, in which the unprotonated amines provide a buffering capacity over a wide range of pHs (Park *et al.,* 2008). Once in the endosomal compartment, PEI can act as a buffer to induce osmotic swelling and endosomal disruption. This is of particular importance to avoid pDNA degradation when the endosome fuses with the lysosomes (Zhang *et al.,* 2008).

Many factors such as molecular weight, branching degree, ionic strength, zeta potential and particle size, affect the efficiency/ cytotoxicity of PEI (Kunath *et al.,* 2003). PEIs with a molecular weight lower than 10 kDa are considered inefficacious in terms of gene transfer; while 25 kDa branched PEI has favourable transfection properties (Godbey *et al.,* 1999b). The branched PEI condenses DNA to a greater extent than the linear PEI. This condensation protects DNA from nuclear digestion in addition to providing compact colloids for endocytosis (Dunlap *et al.,* 1997). While higher molecular weight of PEI show an enhanced DNA condensation profile with higher transfection efficiency, but is also accompanied by an increased cytotoxicity which limits its potential in gene delivery (Godbey *et al.,* 1999c; Fischer *et al.,* 2003). It has been reported that there were two types of cytotoxicity associated with PEI. The first one which is caused by the free PEI occurs immediately upon administration in the circulatory system (Godbey *et al.,* 1999a). The free PEIs interact with several serum components, such as albumin, immunoglobulins and red blood cells which led to huge clusters precipitation and adhere to cell surface (Fischer *et al.,* 1999). This effect will destabilize the plasma membrane and induce an immediate toxicity response. Fortunately, when PEI/DNA complexes are administered, the immediate toxicity is diminished and delayed. The delayed toxicity caused by the PEI/DNA complexes was attributed to the release of DNA from PEI (Lv *et al.,* 2006). Generally, intravenous administration of PEI/DNA polyplexes leads to distribution and accumulation of the polyplexes in the major tissues such as liver, lung, heart, spleen and kidney. Cytotoxic side effects were observed in the lung after intravenous administration of PEI/DNA complexes, which may be due to the

formation of large polyplexes aggregates that accumulate in the narrow lung capillaries (Jeong *et al.,* 2007). Solutions proposed to overcome these problems include the use of several ligands such as polyethylene glycol (PEG), peptides, polysaccharides and antibodies to shield the charge on PEI and reduce the charge-associated toxicity (Lungwitz *et al.,* 2005; Nimesh *et al.,* 2006; Jeudy, *et al.,* 2008; Patnaik *et al.,* 2006).

In vivo, PEI/ DNA complexes have been successfully used in a variety of applications. Significantly reported gene expression was found after application of PEI/ DNA formulations in mouse brain (Goula *et al.,* 1998), and rat kidney (Boletta *et al.,* 1997).

More recently, PEI-based polyplexes have been locally or systemically delivered to lung, liver or target tumours (Lungwitz *et al.,* 2005; Jeudy, *et al.,* 2008). Dong *et al.* (2006) examined the cross-linked PEI with biodegradable linkages small diacrylate [1,4-butanediol diacrylate or ethyleneglycol dimethacrylate (EGDMA)] as potential DNA vector for improved gene delivery. The study showed a 9-fold increase in gene delivery in B16F10 (murine melanoma) cells and a 16-fold increase in 293T (human embryonal kidney) cells, without non-specific toxicity at the optimized condition for gene delivery. Furthermore, the transfection activity of these polyplexes was preserved in the presence of serum proteins.

In another study, higher levels of targeting specificity and gene transfection efficiency with C6 rat glioma and DAOY medulloblastoma tumour cells were obtained by PEGylation of DNA-complexing PEI in nanoparticles functionalized with an Alexa Flour 647 (AF) fluorochrome and the chlorotoxin (CTX) targeting peptide. The results showed that the potential toxicity of this nanovector to healthy tissues was minimized by both the decreased PEI's toxicity due to the biocompatible copolymer and the targeting CTX ligand (Veiseh *et al.,* 2009).

In 2005, Kleeman and his colleagues described a new modification of PEI, in which an oligopeptide related to the protein transduction domain of HIV-1 TAT was covalently coupled to 25kDa PEI via a heterobifunctional PEG spacer resulting in a TAT-PEG-PEI conjugate. Significant higher transfection efficiencies were detected

in vivo for the TAT-PEG-PEI vector utilized for lung cancer therapy, combined by lower toxicity and protection of plasmid DNA (Kleemann *et al.,* 2005).

To reduce the toxicity of PEI, an acid-labile imine linker was employed to design a class of biodegradable PEIs. The study concluded that toxicity was much reduced due to the degradation of the acid-labile linkage in acidic endosomes, and that it could be a useful tool to be employed in PEI-based gene delivery vectors (Kim *et al.,* 2005). The nature of PEI enables the researchers to successfully introduce targeting ligands and/or PEG or other functional moieties to its surface, so that higher transfection efficiency and lower cytotoxicity are achieved. Kircheis *et al.* (1999) conjugated PEGylated PEI polyplexes to tumour targeting ligand transferrin, an asialoglycoprotein. A five-fold increase in the transfection efficiency with lower toxicity was achieved when this conjugate was applied intravenously, in comparison with PEGylated (transferrin-free) PEI polyplexes. The addition of PEG as a co-polymer to mask the charge on PEI and produce sterically stabilized gene vectors is one of the strategies employed to decrease the cytotoxicity of PEI-based gene delivery carrier and improve its biocompatibility. It was reported in several studies that PEGylation extends circulation time and reduces *in vivo* toxicity of PEI-based polyplexes when used in systemic gene delivery (Kircheis *et al.,* 1999; Gref *et al.,* 2000; Neu *et al.,* 2007). Unfortunately, most of these PEGylated polyplexes are suffering from reduced DNA complexation, diminished cellular uptake and/or endosomal escape that result in lower transfection efficiency (Nguyen *et al.,* 2000; Mishra *et al.,* 2004; Park *et al.,* 2005; Zhang *et al.,* 2010). Erbacher *et al.* (1999) reported that steric stabilization of PEG is achieved by creating a PEG layer on the polyplexes surface, thus leading to extended blood circulation time. However, the transfection efficiency of PEGylated PEI polyplexes was significantly lower than that of the corresponding non-modified PEI due to PEG's ability to mask PEI charge properties.

Several strategies were suggested for overcoming these PEG limitations. One of these strategies was the conjugation of a targeting ligand to polyplexes which would enhance the cell specific gene delivery via receptor-mediated cellular uptake (Zhang *et al.,* 2010). Ogris *et al.* (2003) reported a new surface-shielded PEI-based

polyplex delivery system with virus-like characteristics that target gene expression into distant tumour tissues. Polyethyleneimine (PEI) /DNA complexes ('polyplexes') conjugated with the cell-binding ligand transferrin (Tf) or epidermal growth factor (EGF) were used to achieve receptor-mediated endocytosis. PEG was covalently linked to PEI to mask the surface charges of the polyplexes. A stable and non toxic gene vector was successfully synthesized, with 10 to 100 fold higher luciferase marker gene expression levels in tumour tissues than in other organ tissues.

More recently, Zhang *et al.* (2010) combined folate receptor (FR) as a targeting ligand to PEG modified PEI and Minicircle DNA (mcDNA) as a new tumour gene delivery system. mcDNA is a form of supercoiled DNA containing only a gene expression cassette and lacking extraneous plasmid sequences (Darquet *et al.,* 1997). It is generated by site-specific recombination in *E. coli* (Chen *et al.,* 2003). mcDNA was employed in their study by virtue of its improved and extended gene expression's life span (Darquet *et al.,* 1997; Chen *et al.,* 2003). *In vitro* characterization showed stable nano-sized polyplexes with a size ranging from 60-85 nm. A 2-8 fold increase in gene expression in folate receptor-positive cells was obtained using mcDNA. Furthermore, the systemic delivery of the FR modified polyplexes resulted in preferential accumulation of transgenes in folate receptor-positive tumours with 2.3 fold higher gene expression in tumours when mcDNA was used compared to the conventional plasmid. Most importantly, PEG shielding reduced the PEI cytotoxicity significantly.

In another study, Neu *et al.* (2007) investigated the possibility of generating PEI-based gene delivery vectors with improved stability in blood circulation. Low grafted copolymers consist of branched PEI and high molecular weights PEG (25 and 30 kDa) were synthesized. The results demonstrated that the cytotoxicity of PEI was strongly reduced after copolymerization, and PEI-PEG (30 kDa) copolymers exhibited better DNA condensation properties compared to PEI alone. The transfection efficiency of the synthesized copolymers polyplexes was 10 fold higher compared to the control PEI, with marked reduction in the haemolytic activity. Following intravenous administration into mice, PEI-PEG (30 kDa) copolymers resulted in extended circulation time. From their study, it was concluded that PEI

polyplexes prepared using a combined strategy of surface crosslinking and PEGylation seem to provide a novel stable, long circulating vectors.

Apart from PEGylation, other approaches were investigated with the aim of creating a safer and higher transfecting PEI-based gene delivery vector. Rhaese *et al*. (2003) developed a versatile carrier system based on nanoparticles consisting of DNA, human serum albumin (HSA) and PEI with the aim of generating a non-viral delivery system that meets the demands of safety, biocompatibility and enhanced stability. HSA was employed in the study as it is considered non-antigenic as well as biodegradable. It is also utilized in several transfection experiments as it was shown to overcome some of the problems associated with transfection complexes *in vitro* and *in vivo* (Orson *et al.,* 2002). As a major protein component of serum, HSA avoids the problems often encountered with cationic complexes *in vivo*, such as rapid opsonisation by serum proteins (Simoes *et al.,* 2000). The developed vectors showed an optimum transfection efficiency when the particles were prepared at N/P ratios between 4.8 and 8.4. Furthermore, the HSA–PEI–DNA nanoparticles displayed a low cytotoxicity when tested in cell culture and may be suitable for intravenous administration.

In an attempt to merge the desirable properties of branched PEI and alginic acid, Patnaik *et al.* (2006) synthesized a system for efficient gene delivery. Alginic acid is a polysaccharide consisting of β-(1-4)-D-manuronic acid and α-(1-4)-L-guluronic acid. The alginate based DNA delivery systems have been shown to enhance the transgene expression and alleviate the immune response (Sailaja *et al.,* 2002). The positive charge on PEI was partially masked by forming ionic nanocomposite with alginic acid, in aqueous solution, in a simple chemical synthesis. The results from the study showed enhanced transfection efficiency (2-16-fold in comparison to unmodified PEI) in all the cell lines studied, with nearly abolished cytotoxicity, utilizing new generation of modified PEI.

Nimesh *et al.* (2007) followed another approach to systemically modify PEI with the purpose of improving PEI-mediated transfection. The polymer was acylated using acid anhydrides of varying carbon chain length, followed by cross-linking of acylated PEI with a homobifunctional PEG derivative (PEG-bis-P) to form compact

nanoparticles. The positive surface charge on particles decreased with the increase in percentage of acylation and also upon complexation with DNA. The nanoparticles formed stable complexes with DNA and higher weight ratios were required for formation of electro-neutral complexes. Furthermore, these nanoparticles were studied for their gene delivery efficacy on COS-1 cells. It was found that acylated PEI nanoparticles were 5–12-fold more efficient transfecting agents as compared to unmodified PEI and lipofectin with considerable reduction in toxicity of acylated PEI nanoparticles as compared PEI.

Swami *et al.* (2007) reported a different strategy to modify PEI by partially substituting their amino groups with imidazolyl moieties. PEI was cross linked with PEG to synthesize imidazolyl-PEI-PEG nanoparticles (IPP). The imidazolyl substitution enhanced the gene delivery efficacy of PEI by 3-4 fold, with a cationic nanoparticles forming a complex with DNA. In addition, the cytotoxicity of IPP nanoparticles was significantly reduced compared to native PEI.

More recently, Patnaik *et al.* (2010) synthesized a new PEI-based gene delivery vector that exhibits high transfection efficiency with lower cytotoxicity. In their study, the charge-associated cytotoxity of PEI was partially neutralized with anionic hexametaphosphate (HMP) molecules. HPM was used by virtue of its compactness and multipoint attachment sites for electrostatic interactions with PEI amino groups to form nanoparticles. The synthesized PEI-HMP nanoparticles demonstrated a higher transfection rate (1.3 to 6.4 fold) compared to the commercial reagents, with lower cytotoxicity. The decrease in the cytotoxicity was attributed to the presence of oligophosphate moiety, HMP, which shields the positive charge of PEI.

1.3.4.3 Dendrimers

The 20th century has brought advance improvement in polymer synthesis and modification of biodegradable compounds. This paved the way for the synthesis of macromolecules with multiple components and novel architectures that exhibit desirable physicochemical and biological features.

Dendrimers are a new class of synthetic polymers that represent an important part of the emerging nanotechnology and are suitable for a wide range of biomedical and industrial applications (Tomalia, 2005). Dendrimers are defined as globular, monodisperse macromolecules with novel three-dimensional polymeric architecture (Jain *et al.,* 2010). Their name comes from the Greek word *dendron*, meaning ‘tree’, and *meros* meaning ‘part’ that describe the architecture of this polymeric macromolecules which possesses 3-D features resembling a tree.

A typical dendrimer structure consists of three basic domains (Figure 1.11):

- A central core that is either a single atom or a group with at least two identical chemical functionalities where other molecules can be trapped (D'Emanuele *et al.,* 2004).
- An interior layer (known as generation, G) composed of Branches of repeating units radially attached to the core.
- The exterior layers that are composed of many identical terminal functional groups, and this layer plays a key role in the gene complexing abilities of dendrimers (Nanjwade *et al.,* 2009; Dutta *et al.,* 2010).

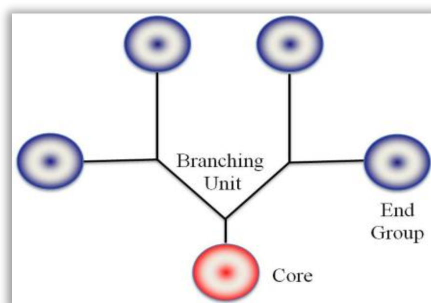

Figure 1.11: Basic structure of dendrimers.

The structure of dendrimers has a great impact on their physical and chemical properties. The prototypical dendrimer starts with an ammonia (NH_3) core that reacts with acrylic acid to produce a tri-acid molecule. This molecule then reacts with ethylenediamine to produce a tri-amine, known as generation 0 (G0) products. This

tri-amine is reacted with acrylic acid to produce a hexa-acid, then with ethylenediamine to produce a hexa-amine (G1), and so on (Figure 1.12).

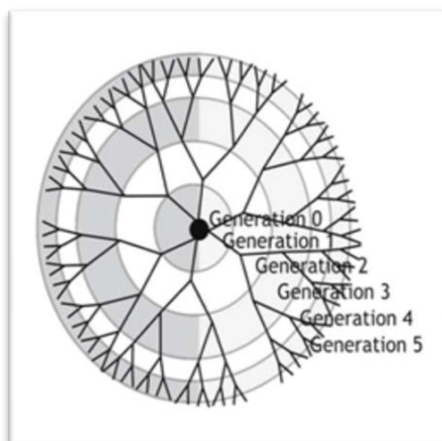

Figure 1.12: Structural elements of dendrimers (*After Dufès et al., 2005*).

This alternation of reactions with acrylic acid and then with ethylenediamine continues until the desired generation is reached. Sugars or other molecules can also be used as the starting core, as long as they have multiple, identical reaction sites. Thus, it is possible to create a surface consisting of multiple amines or multiple acids, following the conjugation of different functional components (Kim *et al.,* 2007).

The first ever report on dendrimers synthesis was described by Vögtel and his group in the late 1970s (Buhleier *et al.,* 1978). A pioneering work was then done on dendrimer synthesis by Denkewalter *et al.* (1981) and Tomalia *et al*. (1985). Dendrimers have generated an increased interest as attractive carriers for gene and drug delivery due to their exceptional molecular structure, such as monodispersity, i.e. being constructed with a well defined molecular structure, low polydispersity index, nanometer size range which can allow easier passage via biological membranes, and well defined shape and multivalency (Parekh, 2007; Dutta *et al.,* 2010). The shape persistence of dendrimers is an essential feature, as it permits the defined placement of functions not on the surface of dendrimers only, but also inside the dendritic scaffold. Multivalency allows the presentation of reactive groups on the

surface of the dendrimers. Thus, more connections between surfaces and bulk materials, such as surface shielding or polymer cross-linking, can be created (Nanjwade *et al.,* 2009). Another feature worth mentioning is that nano-architecture of higher generation dendrimers would serve as synthetic mimics of proteins. The hyper-branched dendritic structure is less compact than a protein, which means the interior is not packed as in typical proteins. Thus dendrimers will show a highly polyvalent surface which exposes much higher functional groups on the surface compared to proteins of similar molecular size (Farin and Anvir, 1991). Dendrimers serve as protein mimics can adapt native (tighter) or denaturated (extended) conformations which depend on several factors such as polarity, ionic strength and pH of the solvent (Nanjwade *et al.,* 2009).

Moreover, dendrimers are easy to prepare and functionalize. Most importantly, the potential use of these materials in drug and gene delivery is defined by the high density of terminal groups that offer multiple attachment sites for drugs conjunction or targeting moieties (Dufès *et al.,* 2005). The exact conformation depends on the nature of the solvent used; in case of G3 (third generation) polypropyleneimine (PPI) dendrimers exhibit an extended conformation in the suitable solvent such as water (polar) or chloroform (acidic), whereas they display a poor crowded conformation in a poor non polar solvent like benzene (Figure 1.13) (Chai *et al.,* 2001; Jones *et al.,* 2003).

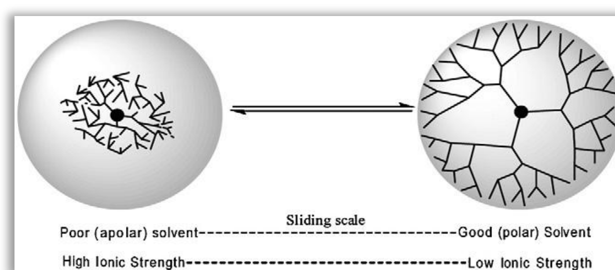

Figure 1.13: Conformation of a dendrimer in polar and apolar solvents (*After Parekh, 2007*).

In contrast to the linear polymers, the viscosity of dendrimer solutions does not increase with mass, but exhibits a maximum at a specific generation (Bosman *et al.,* 1999). This was explained by the way in which dendrimer shape changes with

generation, where lower generations exhibit a more open planar-elliptical shape with transition to a more compact spherical profile for higher generations. This compact shape also decreases the possibility of entanglement, which is characterized in larger classic polymers (Dutta *et al.,* 2010). The presence of several terminal groups enables multiple concurrent interactions with solvents or other molecules and, as a result, dendrimers tend to show elevated solubility and reactivity (Dutta *et al.,* 2010).

Although there are various classes of dendrimers, the ones that have been widely investigated are glycodendrimers and peptide dendrimers. The term glycodendrimers is used to describe dendrimers that include carbohydrates in their architecture. The majority of glycodendrimers have saccharide residues on their outer surface, but glycodendrimers with sugar units as the central core have also been mentioned. In general, glycodendrimers can be classified as carbohydrate-centred, carbohydrate-based and carbohydrate-coated dendrimers (Cloninger, 2002). Peptide dendrimers are broadly defined as radial or wedge-like branched macromolecules consisting of a peptidyl branching core and/or surface functional units covalently attached. Thus, this definition includes any dendrimers with amino acid core, branching unit and surface functional groups or any combination of the three as a peptide dendrimer (Sadler and Tam, 2002).

Since 1979, two major strategies were employed in dendrimer synthesis. The first was introduced by Tomalia, called the "divergent method" in which growth of dendrimers originates from a core site towards the periphery (Figure 1.14) (Tomalia, 1996).

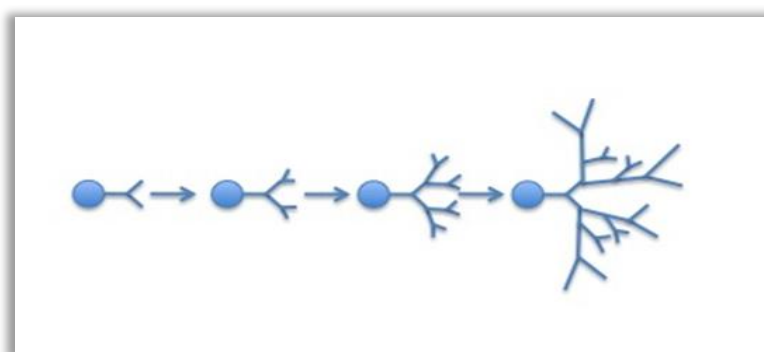

Figure 1.14: Divergent synthesis method.

The second method, established by Hawker and Fréchet follows a "convergent growth process" (Hawker and Fréchet, 1990). In this method, several dendrons are reacted with a multifunctional core to obtain a product in a top-down approach starting from the outermost residues (Figure 1.15).

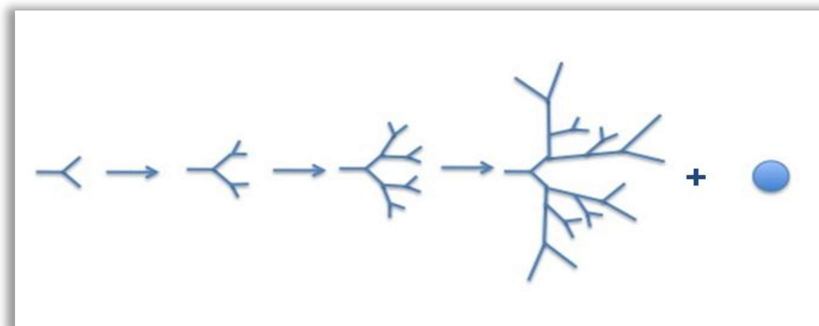

Figure 1.15: Convergent synthesis method.

Most commercially available dendrimers such as the Tomalia's polyamidoamine (PAMAM) dendrimers (Figure 1.16) and polypropyleneimine (PPI) (Figure 1.17) ones are produced by the divergent method (Oliveira *et al.,* 2010). Various studies have explored the role of these two dendrimers as an effective non-viral gene vectors in cancer therapeutics which will be highlighted later in this Chapter.

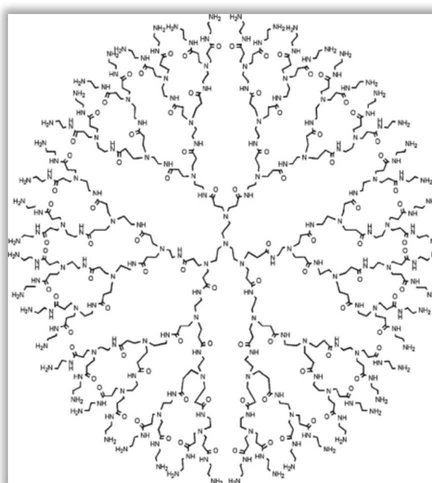

Figure 1.16: PAMAM dendrimer (*After Jain et al., 2010*).

In this work, we exploit the potential of PPI Generation 3 (G3) (also known as DAB 16 and DAB) as a dendritic vector for cancer therapy, thereby; the gene transfer and cytotoxicity of PPI will be detailed further.

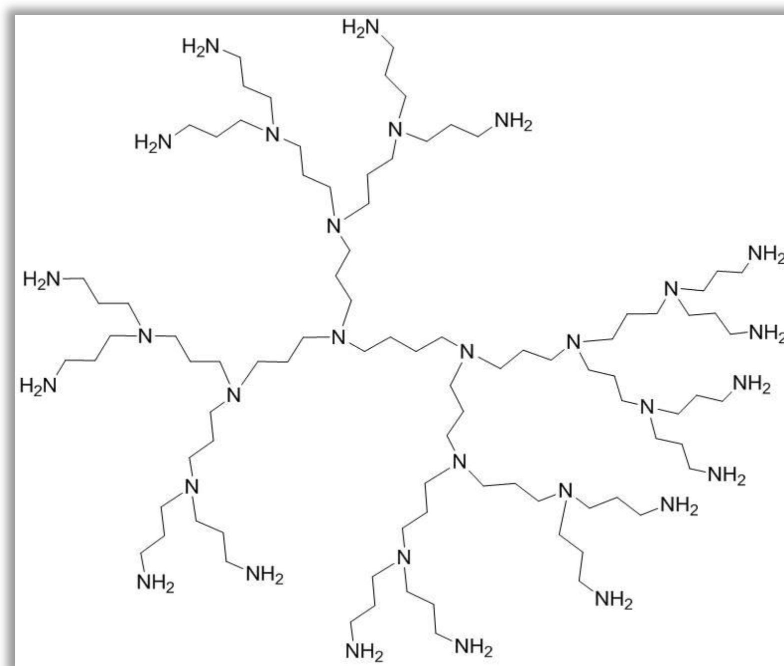

Figure 1.17: PPI dendrimers (G3).

The complexation process between dendrimers and nucleic acids starts by the interaction of dendrimers with all types of nucleic acids such as DNA, RNA and antisense oligonucleotides via electrostatic interactions. The formed complexes will condense the nucleic acid and protect it from degradation (Tang and Szoka. 1997) (Figure 1.18). During complexation, the extended configuration of the nucleic acid is changed and a more compact configuration results, with the cationic dendrimer amines and the anionic nucleic acid phosphates reaching the local charge neutralization resulting in the formation of dendrimer–nucleic acid complexes “polyplexes”. The nature of the complex depends on many factors such as the stoichiometry and concentration of the DNA phosphates and dendrimer-amines, the bulk solvent properties (pH, salt concentration, and buffer strength) and the dynamics of mixing. High ionic strength (i.e elevated amounts of NaCl) interferes with the binding process and appears to help to establish equilibrium. The medium in which

complexes are formed not only affects their morphology but also modifies other properties and even stability *in vivo*. With each increasing dendrimer generation, the number of surface amine groups, which are most likely to bind DNA, effectively doubles (Goula *et al.,* 1998; Dutta *et al.,* 2010).

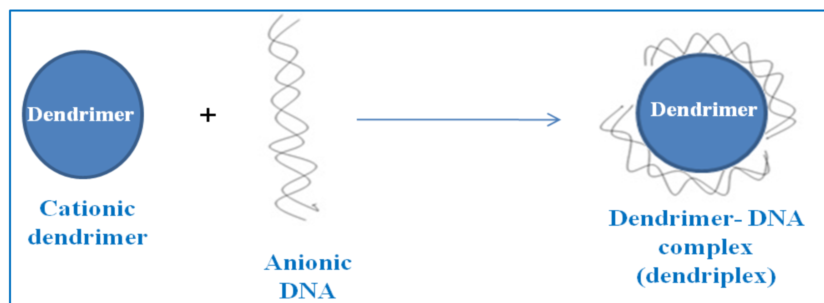

Figure 1.18: Complexation of dendrimer with nucleic acids (*Adapted from Jain et al., 2010)*.

Although dendrimers with their unique features represent an attractive potential in the field of pharmaceutical and biomedical applications, their associated toxicity due to terminal free amine groups and multiple cationic charge, limits their clinical applications (Malik *et al.,* 2000; Jain *et al.,* 2010). Higher generation dendrimers are the most toxic ones. Anionic dendrimers with carboxylated surface and non-charged ones are not cytotoxic (Biricova and Laznickova, 2009). Dendrimers and other cationic macromolecular delivery systems interact in a non-specific way with the anionic biological cell membrane. This non-selective interaction of cationic delivery system causes membrane disruption through formation of nano-holes, membrane thinning and membrane erosion followed by leakage of cytosolic enzymes and cell death (Figure 1.19) As a result, different types of toxicities are observed including cytotoxicity, haemolytic toxicity and haematological toxicity (Jain *et al.,* 2010).

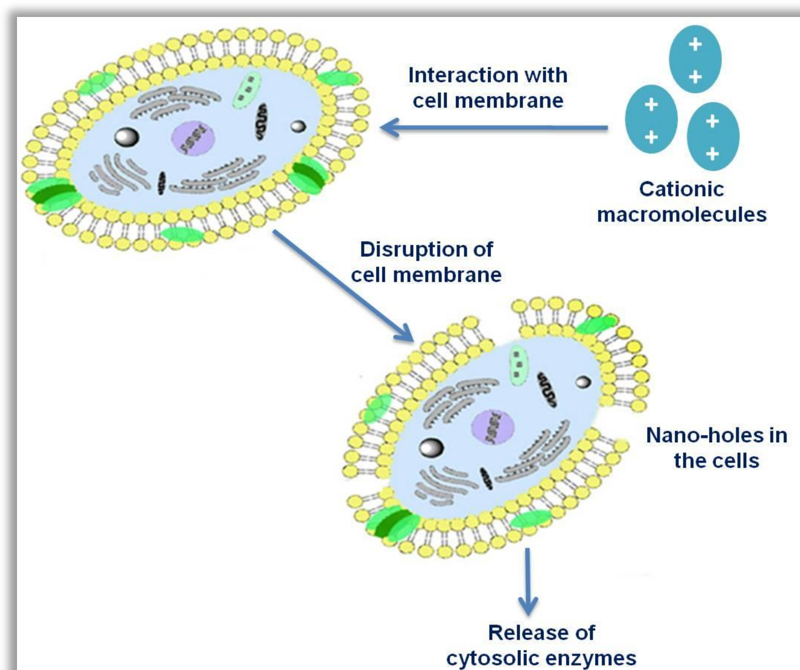

Figure 1.19: Mechanism of interaction of cationic macromolecules with biological membranes (*Adapted from Jain et al., 2010*).

Many functionalization approaches have been investigated to shield the terminal amine groups and thereby reduce the associated positive charge and resultant toxicity (Konda *et al.,* 2001; Bhadra *et al.,* 2005; Gajbhiye *et al.,* 2009). Moreover, functionalization of dendrimers has also been found to introduce valuable information on many other features beneficial in gene delivery, including modification of their physicochemical properties for favouring a suggested particular application (Dutta *et al.,* 2010). Surface modifications of dendrimers result in a change in the overall surface charge, an increase in the molar mass of the product, alterations in the generational, skeletal and substitutional distributions. To enhance the biocompatibility of dendrimers, the total surface charges have to be reduced and partially modified (Svenson, 2009). Total charge and its distribution of modified dendrimers represent an essential role in determining physicochemical properties of the final products and influence their interaction with biological entities in gene delivery applications (Shi *et al.,* 2006).

Regardless of the promising potential of dendrimers in biomedical fields, there is a great need to circumvent the cytotoxicity associated with dendrimers which limits their candidature for clinical applications. Various strategies have been proposed by different scientists to alleviate dendrimer associated toxicity (Figure 1.20) such as the development of biodegradable, less toxic dendrimers (Agrawal *et al.,* 2007), by utilizing polyether dendrimers (Hawker and Frechet, 1990; Malik *et al.,* 2000), polyester dendritic system (Bo *et al.,* 1997; Hirayama *et al.,* 2005), polyetherimine dendrimers (Krishna *et al.,* 2005), polyether-copolyester dendrimers (Carnahan and Grinstaff, 2001; Dhanikula and Hildgen, 2007), phosphate dendrimers (Domanski *et al.,* 2004), citric acid dendrimers (Namazi and Adeli, 2005), melamine dendrimers (Neerman *et al.,* 2004), triazine dendrimers (Chouai and Simanek, 2008).

Surface engineered dendrimers with various functionalities have also been reported to decrease the inherent cytotoxicity of dendrimers and protect the surface amine groups. In fact this strategy is considered one of the best approaches for reducing dendrimer toxicity and was extensively investigated (Konda *et al.,* 2001; Bhadra *et al.,* 2005; Gajbhiye *et al.,* 2009). Surface modification can be performed by many strategies such as PEGylation which reduces the cytotoxicity and haemolytic toxicity of dendrimers by modifying the amine/cationic groups with neutral or anionic functionalities (Bhadra *et al.,* 2003; Jevprasesphant *et al.,* 2003; Stasko *et al.,* 2007). PEGylation enhances other favourable characteristics of dendrimers such as improved biodistribution and pharmacokinetics, increased solubility of dendrimers, improved bioavailability and enhanced gene transfection efficiency and tumour localization (Bhadra *et al.,* 2003; Dufès *et al.,* 2005; Gajbhiye *et al.,* 2009).

Other surface modifications include the incorporation of carbohydrates into the architecture of dendrimers (Bhadra *et al.,* 2005; Agrawal *et al.,* 2007) and the acetylation of dendrimers (Stasko *et al.,* 2007; Kolhatkar *et al.,* 2007). Antibody conjugated dendrimers are another novel strategy to enhance dendrimer performance (Thomas *et al.,* 2004; Shukla *et al.,* 2006). The conjugation of tuftsin (a natural macrophage activated tetrapeptide: Thr-Lys-Pro-Arg) to dendrimers was also proposed as an approach to reduce dendrimer toxicity by many investigators (Gupta

and Haq, 2005; Bai *et al.,* 2008). Folate mediated targeting is another strategy employed in dendrimer modification to target tumour cells by utilizing receptor mediated endocytosis (RME). The concept in using folate is based on the fact that folic acid receptor is overexpressed by many types of tumour cells, such as lung, renal, ovarian, breast and brain metastases (Garin-Chesa *et al.,* 1993; Wang and Low, 1998). This concept has been utilized in dendrimer-mediated cancer therapy (Choi *et al.,* 2005; Singh *et al.,* 2008).

In general, drug conjugation to dendrimer surfaces may also result in reducing their toxicity by protecting the peripheral free amine group of dendrimers (Lee *et al.,* 2006; Kolhatkar *et al.,* 2008). Similarly, complexation of dendrimers with DNA leads to significant reduction in toxicity which was attributed to the protection of dendrimer positive charges by the anionic DNA (Brazeau *et al.,* 1998; Yoo and Juliano, 2000). Another strategy involves the use of anionic or half generation dendrimers that are non-toxic or less toxic compared to full generations (Malik *et al.,* 2000; Bhadra *et al.,* 2003).

Amino acid or peptide-conjugated dendrimers are among the interesting employed strategies to show significant reduction in dendrimer toxicity combined with improvement in their gene transfection efficiency (Choi *et al.,* 2004; Okuda *et al.,* 2004; Kono *et al.,* 2005; Agashe *et al.,* 2006; Yang and Kao, 2007). This particular approach was utilized in this work and will be detailed further.

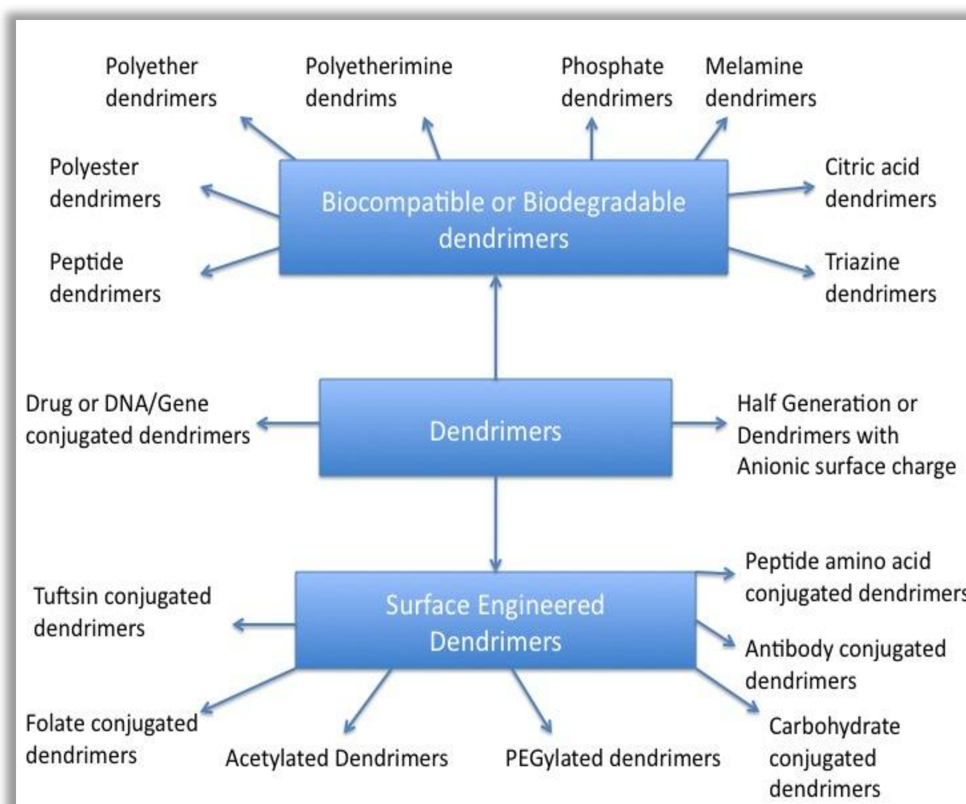

Figure 1.20: Various investigated strategies to reduce dendrimers toxicity (*Adapted from Jain et al., 2010*).

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- ***Polyamidoamine (PAMAM) dendrimers***

Polyamidoamine (Starburst®) dendrimers are spheroidal, cascade polymers. The size and the surface charge of the polymer are controlled by varying the number of generations in the dendrimers synthesis. Many surface modified PAMAM dendrimers are non-immunogenic, water-soluble and possess terminal-modifiable amine functional groups for binding various targeting or guest molecules. PAMAM dendrimers are hydrolytically degradable only under harsh conditions because of their amide backbones. As a result, their hydrolysis proceeds slowly at physiological temperatures (Lee *et al.,* 2005). The internal cavities of PAMAM dendrimers can host metals or guest molecules because of their unique functional architecture, which contains tertiary amines and amide linkages. PAMAM dendrimers are generally prepared by divergent method and products up to generation 10 (G10) have been obtained (Nanjwade *et al.,* 2009).

The use of PAMAM dendrimers for gene delivery was originally reported by Haensler and Szoka (1993). Since then, the family of PAMAM dendrimers is considered the most investigated dendrimers in gene therapy applications (Kukowska-Latallo *et al*., 2000; Arima *et al.,* 2001; Luo *et al*., 2002; Takahashi *et al.,* 2003; Svenson and Tomalia, 2005; Dung *et al.,* 2008; Waite *et al*., 2009; Kuo *et al*., 2010; Han *et al*., 2011).

PAMAM dendrimers have been utilized and examined for gene delivery systems *in vitro* and *in vivo*, extensively in contrast to PPI dendrimers which to the best of our knowledge, have been limited to a small number of investigations.

- ***Polypropyleneimine (PPI) dendrimers***

Polypropyleneimine (PPI) represents the other commercially available dendrimers for drug and gene delivery, which is based on PPI units with butylenediamine (DAB) used as the core molecule. The first cascade structure of PPI was synthesized by Vögtle and co-workers (Buhleier *et al.,* 1978), based on a repetitive reaction sequence of double Michael additions of an amine to acrylonitrile,

followed by the reduction of the nitriles to primary amines. However, large scale synthesis of polypropyleneimine dendrimers has been developed by using a modified Vögtle route, as reported by de Brabander-van den Berg and Meijer (1993). Polypropyleneimine dendrimers contain 100% protonable nitrogens (van Duijvenbode *et al.,* 1998), making them ideally suited as DNA binding and possibly DNA transporting agents. Polypropyleneimine dendrimers of higher generations; G 2–5 (DAB 8, DAB 16, DAB 32, and DAB 64 which contain up to 64 terminal amino groups) were tested as transfecting agents in gene delivery after surface modification (deBrabander *et al.,* 1996; Hussain *et al.,* 2004). Surface-functionalization was found to reduce the haemolytic activity and cytotoxicity of PPI dendrimers (Duncan and Izzo, 2005; Dutta *et al.,* 2007).

In 2002, Zinselmeyer and his co-workers evaluated polypropyleneimine dendrimers (generations 1–5: DAB 4, DAB 8, DAB 16, DAB 32, and DAB 64) as gene delivery systems against human epidermoid carcinoma cell line (A431). The molecular modelling and experimental data of the study revealed that DNA binding increased with dendrimers generation. Cell cytotoxicity was largely generation-dependent, and cytotoxicity followed the trend DAB 64 > DAB 32 > DAB 16 > DOTAP > DAB 4 > DAB 8, whereas transfection efficacy followed the trend DAB 8 =DOTAP = DAB 16 > DAB 4 > DAB 32 =DAB 64. The results obtained revealed that the lower generation (DAB 8- generation 2 and DAB 16-generation 3) polypropyleneimine dendrimers were effective gene delivery systems *in vitro*.

Therefore, in light of these data, modification approaches of the proposed vectors utilizing different techniques can provide an attractive approach for enhancing their transfection efficiency and reducing cytotoxicity, which can limit their clinical gene applications.

In an *in vivo* toxicological study, it was concluded that G5 PPI dendrimers accumulate predominately in liver and cause damage to the liver and hemapoietic system, whereas surface-functionalized dendrimers are devoid of such toxicity (Dutta *et al.,* 2008). In another work, the authors studied the *in vivo* gene transfer activity of PPI (different generations) functionalized as quaternary ammonium gene carriers. Methyl quaternary ammonium derivatives of DAB 4 (generation 1), DAB 8, DAB 16

and DAB 32 (generation 4) were synthesised to give Q4, Q8, Q16 and Q32, respectively. Quaternisation of DAB 8 proved to be crucial in enhancing DNA binding, as evidenced by data from the ethidium bromide exclusion assay and dendrimer–DNA colloidal stability data. This improved colloidal stability had a major effect on vector tolerability, as Q8–DNA formulations were well tolerated on intravenous injection while a similar DAB 8–DNA dose was lethally toxic by the same route. Quaternisation also improved the *in vitro* cell biocompatibility of DAB 16–DNA and DAB 32–DNA dendrimer complexes by about 4-fold, but not that of the lower generation DAB 4–DNA and DAB 8–DNA formulations. The study revealed that the intravenous administration of DAB 16–DNA and Q8–DNA formulations resulted in liver-targeted gene expression as opposed to the lung targeted gene expression obtained with the control polymer-Exgen 500® (linear polyethyleneimine) and a lung avoidance hypothesis was postulated. It was concluded that the polypropyleneimine dendrimers are promising gene delivery systems that may be used to target the liver and avoid the lung. Moreover, the molecular modifications conferring colloidal stability on gene delivery formulations was found to have a profound effect on their tolerability on mice after intravenous administration (Schätzlein *et al.,* 2005).

Self-assembled ternary complexes of PPI dendrimer, cucurbituril, and DNA were evaluated by Lim *et al*. (2002) as an example of a totally self-assembled gene delivery carrier. This novel non-covalent strategy for gene delivery was able to transfect mammalian cells with high efficiency and relatively low cytotoxicity.

Chisholm *et al*. (2009) reported that G3 PPI /DNA nanoparticles are capable of tumour transfection upon systemic administration in tumour-bearing mice. His data highlight the potential of these nanoparticles as a new formulation for cancer gene therapy.

Bhadra *et al*. (2005) conjugated galactose to the surface of (G4 and 5) PPI dendrimers and evaluated their potential as carriers for liver targeting of primaquine phosphate. The authors investigated haemolytic toxicity and effect on haematological parameters of these dendrimers. The results demonstrated that these carbohydrate

coated dendrimers were biocompatible and resulted in significant reduction of haemolytic toxicity compared to uncoated dendrimers.

Agashe *et al*. (2006) examined the cytotoxicity of plain G5 PPI, amino acid protected and carbohydrate-coated PPI dendrimers in two different cell lines, namely human hepatocellular liver carcinoma (HepG2) and monkey kidney fibroblast (COS-7), and observed the effect of terminal functional groups, concentration and incubation time on cytotoxicity. Authors conjugated lactose and mannose to the G5 PPI dendrimers and compared the toxicity profile of these dendrimers with that of the parent dendrimer. For G5 PPI, cell viability was investigated using different concentration of dendrimers over incubation times of 24, 48 and 72 h. Cell viability was found to decrease with increase in concentration and incubation time. The cytotoxicity was found to be concentration and time-dependent for G5 PPI and attributed to the free primary amine groups in G5 PPI and the positive charge associated with them. COS-7 cell lines showed higher cell viability as compared with HepG2 at all concentration and incubation time points. The results obtained from the study demonstrated a substantial reduction in cytotoxicity and haemolytic toxicity was observed with sugar conjugated (lactose and mannose) dendrimers, and statistically insignificant difference in haematological parameters was observed compared to the control dendrimers. Thus, it was concluded that these carbohydrate-coated dendrimers could emerge as newer biocompatible carriers for drug delivery.

In another study, Taratula and his group constructed a gene delivery system based on nanoparticles formulated with G5 PPI dendrimers to improve the effectiveness of systemic delivery of siRNA. The formed siRNA nanoparticles were mixed with a dithiol containing cross-linker molecules followed by coating them with Polyethylene Glycol (PEG) polymer, to provide lateral and steric stability to withstand the aggressive environment in the blood stream. A synthetic analog of Luteinizing Hormone-Releasing Hormone (LHRH) peptide was conjugated to the distal end of PEG polymer to direct the siRNA nanoparticles specifically to the cancer cells. The obtained results demonstrated that this modification and targeting strategy confers the siRNA nanoparticles stability in plasma and intracellular bioavailability, provides for their specific uptake by tumour cells, accumulation of

siRNA in the cytoplasm of cancer cells, and efficient gene silencing. In addition, *in vivo* body distribution data confirmed high specificity of the planned targeting delivery strategy which created the basis for the prevention of adverse side effects of the treatment on healthy organs (Taratula *et al.,* 2009).

Hao *et al*. (2009) investigated the effect of G3 PPI nanoparticles modified by Pluronic (P123) for gene delivery. Pluronic P123 is an inert carrier known for promoting various functional alterations in cells to increase the delivery and transfection efficiency of nucleic acid medicine. The prepared P123-PPI/DNA nanoparticles showed lower cytotoxicity compared to G3 PPI alone. The study concluded that the addition of free P123 during the preparation of P123-PPI/DNA nanoparticles could significantly enhance the transfection efficiency of human lung cancer cells (SPC-A1) in the presence of 10% foetal bovine serum. Therefore, P123-PPI/DNA complex nanoparticles may be a safe, efficient and promising cationic conjugate for gene delivery.

Recently, Russ *et al.* (2008) grafted-PPI (G2 and G3) dendrimers with either oligoethylenimine (OEI) via ester degradable or G2 PPI dendrimer as surface modification. They replaced the branched non-toxic OEI core unit with non-toxic low generation polypropyleneimine (PPI) dendrimers as central moiety and investigated their potential as vectors for gene delivery both *in vitro* and *in vivo*. *In vitro* transfection studies with the polyplexes were performed using both Neuro2A neuroblastoma cells and B16F10 murine melanoma cells. Results indicated that none of the compounds was cytotoxic, and grafting of either G2 or G3 core units with OEI led to enhanced transfection efficiency, regardless of the core generation. Increase of the generation of dendrimers did not improve transfection efficiency, *in vitro*. In contrast to previously published reports about PEI-induced erythrocyte aggregation, their work showed that none of the developed vectors induced erythrocyte aggregation. *In vivo*, a clear influence of the dendrimers in the tumour gene expression levels was observed, that is, higher dendrimer core generation increased gene expression, demonstrating that surface-functionalization and incorporation of ethyleneimine core are the main factors contributing to improve biocompatibility and transfection efficiency.

Tziveleka and his group (2007) investigated whether partially or fully surface-functionalized of G4 PPI dendrimers with guanidinium groups were able to condense plasmid DNA. *In vitro* studies using human embryonic kidney (HEK 293) and the monkey kidney fibroblast (COS-7) cells showed that complete replacement of primary amino groups with the hydroxylated moieties resulted in complete loss of transfection efficiency. Contrarily, functionalization of the dendrimers with guanidinium groups enhanced transfection efficiency. This enhancement was dependent on the number of guanidinium groups at dendrimer periphery and cell type. The fully guanidinylated-modified dendrimer showed the best transfection efficiency, which was attributed to an accumulation of the guanidinium group at the dendrimeric surface, improving their penetrating ability. However, cytotoxicity studies demonstrated that increase in the degree of guanidinylation increases the dendrimers cytotoxicity (Tziveleka *et al.,* 2007).

Stasko *et al*. (2007) prepared fluorescently labelled derivatives of G5 PPI dendrimers with acetylation and evaluated their cytotoxicity and membrane disruption in cultured human umbilical vein endothelial cells (HUVEC). They found a significant reduction in the acute cytotoxicity of cationic primary amine containing dendrimers, whereas the plain PPI dendrimers demonstrated drastic time-dependent changes in the plasma membrane permeability and prominent cytotoxicity.

For a successful gene delivery, it is a matter of great importance to develop an effective and safe gene delivery vector. To this end, several strategies have been investigated. Among them, the conjugation of ligands, namely amino acids to gene delivery systems seems particularly promising.

1.3.4.4 Amino acids in gene delivery

Amino acids are indispensable for protein synthesis, which are vital for cell growth and proliferation in both normal and tumour cells (Christensen, 1990; McGivan and Pastor-Anglada, 1994). Amino acid transport across the plasma membrane is mediated through amino acid transporters located on the plasma membrane. Among the amino acid transport systems, the system L-amino acid

transporter, which is a Na+-independent neutral amino acid transport system, is a key route for providing living cells including tumour cells with neutral amino acids including several essential amino acids (Christensen, 1990). Recent studies have revealed that amino acids, especially leucine, are signalling molecules which regulate cellular metabolism, gene expression, cell growth and cell survival (Hay and Sonenberg, 2004; Wullschleger *et al.,* 2006; Ishizuka *et al.,* 2008).

This work investigates the possibility of synthesizing and characterizing nanocarrier systems conjugated to three amino acids: arginine (Figure 1.21), lysine (Figure 1.22) and leucine (Figure 1.23) that can act as a vehicle to deliver therapeutic genes to tumour tissues, without compromising safety issues.

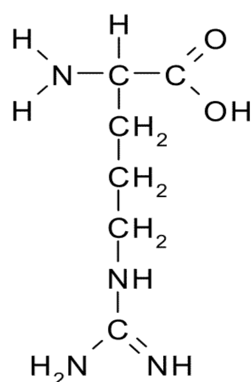


Figure 1.21: Arginine.

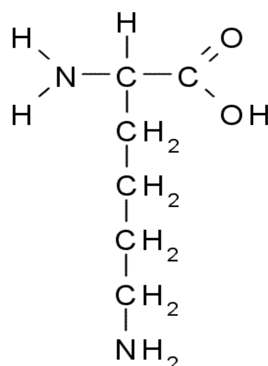


Figure 1.22: Lysine.

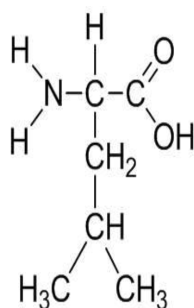


Figure 1.23: Leucine.

It has recently been demonstrated that some peptide sequences known as transduction domains or membrane translocation signals containing positively charged amino acid residues such as arginine and lysine, have been reported to have a high cell-penetrating ability (Choi *et al.,* 2004; Okuda *et al.,* 2004; Kim *et al.,* 2007; Canine *et al.,* 2008) and are known to efficiently deliver molecules (Zhao and Weissleder, 2004; Brooks *et al.,* 2005).

Many biologically active compounds, including various large molecules, need to be delivered intracellularly to yield their therapeutic action inside the cytoplasm or the nucleus. However, the lipophilic nature of the biological membranes restricts the direct intracellular delivery of such compounds. In addition, large molecules such as DNA, which are taken up via endocytosis (Vagara *et al.,* 2000) and transferred within endosomes, are subjected to degradation by lysosomal enzymes. So, although many compounds show promising potential *in vitro*, their application is limited *in vivo* due to bioavailability problems. A novel strategy to deliver these molecules involves conjugating them to peptides that can translocate through the cellular membranes, thereby enhancing their delivery inside the cell.

Over the past decade, several proteins and peptides have been found to cross the cellular membranes by a process called “protein transduction”, allowing them to deliver substances into the cytoplasm and/or the nucleus. These proteins and peptides have been used for intracellular delivery of various substances with molecular weights several times higher than their own (Schwarze and Dowdy, 2000). More accurately, their ability to translocate across the plasma membranes is confined to

short sequences of less than 20 amino acids, which are highly rich in basic residues. Such sequences are called "Protein Transduction Domains (PTDs)" or "Cell-Penetrating Peptides (CPPs)" (Gupta *et al.,* 2005). CPPs are divided into two classes: the first class consists of amphipathic helical peptides, such as transportan and model amphipathic peptide (MAP), where lysine (Lys) is the main contributor to the positive charge, while the second class includes arginine (Arg)-rich peptides (Hallbrink *et al.,* 2001).

Based on various literature reports on the significance of these positively charged amino acids to the polymeric gene delivery strategies (Nakanishi *et al.,* 2003; Gao *et al.,* 2008) we CAN hypothesize that the conjugation of these two amino acids can lead to enhancement in gene transfection efficiency of PEI and DAB polyplexes.

Okuda *et al.* (2004) reported higher transfection efficiency with low cytotoxicity of modified poly(l-lysine) dendrimer 6th generation (KG6) into several cultivated cells. The authors investigated the effect of substituting terminal cationic groups on the gene delivery into CHO cells (a Chinese hamster ovary cell line), COS-7 cells (a monkey kidney cell line), and HeLa cells (a human cervix cell line), by replacing the terminal amino acids (lysine) with arginines (KGR6) and histidines (KGH6), respectively. DNA-binding analysis showed that KGR6 and KG6 bind strongly to the plasmid DNA, whereas KGH6 showed reduced binding ability. KGR6 exhibited 3-12-fold increase in transfection efficiency into several cultivated cells compared to KG6. In contrast, KGH6 showed no transfection efficiency. This was attributed to the membrane permeability and nuclear localization ability of arginine residues conjugated to the periphery of the dendrimers.

Choi *et al.* (2004) designed a novel type of arginine-rich PAMAM as a potent non-viral gene delivery carrier. The primary amines located on the surface of PAMAM were conjugated with L-arginine to generate an L-arginine grafted-PAMAM dendrimer (PAMAM-Arg). An L-lysine-grafted-PAMAM dendrimer (PAMAM-Lys) was also synthesized and compared as a control reagent. The polymers were found to self-assemble electrostatically with plasmid DNA, forming nano-size complexes around 200 nm. PAMAM-Arg showed enhanced gene

expression in HepG2 (Human embryonic kidney 293) cells and Nuero 2A (mouse neuroblastoma) cells as well as for primary rat vascular smooth muscle cells in comparison with native PAMAM and PAMAM-Lys. The above results led the authors to conclude that the outstanding transfection efficiency with relatively low cytotoxicity and ease of preparation would make PAMAM-Arg a promising non-viral vector for both *in vitro* and *in vivo* use. Particularly, PAMAM-Arg may be employed as a dendritic carrier molecule and could entangle cargo molecules such as small molecules, peptides, proteins, oligonucleotides, and plasmids lacking cell penetrating or plasma membrane crossing capability.

Kim and his group (2006) investigated the transfection efficiency of a cationic arginine-grafted polyamidoamine (PAMAM-Arg) dendrimer in primary cortical cultures, which are known to be extremely vulnerable to exogenous gene transfection. PAMAM-Arg/DNA complexes exhibited significantly high transfection efficiencies and low cytotoxicity in primary cortical cells, as compared to other gene carriers such as, native PAMAM, branched polyethyleneimine (PEI), and Lipofectamine. Efficient transfection was not limited to neurons but extended to all three glial cells, astrocytes, microglia and oligodendrocytes, present in these primary cortical cultures. The authors concluded that the numbers of green fluorescent protein (GFP)-positive and HMGB1-negative cells indicated that PAMAM-Arg/shRNA-expressing plasmid complex suppressed target gene expression in over 40% of cells, which is the highest level achieved in primary cortical culture by any gene carrier, which demonstrates the potential of PAMAM-Arg for mediating gene delivery to primary neuronal cells.

In 2007, Yang and Kao conjugated Starburst® anionic G3.5 and cationic G4 PAMAM dendrimers with arginine-glycine-aspartate (RGD) peptides. They investigated cellular internalization of dendrimers by Fluorescein isothiocyanate (FITC)-conjugated PAMAM dendrimers in adherent fibroblasts and reported that anionic G3.5-based dendritic RGD clusters showed no negative effect on fibroblast viability as well as for cationic G4-based dendritic RGD clusters at lower concentrations. According to the authors, these amino acids dendritic RGD clusters hold great potential for tissue engineering as well as in drug delivery.

Kim *et al.* (2007) synthesized arginine-conjugated polypropyleneimine dendrimer G2 (DAB-8), PPI2-R and investigate its potential for gene delivery systems. The investigated PPI2- R condensed plasmid DNA into particles of 200 nm at a weight ratio of 4 completely, and PPI2-R polyplexes showed a fluorescence of less than 10% over a charge ratio of 2 as observed by PicoGreen reagent assay, suggesting its good DNA condensing ability. Transfection efficiency of PPI2-R was found to be high comparable to that of PEI 25kD and to be 8–214 times higher than that of unmodified PPI2 on HeLa and 293 cells. In addition, PPI2-R showed 4 fold higher transfection efficiency than PEI, treating with 10 mg pDNA because of its low cytotoxicity on HeLa cells. Finally, PPI2-R showed enhanced transfection efficiency 2–3 times higher than PEI on HUVECs, showing its potency as a gene delivery carrier for primary cells. These results demonstrate that arginine-conjugation of PPI2 is successful in developing a low toxic and highly transfection efficient gene delivery carrier.

Gao *et al.* (2008) investigated the characterization of arginine-chitosan (Arg-Cs)/DNA self-assembled nanoparticles (ACSNs) and transfection efficiency in HEK 293 and COS-7 cells. Chitosan (Cs) is a natural cationic polysaccharide that has shown potential as non-viral vector for gene delivery because of its biocompatibility and low toxicity. However, chitosan used for gene delivery is limited due to its poor water solubility and low transfection efficiency. The authors hypothesized that conjugating a positively charged amino acid residue (arginine) to chitosan nanoparticles may improve transportation into cells. The results generated from their study showed that the particle size and zeta potential of ACSNs prepared with different N/P ratios were 200–400 nm and 0.23–12.25 mV, respectively. The *in vitro* transfection efficiency of ACSNs showed dependence on pH of transfection medium, and the highest expression efficiency was obtained at pH 7.2. Moreover, the results showed that the chitosan linking arginine through NH_2 group could improve its water solubility and enhance its gene transfection efficiency. The transfection efficiency of arginine conjugated chitosan nanoparticles was much higher than that of chitosan/DNA self-assemble nanoparticles. These results suggested that ACSNs could be a safe and effective non-viral vector for gene delivery.

Yamanouchi *et al.* (2008) developed a novel family of aqueous soluble synthetic biodegradable poly(ester-amide)s (Arg-PEAs) made from three building blocks: amino acids (Arg), diols and diacids, and evaluated them for their bio safety and capability to transfect rat vascular smooth muscle cells for vascular therapy. Arg-PEAs showed high binding capacity toward plasmid DNA. All Arg- PEAs transfected smooth muscle cells with an efficiency that was comparable to the commercial transfection reagent Superfect®, but unlike Superfect®, Arg-PEAs had minimal adverse effects on cell morphology, viability or apoptosis. It was demonstrated that Arg-PEAs were able to deliver DNA into nearly 100% of cells under optimal polymer-to-DNA weight ratios, and that such a high level of delivery was achieved through an active endocytosis mechanism. A large portion of DNA delivered, however, was trapped in acidic endocytotic compartments, and subsequently was not expressed. These results suggest that with further modification to enhance their endosome escape, Arg-PEAs can be attractive candidates for non-viral gene carriers owning to their high cellular uptake nature and reliable cellular biocompatibility. It is worth mentioning that for the case of linear polymers, arginine-modified chitosan (Gao *et al.,* 2008) and arginine- based poly(ester-amide)s (Yamanouchi *et al.,* 2008) high transfection efficiency were obtained indicating that these conjugated arginines still have an excellent gene delivery capabilities even though they do not exist as oligo-peptide forms (Kim *et al.,* 2009).

More recently, Kim *et al.* (2009) investigated arginine-grafted bioreducible poly(CBA-DAH) (ABP) polymer for non-viral gene delivery systems. The study showed that the synthesized polymer ABP was able to condense pDNA into nano-sized (less than 200 nm) with positive charge. It was found the biodegradation of ABP by reductive cleavage can facilitate the efficient release of pDNA from polyplexes and reduce its cytotoxicity. The cytotoxicity of ABP was examined to be very low, as anticipated. ABP showed greatly enhanced transfection efficiency in comparison with unmodified poly(CBA-DAH) and PEI 25k in mammalian cells. The cellular uptake profile of ABP polyplex revealed no significant difference from that of poly(CBA-DAH), suggesting that ABP can act as an endosome buffer itself, and the enhanced transfection efficiency of ABP is not induced by its high cellular

penetrating ability but may be mediated by other factors such as good nuclear localization capability.

Furthermore, many studies have established that hydrophobic moieties affect transfection activity of cationic polymers and have displayed promising results (Han *et al.,* 2001; Wang *et al.,* 2002; Kono *et al.,* 2005). Upon entering cells, the carriers must pass through a hydrophobic lipid-based plasma membrane. Lipid-containing carriers are suggested to have an enhanced compatibility with the plasma membrane, and commercially available, lipid-containing transfecting agents, INTERFERin® and Lipofectamine 2000® are more efficient in delivering siRNA, in comparison to jetPEI® and Metafectene®. Apparently, the hydrophobic moieties could improve the complex–plasma membrane interactions, and facilitate the endocytosis (Alshamsan *et al.,* 2009). In addition, several hydrophobic moieties such as linear fatty acids (Lorenz *et al.,* 2004; Wolfrum *et al.,* 2007), lithocholic acid (Lorenz *et al.,* 2004), cholesterol (Lorenz *et al.,* 2004; Soutschek *et al.,* 2004; Morrissey *et al.,* 2005), and α-tocopherol (Nishina *et al.,* 2008) have been conjugated to siRNA to enhance gene delivery (Jeong *et al.,* 2009; Tseng *et al.,* 2009). Toward this end, cationic polymers have also been substituted with various hydrophobic molecules to improve their gene delivery efficiency. Similar to electrostatic forces, hydrophobic interactions play an essential role in the gene delivery procedure, and the incorporation of hydrophobic chains can affect not only the interaction with the plasma membrane, but interactions at most steps during the whole gene delivery process (Liu *et al.,* 2010). In addition to electrostatic forces, hydrophobic interactions may also be involved in the process of complex formation between genes and hydrophobized polycations, as the inclusion of hydrophobic functionalities might cause a supportive binding of genetic materials (Kuhn *et al.,* 1999; Liu *et al.,* 2010).

In a study performed to deliver siRNA using hydrophobically oleic and stearic acid-modified PEIs, Alshamsan *et al.* (2009) suggested that electrostatic interactions might not be the only mechanism behind the complex formation of the modified PEIs with siRNA. According to the zeta potential results, the expected increase in the net surface charge proportional to the PEI ratio was observed only with the PEI/siRNA complexes. With the modified PEIs/siRNA, and despite the

increasing PEI ratio in the formulation, the variability in surface charge was attributed to the relatively flexible three-dimensional conformation of the grafted fatty acids. The flexibility of the fatty acids is able to create a non-uniform surface charge distribution on the particles. Although a reduction in zeta potential with increasing PEI content was not expected, it could be explained by the incremental increases in the non-cationic fatty acid content. Therefore, the flexibility of the aliphatic fatty acids was suggested to allow for physical encapsulation of siRNA, which could explain the superior condensing and protective effect of the modified PEIs over native PEI, despite the variable zeta potentials.

Another beneficial point for the hydrophobic modifications is the enhanced adsorption to cell membrane (Liu *et al.,* 2010). It was reported that hydrophobic interaction is known to play a key role in the binding of amphiphilic compounds to biological lipid membranes. Moreover, the adsorption can induce subsequent endocytosis. In general, the uptake of polyplexes into cells is said to be by adsorptive endocytosis (Nishikawa and Huang. 2001). Up to this point, the hydrophobic modifications of polycations should enhance their adsorption to the cell membrane, and facilitate adsorptive endocytosis (Wong *et al.,* 2007). The cellular entry of hydrophobized polycation/gene complex through nonspecific adsorptive endocytosis is shown in (Figure 1.24). Incani *et al*. (2007) found that the impartment of palmitic acids to PEI and PLL led to a higher binding of the hydrophobized polymers to bone marrow stromal cells, in comparison to native polymers.

Several reports have demonstrated the ability of hydrophobic modifications to alleviate serum inhibition occurred with cationic polycations (Nguyen *et al.,* 2000; Kuo *et al.,* 2003; Bromberg *et al.,* 2005; Eliyahu *et al.,* 2005).

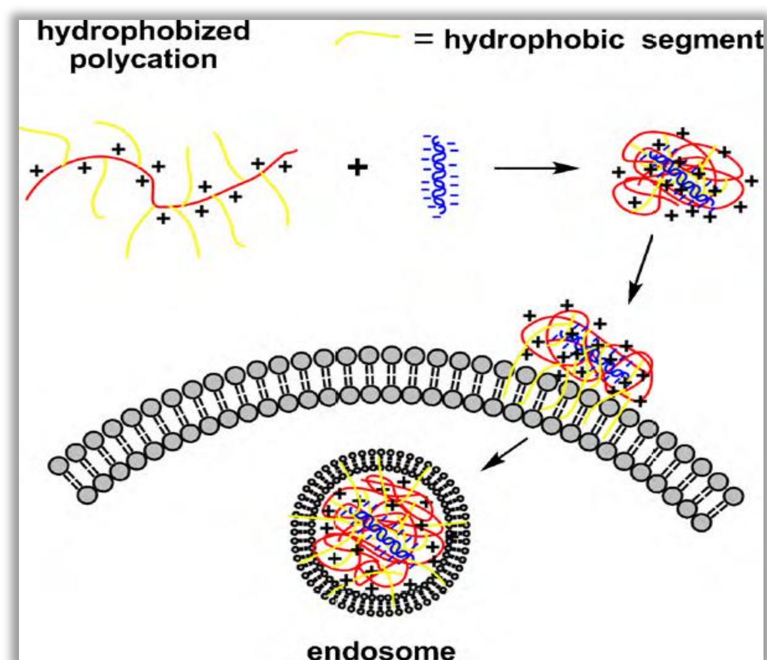


Figure 1.24: Cellular entry of modified hydrophobized cationic gene carriers via adsorptive endocytosis (*After Zhang et al., 2010*).

Another favourable characteristic to hydrophobic modification is their ability to facilitate gene dissociation from the polycation carriers. The hydrophobic modification is expected to restrain the dissociation of vector/gene complexes to a much lesser degree than would the ionic interactions between cationic vector and anionic gene. This may lead to higher transfection efficiency, compared to polymer systems that only utilize ionic interactions (Kurisawa *et al.,* 2000).

The effects of hydrophobic modifications of polycations on cytotoxicity have been investigated by many groups. Tian *et al*. (2007) reported reduced cytotoxicity of PEI after introducing a biocompatible hydrophobic poly(γ-benzyl L-glutamate) (PBLG) moiety, and hypothesized that the PEI-PBLG (PP) shielded the high positive charge density on primary amine groups of PEI. The results showed that PP can effectively condense pDNA into nanoparticles (96 nm) smaller than those of PEI/DNA, which was caused by the hydrophobic PBLG segments compressing the PP/DNA complex particles in aqueous solution. The *in vitro* transfection efficiency of PP/pDNA complexes improved a lot in HeLa cells, Vero cells and 293T cells as

compared to that of PEI-25K by the expression of Green Fluorescent Protein (GFP) as determined by flow cytometry. Cytotoxicity studies by MTT assays suggested that the modified PP had much lower toxicity than PEI. The authors concluded that the water-soluble PP copolymer showed considerable potential as carriers for gene delivery.

In another study, Agashe *et al.* (2006) reported that phenylalanine and glycine amino acids conjugated dendrimers show a significant reduction in toxicities associated with dendrimers. Conjugation of these amino acids to G5 PPI dendrimers showed a considerable reduction in haemolytic toxicity. In addition, these amino acid functionalized dendrimers did not demonstrate any concentration- and time-dependent cytotoxicity. Many techniques have been investigated for decoration of dendrimers with amino acids and peptides at their surface.

Kono *et al*. (2005) designed G4 PAMAM dendrimers with phenylalanine or leucine residues at their periphery to enhance efficient gene transfection through synergistic proton sponge effect. They found higher transfection efficiency in addition to lower cytotoxicity with phenylalanine-modified dendrimers in contrast with some commonly applied transfection agents.

In summary, the interest in gene therapy has arisen recently because of its great potential in treating not only inherited diseases, but also acquired conditions and most importantly, cancer. The key point in gene therapy is gene transfer to deliver therapeutic genes into the desired cells, which is followed by gene expression. An ideal delivery vector is essential, as the extracellular barriers (phagocytosis, degradation) as well as intracellular barriers (lack of proper recognition properties, lysosomal degradation, and dissociation from the vectors) may limit or prevent gene delivery, transcription and translation (Kabanov, 1999; Liu *et al.,* 2010). Thus, non-viral gene therapy is only possible if an improved gene expression is achieved in a way comparable to the efficient viral ones.

1.4 Aims and objectives

Currently, developing a safe and efficient delivery system is a major challenge for gene therapy. The optimal delivery strategy aims to design a carrier that delivers genes specifically to the desired tissue site, facilitate the cellular uptake of genes within target cells and promote efficient intracellular trafficking without exerting any immunogenicity or toxic side effects. While viral carriers can produce efficient gene expression, they also have serious disadvantages, such as high immunogenicity, random genomic integration, and severe limitations in the size of foreign transgenes that they can carry (Smith, 1995). In contrast, non-viral vectors have low immunogenicity, greater adaptability, the capacity to handle larger sizes of genes, and a potential for large-scale manufacture. On the other hand, the non-viral carriers have some problems in the areas of substantially low gene expression and toxicity (Liu *et al.,* 2010).

Therefore, this work focuses on designing non-viral nanocarrier systems that can deliver therapeutic genes in a safe and effective way. We hypothesize that the grafting of amino acids to the non-viral delivery systems (polyplexes) may significantly improve the efficacy of gene expression and reduce the toxicity of delivery systems, currently a limiting factor in the development of gene therapeutic strategies. To this end, the aims and objectives of this work were:

1. To prepare and characterize amino acid-bearing PEI and DAB polyplexes.
2. To evaluate their transfection and therapeutic efficacies *in vitro* on A431 human epidermoid carcinoma and T98G human glioblastoma cell lines.
3. To evaluate their transfection efficacy *in vivo* after intravenous administration in mice bearing A431 tumours.

In Chapter 2, the physical characteristics of the prepared systems were investigated utilizing NMR analysis to confirm the conjugation of the amino acids to the systems. Size and zeta potential were measured and optimized. The degree of DNA accessibility following complexation with the amino acid-bearing PEI or DAB formulations was assessed by PicoGreen® and gel retardation assay.

Chapter 3 and 4 investigate the *in vitro* and *in vivo* evaluations of the transfection and therapeutic efficacies of those amino acids-bearing PEI and DAB systems. *In vitro*, the transfection efficacy of the DNA carried by the amino acid-bearing PEI or PPI was assessed with a plasmid coding for β-galactosidase (pCMV βgal), using a β-galactosidase transfection assay. Imaging of the cellular uptake of the DNA carried by amino acid-bearing PEI or DAB was done by confocal microscopy and compared to the control. Anti-proliferative effect was subsequently assessed using a standard colorimetric MTT assay. The *in vivo* evaluations aim at investigating the gene expression in mice bearing subcutaneously implanted A431 tumours that were treated intravenously with a single injection (via tail vein injection) of amino acid-bearing and control PEI or DAB carrying β-galactosidase expression plasmid and analyzed for their β-galactosidase levels. The biodistribution of gene expression was also visualized by bioluminescence imaging, using an IVIS Spectrum.

Chapter 2

Preparation and characterization of amino acid-bearing polyethyleneimine and polypropyleneimine polymers

2.1 Introduction

Polymeric gene delivery carriers that have multi-functional groups modified with different moieties have gained recognition as effective gene transfer vectors (Park *et al.,* 2006; Kim *et al.,* 2009). Among non-viral carriers, PEI and lower generation 3 polypropyleneimine PPI (DAB) have gained increased attention as they mediate gene transfection via self-assembled complexes of polyplexes which condense DNA into small particles suitable for gene delivery (Kim *et al.,* 2007; Fujita *et al.,* 2008).

Despite the advantages of these two vectors, their applications to human gene therapy have been limited because of their unsatisfactory transfection ability and/or cytotoxicity. Several strategies have been utilized to improve the current status of non-viral polymeric gene delivery systems (Jain *et al.,* 2010; Liu *et al.,* 2010). In order to develop non-toxic efficient polymeric carriers, surface modification of PEI and DAB with amino acids seems particularly promising (Choi *et al.*, 2004; Fujita *et al.*, 2008; Russ *et al*., 2008; Gao *et al.*, 2008; Kim *et al.*, 2008; Canine *et al.*, 2008; Kim *et al.,* 2009). Three amino acids were employed in this study for the delivery of plasmid DNA: arginine, lysine and leucine. Arginine and lysine residues have been conjugated to PAMAM (Choi *et al.,* 2004; Nam *et al.,* 2008), chitosan (Gao *et al.,* 2008), G2 PPI (Kim *et al.*, 2007) and have shown greatly enhanced transfection efficiency compared to the native polymers. The enhancement in the transfection efficiency was attributed to the excellent membrane permeability and nuclear localization ability of arginine residues conjugated to the periphery of the polymers. Many studies have established that hydrophobic moieties affect the transfection activity of cationic polymers (Kurisawa *et al.,* 2000; Wang *et al.,* 2002; Kono *et al.,* 2005; Tian *et al.,* 2007; Liu *et al.,* 2010). The hydrophobic characteristics of the gene vectors will influence the formation and the stability of complexes between the carriers and DNA as they enhance interactions between the carriers and cells (Kono *et al.,* 2005). In addition, surface modification with hydrophobic moieties is a well demonstrated approach employed to alleviate the cytotoxicity of cationic carriers (Tian *et al.,* 2007; Liu *et al.,* 2010). Based on the previous literature, the hydrophobic amino acid leucine was also used in this study.

The aim of this chapter is therefore to investigate the influence of conjugating amino acids to branched PEI (25kDa) and G3 diaminobutyric PPI dendrimers (DAB) polyplexes on DNA condensation, their size, surface charges and morphology. To achieve this, the first part of this study was the synthesis of amino acids modified polyplexes derived from PEI and DAB. As model delivery nano-systems, we chose to use PEI and DAB, as they have been widely used for non-viral transfection *in vitro* and *in vivo* and combine strong DNA compaction capacity with an intrinsic endosomolytic activity, also known as the proton sponge effect. In particular, generation 3 diaminobutyric polypropyleneimine (DAB) has been used previously *in vivo* and showed highly promising potentials in cancer therapy (Schätzlein *et al.,* 2005; Koppu *et al.,* 2010). Based on literature reviews establishing the role of amino acids in gene delivery for cancer therapeutics, we investigated if arginine, lysine and leucine-bearing PEI or DAB would lead to an improved transfection efficacy by synergistic actions of the proton sponge effect and interactions with the cellular membranes.

Prior to biological studies, this Chapter will investigate the physical characteristics of the prepared systems. Structural characterization will be carried out using Nuclear Magnetic Resonance (NMR) spectroscopy. The propensity of the polymers used to self-assembly was assessed using fluorescence spectrophotometry and transmission electron microscopy. Size and zeta potential of the prepared systems were assessed using photon correlation spectroscopy.

2.1.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is one of the most important techniques used for structural elucidation of organic compounds. This technique is based on the fact that some elements have isotopes with nuclear spin. The atomic nucleus employed for NMR spectroscopy in this work is ^{1}H (proton, 99.98% natural abundance) which has been utilized since the beginnings of NMR spectroscopy and is considered the most sensitive to detection by NMR (Williams and Fleming, 1995; Akitt and Mann, 2000). Proton NMR (also known as ^{1}H NMR) is the application of nuclear magnetic resonance in NMR

spectroscopy with respect to hydrogen-1 nuclei within the molecules of a substance, in order to determine the structure of its molecules.

When a compound containing ^{1}H is placed or positioned between the poles of an applied magnetic field, the atomic nuclei in the compound will be spinning to generate their own magnetic field. They will then be irradiated with electromagnetic radiation of the correct frequency and the nuclei will undergo a change in their spin state due to energy (radio waves) being absorbed. Upon returning to their original state, the nuclei transmit this energy, which is detected by a receiver coil (Fessenden and Fessenden, 1990).

According to their immediate environments, different atoms will absorb at different magnetic field levels to yield a characteristic NMR spectrum, or map of the compound of interest. This map is known as a proton NMR spectrum and is a plot of intensity of the absorption of radio waves energy *versus delta* values (δ). Each δ value defines the resonance position of a given atom in the spectrum expressed in units of radiofrequency (1 δ = 1 part/million of the instrument's radio-frequency). The zero value on the scale represents the frequency at which a standard compound, $(CH_3)_4Si$ (tetramethylsilane, or TMS), would absorb. This is used as a reference point. Most protons in organic compounds absorb radiofrequency and shift the signals to the left of the δ=0 value (also expressed as a shift downfield), and the distance from this value to the δ values for each signal in the spectrum is giving rise to the signal. The presence of an electronegative atom in the vicinity of a proton results in a deshielded (less electron dense) proton and less energy (smaller applied field) is required to produce a change in its spin states. Consequently, a deshielded proton will yield a signal further downfield from a proton which is not deshielded. In addition, the relative areas under the principal signals are proportional to the numbers of protons giving rise to the signal (Fessenden and Fessenden, 1990).

2.1.2 Plasmid DNA preparation

Plasmids are self-replicating, extrachromosomal circular dsDNA molecules found in almost all bacteria species. Plasmids carry genes that specify a range of functions including antibiotic resistance. Since the 1970s, vectors for propagation, manipulation and delivery of a specific DNA sequence have been constructed with fragments from *Escherichia coli* (*E. coli*) plasmids. All plasmid vectors contain three common features: a replicator, a selectable marker (usually a gene encoding resistance to a particular antibiotic) and a cloning site. Transformation of *E. coli* with a high copy number plasmid can produce hundreds of clones per bacterial cell. These can be readily separated out from chromosomal DNA, owing to their relatively small size (Feinbaum, 1998).

The process of plasmid preparation and purification is performed according to the follow procedure. Plasmid DNA encoding β-galactosidase (pCMV β-Gal) was grown in *Escherichia coli* (using 100 mg/mL ampicillin as selective antibiotic) at 37° C with shaking in complete Luria-Bertani (L-broth) medium containing essential nutrients in addition to ampicillin to allow efficient proliferation (according to QIAGEN manufacturer's instructions). The culture was then harvested by centrifugation for 12-16 h after incubation, achieving a density of ~ $2x10^9$ cells / mL (Wilfinger *et al.,* 1997).

The next step is the extraction of plasmid DNA which is carried out via cell lysis. Cell lysis can be performed by exposing the cells to chemical agent usually ethylenediamine tetraacetate (EDTA) that affect the integrity of bacterial cell membranes. EDTA works by removing magnesium ions which is essential for the cellular envelope's structural integrity. It also prevents DNA degradation by inhibiting the appropriate cellular enzymes. However, anionic detergent sodium dodecyl sulphate (SDS) is often used in combination with NaOH, and is called alkaline lysis. The high pH (pH 12) serves to denature genomic DNA by breaking down hydrogen bonds of all DNA molecules except those in the supercoiled state. RNA molecules also have the ability to compete with DNA for resin binding sites: therefore RNase is added to the lysis mixture. Sodium dodecyl sulphate (SDS) is used due to its ability to remove lipid molecules from the membrane and hence

produce cell lysis. Upon bacterial cell lysis, a cell extract is produced which must be purified to remove the cellular components and collect the purified plasmid DNA.

The purification process of the plasmid DNA was carried out using a QIAGEN endotoxin-free Giga Plasmid Kit according to the manufacturer's instructions. Removal of cellular components such as cell membrane debris, RNA, proteins and carbohydrates can be easily carried out using an anion-exchange column. The presence of these components may cause fever and endotoxin shock syndrome. In addition, they may sharply reduce cell transfection efficiency (Feinbaum, 1998). The basic concept of anion-exchange column chromatography is based on the fact that DNA, unlike other components in a cell extract, carries a relatively strong negative charge. The column employed contains a resin that has molecules with high density positive charges that form strong electrostatic interactions with the negatively charged nucleic acid molecules. The neutral and positively charged contaminants are then washed through the column using a low salt buffer. To elute the DNA, a high salt buffer is used to destabilize the ionic interactions between the DNA and resin particles. Finally, DNA can be precipitated using ethanol and collected by centrifugation to yield a concentrated DNA sample.

The quantity of the purified plasmid DNA was measured by UV absorbance spectrophotometry using a GeneQuant RNA/DNA calculator at a wavelength of 260 nm, adjusted for non-specific absorbance at 320 nm. The relationship of A260 of 1.0 for a 1 cm path length = 50 $\mu g.mL^{-1}$ pure DNA was used. The total yield is calculated by multiplying the DNA concentration by total sample volume. The A260/ A280 ratio is a reliable estimate of DNA purity with few limitations, and a value of 1.8-2.0 represents a high-quality DNA sample (Wilfinger *et al.,* 1997).

2.1.3 DNA complexation studies

In gene delivery, the vector used has to be able to condense DNA. The cationic moieties of the vectors such as amino groups will react with the phosphate groups of DNA via electrostatic interactions. This results in decreasing internal charge repulsions within the DNA backbone and consequently DNA condensation

(Labat-Moleur *et al.,* 1996). DNA condensation is an essential step in developing a gene delivery vector for systemic applications that produce stable complexes with variable molecular properties (size and charge) to yield a more desirable biodistribution and cellular uptake that ultimately lead to a better gene expression (Singer *et al.,* 1997).

Apart from enhancing cellular uptake of the genetic materials, the gene delivery vehicles have to protect the DNA from degradation by the hostile environment of circulation (Gao and Huang, 1995). In this study, to ensure that PEI and DAB systems were capable of complexing DNA, both gel retardation and PicoGreen® assay studies were carried out.

2.1.3.1 Gel retardation assay

The DNA condensation ability of gene delivery systems is evaluated by an agarose gel retardation method that uses ethidium bromide as a DNA intercalator. This method is based on the concept that upon intercalation of ethidium bromide (a planar aromatic dye) with DNA, a significant increase in the fluorescence intensity of ethidium bromide is observed. When DNA condensation occurs, the ethidium bromide is excluded from binding to the DNA, and a decrease in fluorescence intensity of ethidium bromide is observed. When applied to agarose gel under the influence of an electric field, the DNA/vector complexes as well as free DNA migrate through the gel at different rates dependent on the molecular weight and DNA conformation, the applied voltage and the composition of the electrophoresis buffer (Budelier and Schorr, 2001). The negatively charged substances will move through the agarose gel, while the positively charged and neutral ones will remain stationary at the origin. Visualisation of the distance of migration of plasmid bands using UV irradiation in the presence of ethidium bromide allows identification of the PEI or PPI amine: DNA phosphate molar ratio at which charge neutralisation begins to alter DNA mobility and the ratio at which the overall complexation process is complete. A fully stable condensed complex will have the ability to prevent the binding of ethidium bromide to DNA. Only when DNA is relaxed or released due to

the collapse of the complex induced by the electric current, is the ethidium bromide able to intercalate with the DNA, produce fluorescence and enable DNA visualisation on the agarose gel under UV illumination (Gershon *et al.,* 1993).

2.1.3.2 PicoGreen® assay

Fluorescent probes that interact with nucleic acids represent an important role in biophysical studies of biological macromolecules and their complexes, in a variety of biomedical assays and bioanalytical techniques (Dragan *et al.,* 2010). PicoGreen® is a fluorescent probe which binds DNA and forms a highly luminescent complex when compared to the free dye in solution. It is considered to be the most prominent fluorescent dye introduced into biomedical research nearly a decade ago (Ahn *et al.,* 1996; Singer *et al.,* 1997).

In this study, the degree of DNA accessibility following complexation with the amino acid-bearing PEI and DAB systems was assessed by PicoGreen® assay. The fluorescence of PicoGreen® significantly increases on intercalation with double stranded DNA. The electrostatic interaction between the anionic DNA and cationic groups of the polymer on formation of the DNA-cationic polymer-amino acids nanocomplex condenses the DNA and reduces the number of PicoGreen® binding sites, ultimately reducing the fluorescence intensity for the PicoGreen® solution.

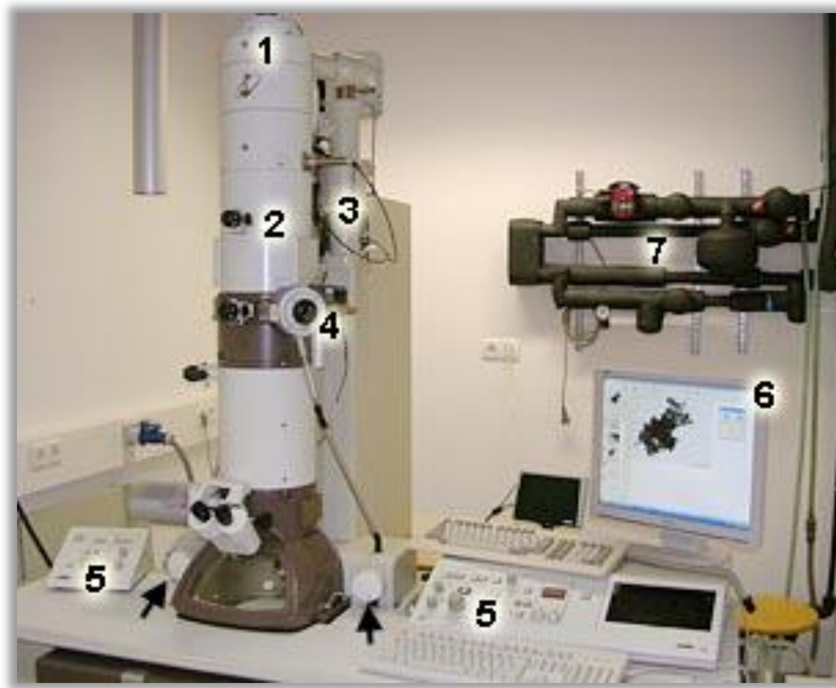
2.1.4 Fluorescence spectrophotometry

Fluorescence spectrophotometry is a type of electromagnetic spectroscopy which analyzes fluorescence from a sample. It is considered to be one of the most valuable fast analytical techniques due to its simplicity, specificity and sensitivity (Connors, 2002). It involves using a beam of light, usually ultraviolet light, that excites the molecules from its ground electronic state to an excited electronic state by absorbing energy in the form of visible or ultraviolet light, and causes them to emit this energy as radiation (light of a lower energy), thus returning to ground state. The emitted radiation is called fluorescence (Connors, 2002).

2.1.5 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a technique well utilised in the biological and physical sciences for structural characterization, especially in nano-sized samples (1-1000 nm). TEM were developed in the 1950s (Williams and Carter, 1996) as direct derivative of light microscopes which produce limited images resolution. TEM uses electron illumination instead of visible light (Watt, 1997). When a beam of electrons are transmitted across thin specimens (< 1μm), images are generated based on the interaction between electrons and the specimen (Figure 2.1).

The TEM imaging process is obtained when a well-defined beam of electrons is generated from a heated tungsten filament contained in the electron gun, with a typical accelerating voltage of 20-200 kV. A condenser lens is in place to focus the electron source. The electron beam is then passed through the specimen stage, in which samples are supported on a thin circular metallic mesh or specimen grid usually 3 mm in diameter. Before collecting the images via a photographic recording camera, a projector lens is optimized to reduce or magnify the images. This is the most important lens in TEM since it focuses and constructs the images (Watt, 1997).



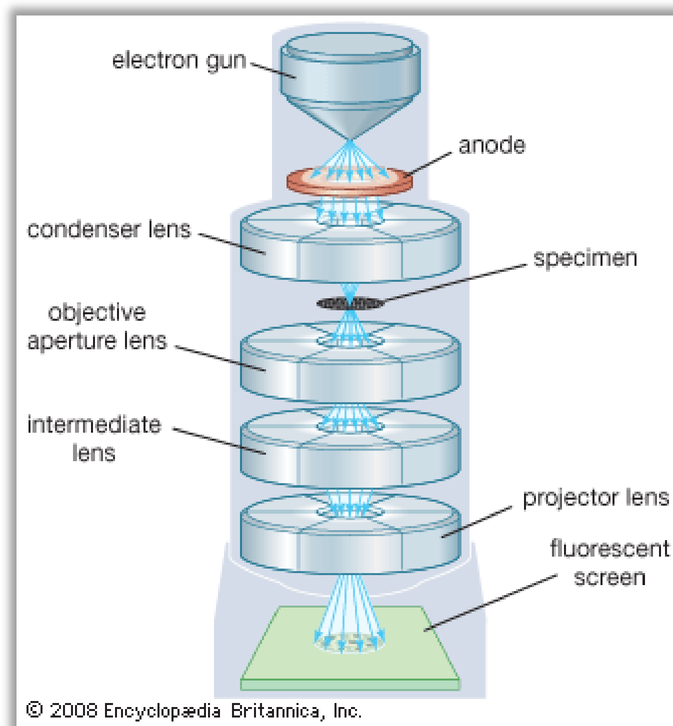


Figure 2.1: A brief illustration of TEM. (1) Electron gun, (2) Condensor lens, (3) Vacuum pumps, (4) Specimens, (5) Operation panels, (6) Image display, (7) Cooling system. (*Adapted from Encyclopedia Britanica, Inc.*).

2.1.6 Photon correlation spectroscopy (PCS)

Size distribution and zeta potential are essential factors in the characterization of any vector/DNA complex gene delivery systems that determine the behaviour of these systems *in vitro* and *in vivo*. Photon correlation spectroscopy (PCS) or dynamic light scattering employs time variation of scattered light from suspended particles under Brownian motion to obtain their hydrodynamic size distribution. It is considered the most popular technology in sizing nanoparticles. It utilizes a laser beam to probe a small volume of a particle suspension (Malvern Instruments Ltd., 2004). As the particles are undergoing Brownian motion, interference between scattered light produces a fluctuation in the intensity with time at the detector (normally a photomultiplier). This temporal fluctuation, containing information on the motion of particles, is registered and processed by a digital correlator (Filella *et al.,* 1997). Brownian motion is a constant random movement of particles due to the interaction with solvent molecules that surround them and the larger the particles, the slower this motion. PCS calculates the diameter of particles (usually ranging from 1 to 5000 nm) from the velocity of Brownian motion (known also as translational diffusion coefficient) under controlled temperature and viscosity. As the diameter obtained by PCS refers to how a particle diffuses within a fluid, measurements were referred to as hydrodynamic diameter (d (H)).

$$d\,(H) = (\kappa T)/(3\,\pi \eta D)$$

where, d (H) = hydrodynamic diameter, κ = Boltzmann's constant, T = absolute temperature, η = viscosity, D = translational diffusion coefficient.

The surface charge of a particle dispersed in a suspension is determined by the ionization state of the ionizable groups on the particle surface and the pH of the medium (Shaw, 1991). When such a charged solid surface is in contact with a liquid phase, an electrical potential develops at the interface. The liquid layer surrounding the particle exists as two parts: an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated (Figure 2.2) (Goddard and Hotchkiss, 2007).

Although ions in the Stern layer are fixed in place, ions in the diffuse or mobile layer are free to migrate. The common practice is to determine the electric potential of a particle at a location away from the particle surface, somewhere in the diffuse layer. The plane between the Stern layer and diffuse layer is called the shear plane. The potential at this plane is called the zeta potential which is a very important parameter for colloids or nanoparticles in suspension (Bayraktar and Pidugu. 2006). Its value is closely related to suspension stability and particle surface morphology. Therefore it is widely used in product stability studies and surface adsorption research (Delgado *et al.*, 2007).

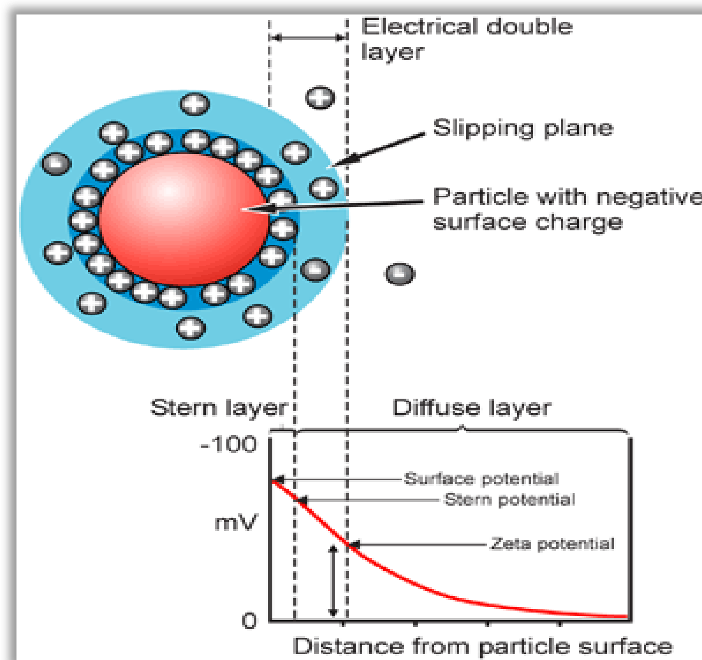


Figure 2.2: Illustration of the electrical double of a particle and the measurement of zeta potential using photon correlation spectroscopy (*After Malvern Instruments Ltd., 2004*).

2.2 Materials and Methods

2.2.1 Materials

Materials	Supplier
Branched polyethyleneimine (PEI) (25 kDa)	Sigma-Aldrich, UK
Polypropyleneimine (DAB)	Sigma-Aldrich, UK
D-Arginine (Arg)	Sigma-Aldrich, UK
D-Lysine (Lys)	Sigma-Aldrich, UK
D-Leucine (Leu)	Sigma-Aldrich, UK
Dimethylsuberimidate dihydrochloride (DMSI)	Sigma-Aldrich, UK
Triethanolamine	Sigma-Aldrich, UK
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich, UK
Deuterated water (D_2O)	Sigma-Aldrich, UK
Sephadex G75	Sigma-Aldrich, UK
Dextrose	Sigma-Aldrich, UK
Magnesium chloride	Sigma-Aldrich, UK
Mercaptoethanol	Sigma-Aldrich, UK
Quanti-iT™ PicoGreen®	Invitrogen, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, UK
Isopropranol	Sigma-Aldrich, UK
Sodium hydroxide	Sigma-Aldrich, UK
Hydrochloric acid	Sigma-Aldrich, UK
Ampicillin	Sigma-Aldrich, UK
Methanol	BDH Laboratories, UK
EndoFree Plasmid Giga Kit	Qiagen, UK
pCMVsport β-Galactosidase	Invitrogen, UK
Agarose; electrophoresis grade	Continental Laboratory Products (Northampton, UK).
Tris-(hydroxymethyl) aminomethane (TRIS) base	Sigma-Aldrich, UK
Phosphoric acid	BDH Laboratories, UK
Boric acid	Sigma-Aldrich, UK

(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) free acid, 1M (sterile)	Sigma-Aldrich, UK
Hyperladder I	Bioline Ltd, UK
5 x Loading Buffer	Bioline Ltd, UK
Glacial acetic acid	BDH Laboratories, UK
Coomassie Brilliant Blue G	Sigma Aldrich, UK

2.2.2 Synthesis of PEI and DAB polyplexes conjugated to amino acids

Amino acid coupling to PEI was performed by using dimethylsuberimidate (DMSI) as an imidoester cross-linking agent in a similar manner to that reported for transferrin-bearing dendrimers and vesicles (Dufès *et al.,* 2000; Koppu *et al.,* 2010). PEI (8.6 mg, 10 mg, 11 mg respectively for PEI-Arg, PEI-Lys, PEI-Leu) was dissolved in 2 mL triethanolamine HCl buffer (pH 7.4, 2 mL) at 25 °C and mixed thoroughly. Twelve mg dimethylsuberimidate (DMSI) were then added and stirred for one hour at room temperature. Finally, arginine, leucine or lysine (6mg) was added and stirred for another two hours at 25 °C.

Polypropyleneimine dendrimer generation 3 (DAB) was conjugated to arginine, lysine and leucine by using DMSI as a cross-linking agent as previously described (Koppu *et al.,* 2010; Aldawsari *et al*., 2011). DAB (5.7 mg, 6.9 mg, 7.7 mg respectively for DAB-Arg, DAB-Lys, DAB-Leu) was added to arginine, leucine or lysine (6 mg) and DMSI (12 mg) in triethanolamine HCl buffer (pH 7.4, 2 mL). The coupling reaction was allowed to take place at 25 ^{o}C for 2 h whilst stirring.

For both systems, the final product was purified by size exclusion chromatography using a Sephadex G75 column and freeze-dried (Epsilon 2-4 LSC freeze dryer, SciQuip Ltd, UK). The grafting of the amino acids to DAB was assessed by ^{1}H NMR spectroscopy using a Jeol Oxford NMR AS 400 spectrometer.

2.2.3 ^{1}H NMR

The prepared PEI and DAB systems bearing amino acids were then dissolved in deuterated water (D_2O) at a concentration of 4 mg/mL. The grafting of the amino acids to PEI and DAB was assessed by ^{1}H NMR and ^{1}H-^{1}H correlation Spectroscopy (COSY) experiment, using a Jeol Oxford NMR AS 400 spectrometer.

2.2.4 Plasmid preparation

Plasmids were grown in *E. coli* in the presence of the selective antibiotic ampicillin (50 μg mL^{-1}). Plasmid purification was conducted using the endotoxin-Free Giga Plasmid Kit in accordance with the manufacturer's instructions.

2.2.5 DNA condensation experiments

2.2.5.1 Gel retardation assay

DNA condensation ability of the amino acid-bearing DAB was also examined by agarose gel retardation assay. The prepared PEI and DAB polyplexes were prepared at a final DNA concentration of 20 μg/mL. After mixing with loading buffer, the samples (10 μl) were loaded on a 1X Tris-Borate-EDTA (TBE) (89 mM Tris base, 89 mM boric acid, 2 mM Na_2-EDTA, pH 8.3) buffered 0.8% (w/v) agarose gel containing ethidium bromide (0.4μg/mL), with 1x TBE as a running buffer. The DNA size marker was HyperLadder I (Bioline, UK). The gel was run at 50V for 1h and then photographed under UV light.

2.2.5.2 PicoGreen® assay

The degree of DNA accessibility following complexation with the amino acid-bearing DAB polymers was assessed by PicoGreen® assay, performed according to the protocol from the supplier. PicoGreen® reagent was diluted 200-fold in Tris-EDTA (TE) buffer (10 mM Tris, 1mM EDTA, pH 7.5) on the day of the experiment. One mL of complex polymer-DNA at various polymer : DNA weight ratios (20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1, 0:1) was added to 1 mL PicoGreen® solution. The DNA concentration in the cuvette (10 μg/mL) was kept constant throughout the experiment. The fluorescence intensity of the complexes in the presence of PicoGreen® were analyzed at various time points with a Varian Cary Eclipse Fluorescence spectrophotometer (Palo Alto, CA) (λ_{exc} : 480 nm, λ_{em} : 520 nm). Results were represented as percentage of DNA condensation (= 100 - % relative

fluorescence to DNA control) and compared with those obtained for polymer: DNA weight ratio 5:1 (*n=4*) according to the following equation:

$$F\gamma = (F_t - F_{PG}) / (F_0 - F_{PG})$$

where

F_t = fluorescence of the polymer / DNA complexes.

F_0 = fluorescence of the DNA alone.

F_{PG} = fluorescence of PicoGreen®.

Data are represented as means of four measurements (± S.E.M) and the values were plotted using Microcal Origin® v 6.0 software.

2.2.6 Transmission Electron Microscopy (TEM)

Nanoparticles of Arg-, Lys- and Leu- bearing PEI and DAB complexed with DNA were also visualized by transmission electron microscopy as previously described (Dufès *et al*., 2000). TEM was performed at College of Medical, Veterinary and Life Sciences, University of Glasgow. Formvar/Carbon-coated 200 mesh copper grids were glow discharged and specimens in distilled water were dried down with filter paper to a thin layer onto the hydrophilic support film. Twenty microlitres of 1% aqueous methylamine vanadate stain (Nanovan; Nanoprobes, Stony Brook, NY, USA) was applied and the mixture dried down immediately using filter paper. Dried specimens were imaged with a LEO 912 energy filtering transmission electron microscope operating at 120kV. Contrast enhanced, zero-loss energy filtered digital images were recorded with a 14 bit /2K CCD camera.

2.2.7 Size and zeta measurements

PEI and DAB / DNA complexes conjugated to the three amino acids were analyzed for size and zeta potential by photon correlation spectroscopy and laser Doppler electrophoresis using a Malvern Zetasizer Nano-ZS (Malvern Instruments,

Malvern, UK) equilibrated at room temperature (25 °C). Samples were prepared as in section 2.2.2 and were diluted 1:100 in 5% dextrose (adjusted to pH 7.4). Samples were then transferred to a capillary cell and all the size and zeta measurements were performed in triplicate.

2.2.8 Statistical analysis

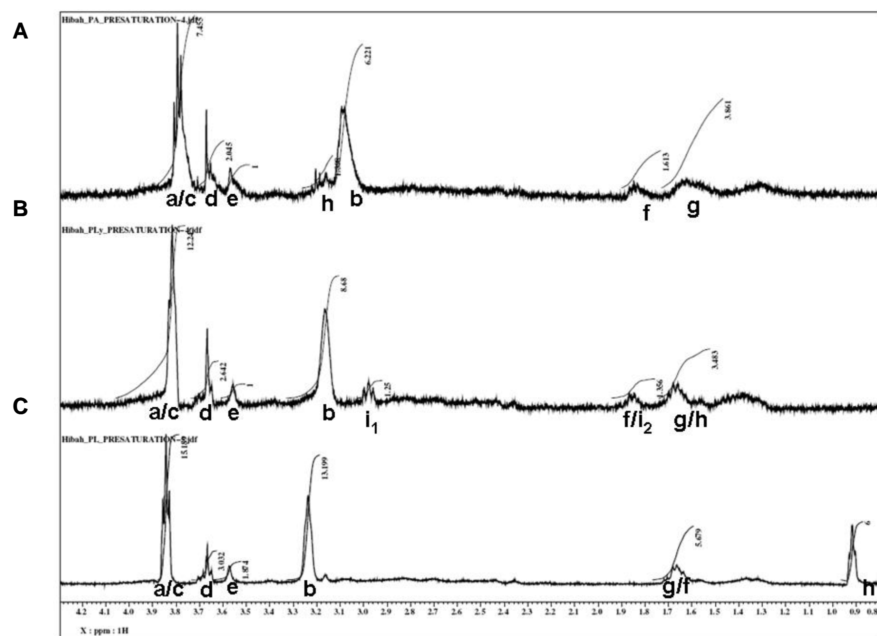
Results were expressed as means ± standard error of the mean (S.E.M). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison post-test (GraphPad Prism software). Differences were considered as significant when $P < 0.05$.

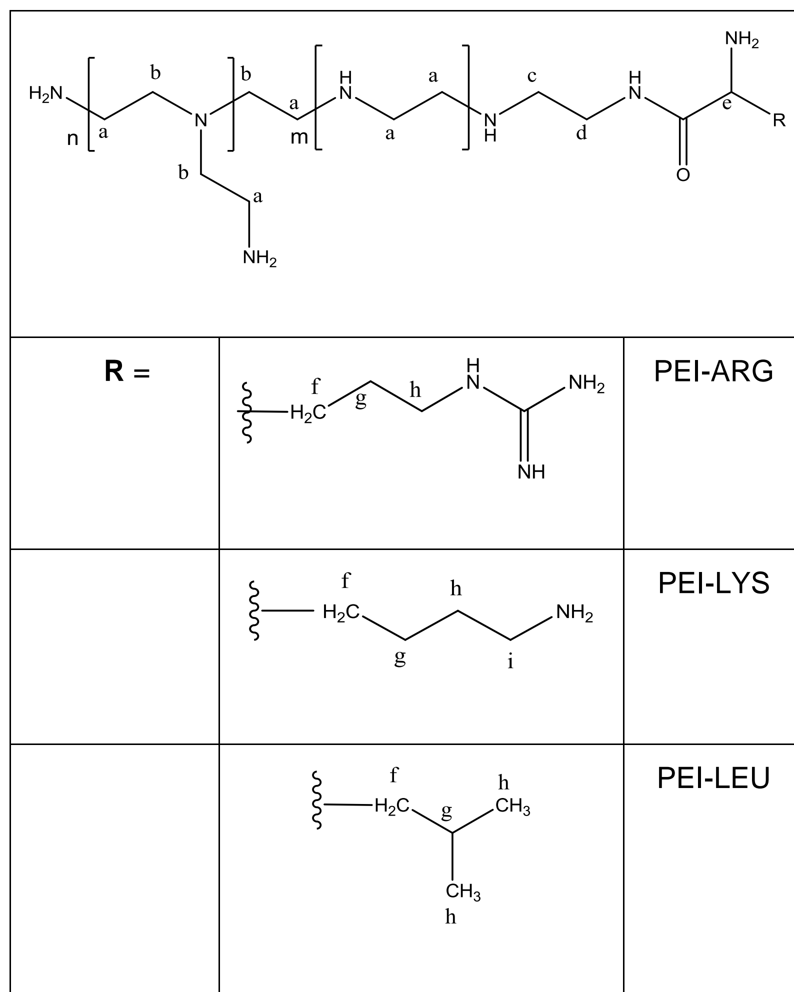
2.3 Results

2.3.1 ^{1}H NMR

The synthesis of PEI-Arg, PEI-Lys and PEI-Leu was achieved by an easy one-step process and was confirmed by ^{1}H NMR spectrum (Figure 2.3), as follows : ^{1}H NMR (D_2O): δ PEI (HN-**CH_2**-CH_2) = 3.80; PEI (N-**CH_2**-CH_2) = 3.10; PEI (O=CHN-**CH_2**-CH_2) = 3.66; δ arginine (1Hα) = 3.57; δ arginine (2Hβ) = 1.85; δ arginine (2Hγ) = 1.64; δ lysine (1Hα) = 3.57; δ lysine (2Hβ) = 1.87; δ lysine (4H) = 1.70-1.25; δ lysine (H_2N-**CH_2**) = 2.98 and 1.87; δ leucine (1Hα) = 3.57; δ leucine (2Hβ/ 1Hγ) = 1.68; δ leucine (2CH_3) = 0.92.

The number of amino acids residues conjugated to PEI was calculated by comparing the integration of the NMR proton peaks of the amino acids with that of the polymer backbone. For consistency, ratios between the integral of the amide linked methylene group at δ 3.66 and to that of the unbound ethylenimine units at δ 3.80 and 3.10 was determined. The amino acids Arg, Lys and Leu were conjugated to PEI at a level of 62 ± 3 μg /mg PEI-Arg, 91 ± 2 μg/mg PEI-Lys and 74 ± 1 μg/mg PEI-Leu. Percentage conjugation of the amino acids with the polymer was found to be 14.95%, 12.63%, and 10.68% for arginine, leucine and Lysine, respectively. The molecular weights of amino acids-PEI were then calculated to be 29 kDa for arginine and 28 kDa for both Lysine and leucine. All formulations produced high yields: 97.9% for PEI-Arg, 98.3% for PEI- Leu and 97.4% for PEI-Lys, respectively.





A

B

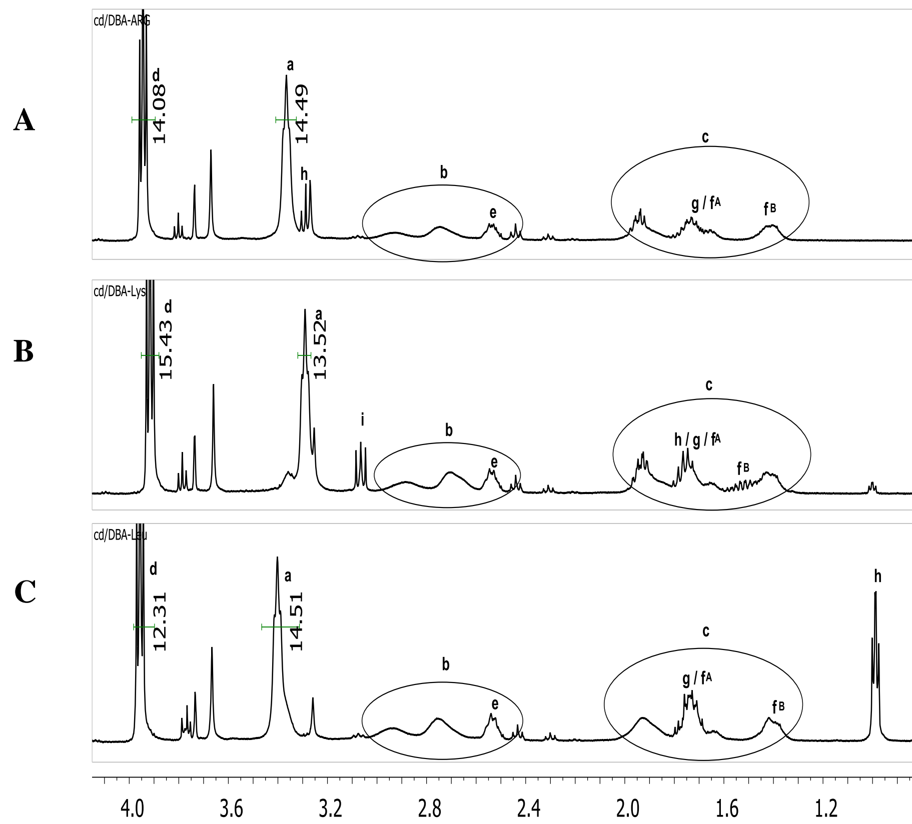
C

Figure 2.3: ^{1}H NMR spectra of PEI-arg (A), PEI-Lys (B) and PEI-Leu (C).

The synthesis of DAB-Arg, DAB-Lys and DAB-Leu was confirmed by ^{1}H NMR (Figure 2.7) and spin systems for each moiety were confirmed by ^{1}H-^{1}H COSY.

^{1}H NMR (D_2O) δ: DAB-(H_2N-**C*H₂***-CH_2-CH_2-N-) = 3.20-3.45; DAB (-N-**C*H₂***-CH_2-**C*H₂***-N-) = 2.45-3.00; DAB (O=CHN-**CH₂**-CH_2) = 3.85-4.00; Arg (1Hα) = 2.54; Arg (2Hβ) = 1.66 and 1.41; Arg (2Hγ) = 1.74; Arg (2Hω) **=** 3.23, triplet ; Leu (1Hα) = 2.54; Leu (2Hβ) = 1.65 and 1.41; Leu (2Hγ) = 1.75; Leu (2CH_3) = 0.99 (overlapping doublets); Lys (1Hα) = 2.54; Lys (2Hβ) = 1.71 and 1.54; Lys (2Hγ/2Hω) = 1.74; Lys (H_2N-**C*H₂***) = 3.06, triplet.

The characteristic broad triplet peak for the CH_2 adjacent to peripheral primary amino group of DAB at δ 2.65 was shifted to 3.20-3.45 ppm in the NMR spectrum of a conjugated DAB-amino acid analogue. An additional triplet at the region of δ 3.85-4.00 was observed which is compatible with methylene protons adjacent to an amide unit indicating that some of the peripheral amino groups had formed an amide linkage with DAB. These results demonstrated that DAB has been successfully conjugated with the respective amino acids. ^{1}H NMR spectra showed that 50% of the surface group of dendrimer DAB-16 is bound to 8 units of amino acids as signified by the ratio of the integrals of resonances at ca. δ 3.90 and 3.30 for methylene units (**d** and **a**) attached to the amide-linked bound amino acid and unbound free amine, respectively. Percentage conjugation of the amino acids with the polymer was found to be 49.28, 45.90, and 53.49 for arginine, leucine and lysine, respectively. The molecular weights of amino acids-DAB were then calculated to be 2.9kDa for arginine, 2.6KDa for leucine and 2.7KDa for Lysine. All formulations produced high yields: 99.1% for DAB-Arg, 98.9% for DAB- Leu and 99.9% for DAB-Lys, as previously observed when preparing amino acid-bearing PEI and transferrin-bearing DAB using the same simple one-step synthesis (Koppu *et al.,* 2010; Aldawsari *et al*., 2011).



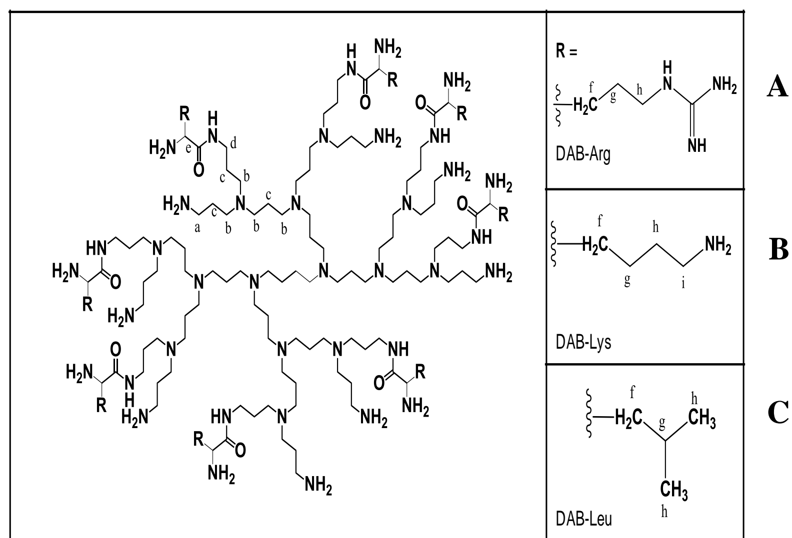


Figure 2.4: ^{1}H NMR spectra of DAB-Arg (A), DAB-Lys (B) and DAB-Leu (C).

2.3.2 DNA condensation experiments

2.3.2.1 Gel retardation assay

As previously stated, an agarose gel retardation assay can be used to visualize the N/P ratio at which DNA binding by a carriers begins to retard DNA migration (the point at which DNA collapse, or condensation start, usually occur when approximately 90% of DNA phosphate group charges are neutralised by the carrier) and the ratio at which mobile pDNA can no longer be detected on the gel (completion of the complexation process).

Based on the results obtained, the gel retardation assay confirmed the complete or partial DNA condensation by the three amino acid-bearing PEI, as demonstrated by the DNA condensation experiment. For PEI-Arg (Figure 2.5 A), at polymer: DNA weight ratios of 20:1, DNA was fully condensed, thus preventing ethidium bromide to intercalate with DNA. No free DNA was therefore visible at this ratio. However, DNA was partially condensed by PEI-Arg polymer for the other polymer: DNA ratios, thus allowing ethidium bromide to intercalate with DNA. A band corresponding to free DNA was therefore visible. At a polymer: DNA weight ratio of 10:1, PEI-Arg was able to retard plasmid DNA. PEI-Lys (Figure 2.5 B) was able to completely condense DNA at polymer: DNA weight ratios higher than 5:1, no free DNA was therefore visible at these ratios. However, DNA was partially condensed by the polymer for the other polymer: DNA ratios. It can retard DNA at a polymer: DNA weight ratio of 2:1.

PEI-Leu was able to completely condense DNA at polymer: DNA weight ratios higher than 5:1, no free DNA was therefore visible at these ratios. As seen in Figure 2.5 (C), PEI-Leu can retard DNA at polymer: DNA weight ratios smaller than 2:1.

These results demonstrated that complexes can be formed through electrostatic interactions between DNA and amino acid-bearing PEI polyplexes.

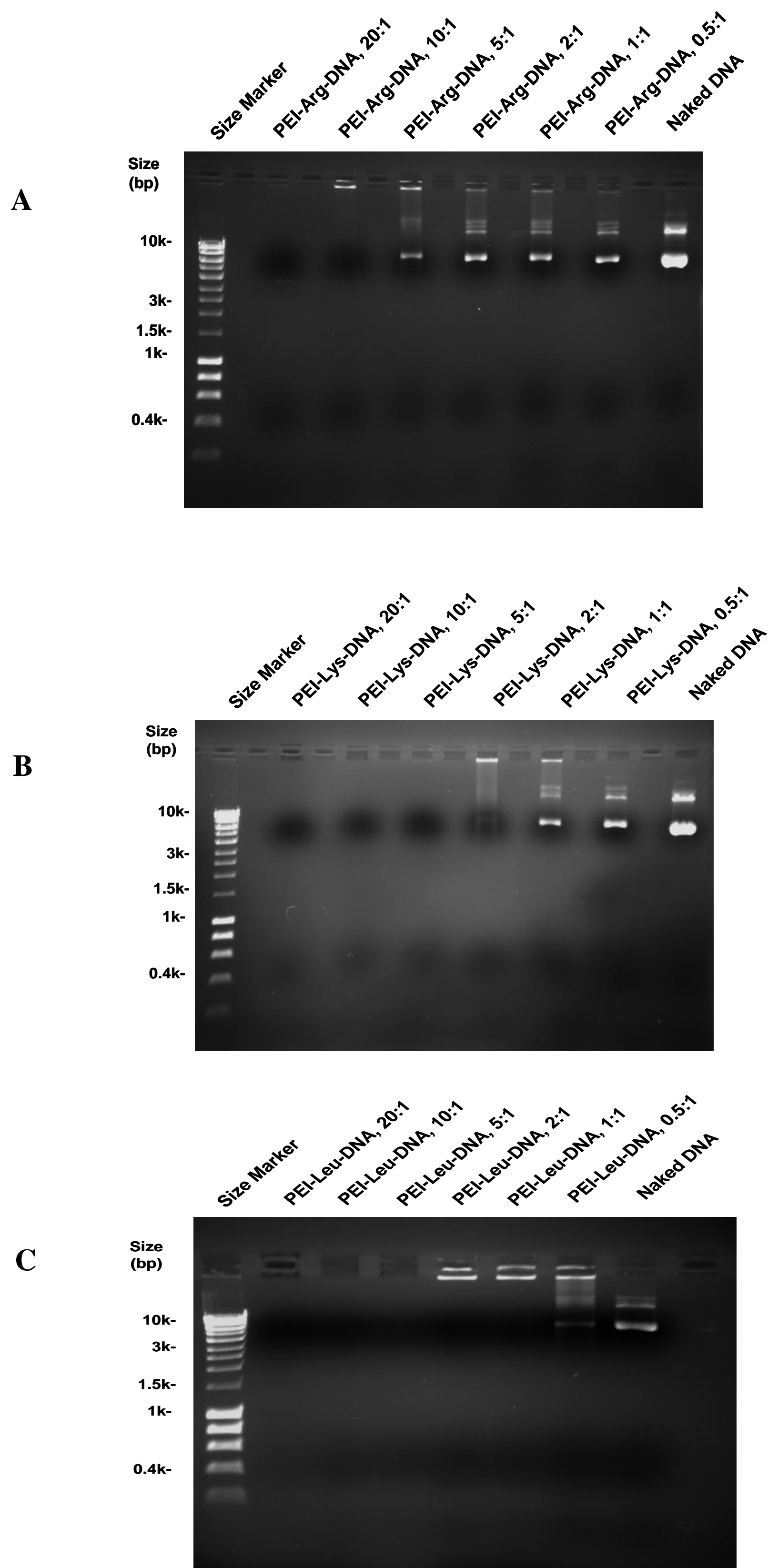


Figure 2.5: Gel retardation assay of (A) arginine, (B) lysine and (C) leucine-bearing PEI polyplex.

A gel retardation assay confirmed the complete and partial DNA condensation by the 3 amino acid-bearing DAB dendrimers (Figure 2.6). At dendrimer: DNA weight ratios of 20:1 and 10:1, DNA was fully condensed by DAB-Arg, thus preventing ethidium bromide to intercalate with DNA. No free DNA was therefore visible at this ratio. DNA was however partially condensed by DAB-Arg dendrimer for the other dendrimer: DNA ratios. Ethidium bromide could therefore intercalate with DNA and a band corresponding to free DNA was visible. DAB-Lys and DAB-Leu seemed to be able to condense DNA at dendrimer: DNA ratios higher than 1:1, as free DNA bands were only visible at a dendrimer: DNA ratio of 0.5:1. These results demonstrate that amino acid-bearing DAB can condense DNA via electrostatic interactions between the positive charges of amino acid-bearing DAB and the negative charges of DNA phosphates.

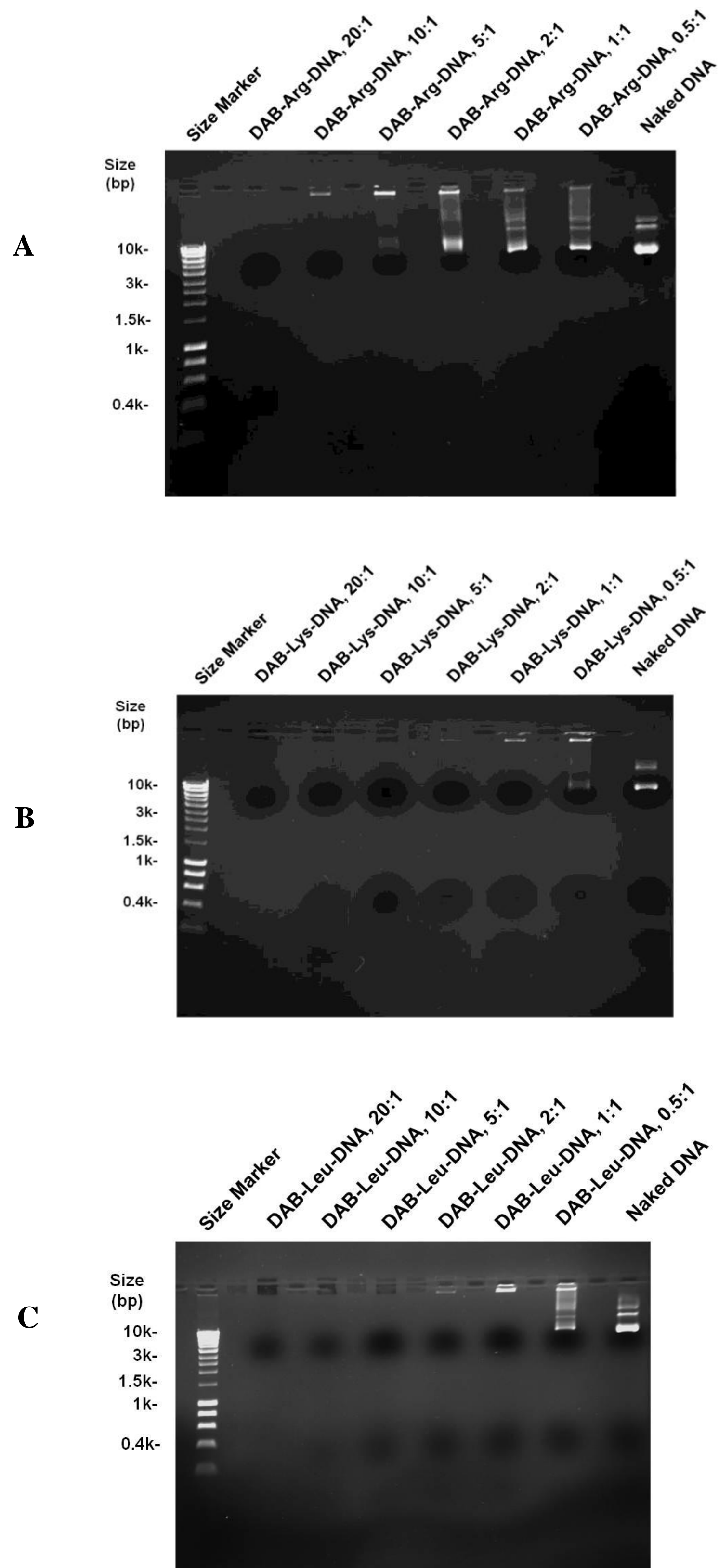


Figure 2.6: Gel retardation assay of (A) arginine, (B) lysine and (C) leucine-bearing DAB polyplex.

2.3.2.2 PicoGreen® assay

Another rapid and sensitive assay for detecting double-stranded DNA in solution is described by Singer *et al.* (1997), and is considered extremely important in a wide variety of biological applications. This assay employs a new dye, PicoGreen® reagent, which become intensely fluorescent upon binding nucleic acid.

For PEI polyplexes, the three amino acid-bearing PEI polymers were able to condense more than 80% of DNA, at polymer: DNA weight ratios higher than 2:1 for PEI-Arg, 0.5: 1 for PEI-Lys and 1:1 for PEI-Leu (Figure 2.7). DNA condensation increased with increasing weight ratios and was almost complete at a polymer: DNA weight ratio of 20:1 for the three polymers. It occurred almost instantaneously and was found to be stable for at least 24h. At the polymer: DNA ratio of 5:1, optimal for PEI polyplex, the DNA condensation was either improved for PEI-Arg and PEI-Leu, or similar for PEI-Lys compared to that observed with PEI, which was about 90% independently of the duration.

The three amino acid-bearing DAB were able to condense more than 70% of the DNA at dendrimer: weight ratios of 2:1 or higher (Figure 2.8). DNA condensation occurred almost instantaneously and was found to be stable over at least 24h. It increased with increasing weight ratios and was almost complete at a dendrimer: DNA weight ratio of 20:1 for the 3 dendrimers. The DNA condensation observed for dendrimer: DNA weight ratios of 2:1 or higher was much higher than that observed for the unmodified dendrimer, which was of 60% at its best and decreasing with time.

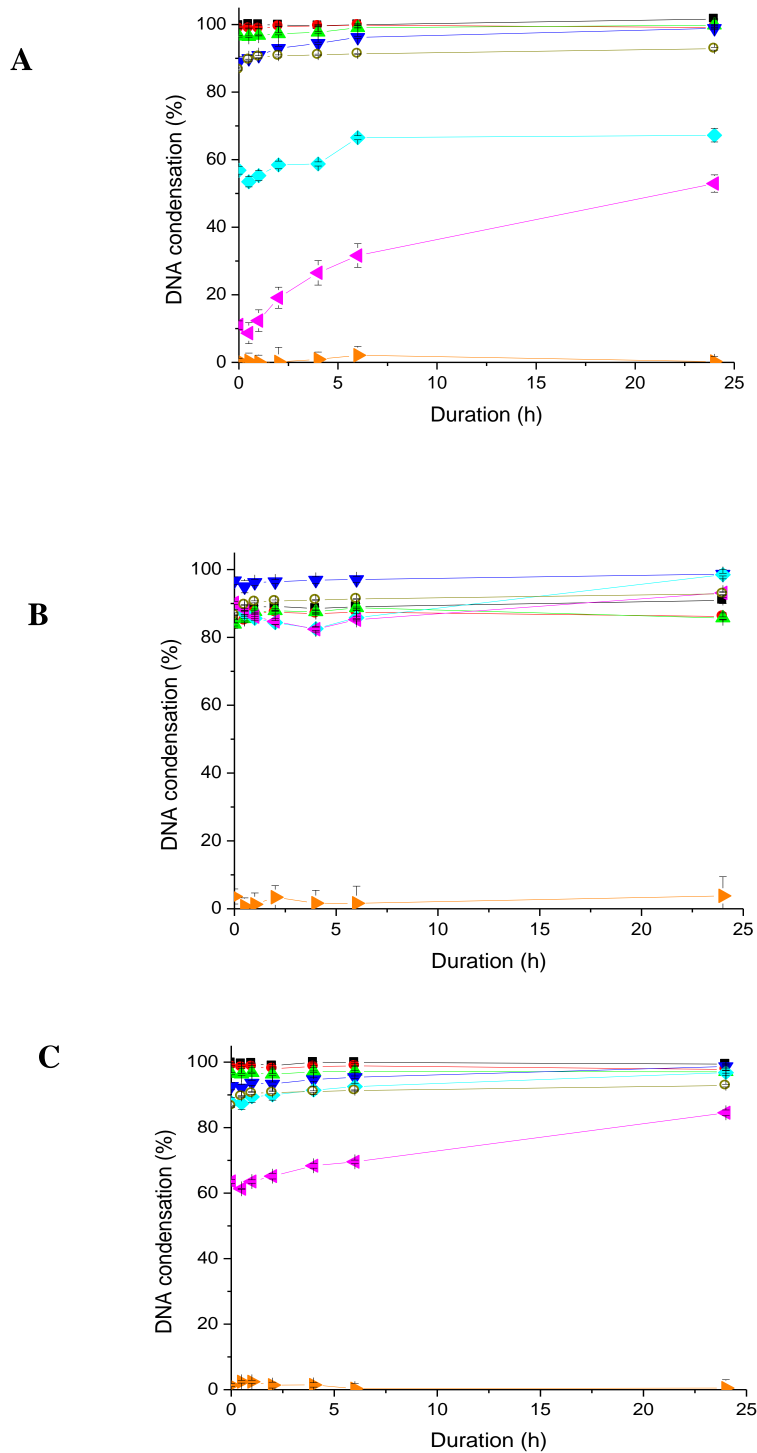


Figure 2.7: DNA condensation of PEI-Arg (A), PEI-Lys (B) and PEI-Leu (C) polyplexes using PicoGreen® reagent at various durations and polymer: DNA weight ratios : 20:1(■, black), 10:1 (●, red), 5:1 (▲, green), 2:1 (▼, blue), 1:1 (♦, cyan), 0.5: (◄, pink), DNA only (►, orange), (empty symbol, dark yellow: PEI-DNA, polymer: DNA weight ratio: 5:1). Results are expressed as mean ± SEM *(n= 4)*.

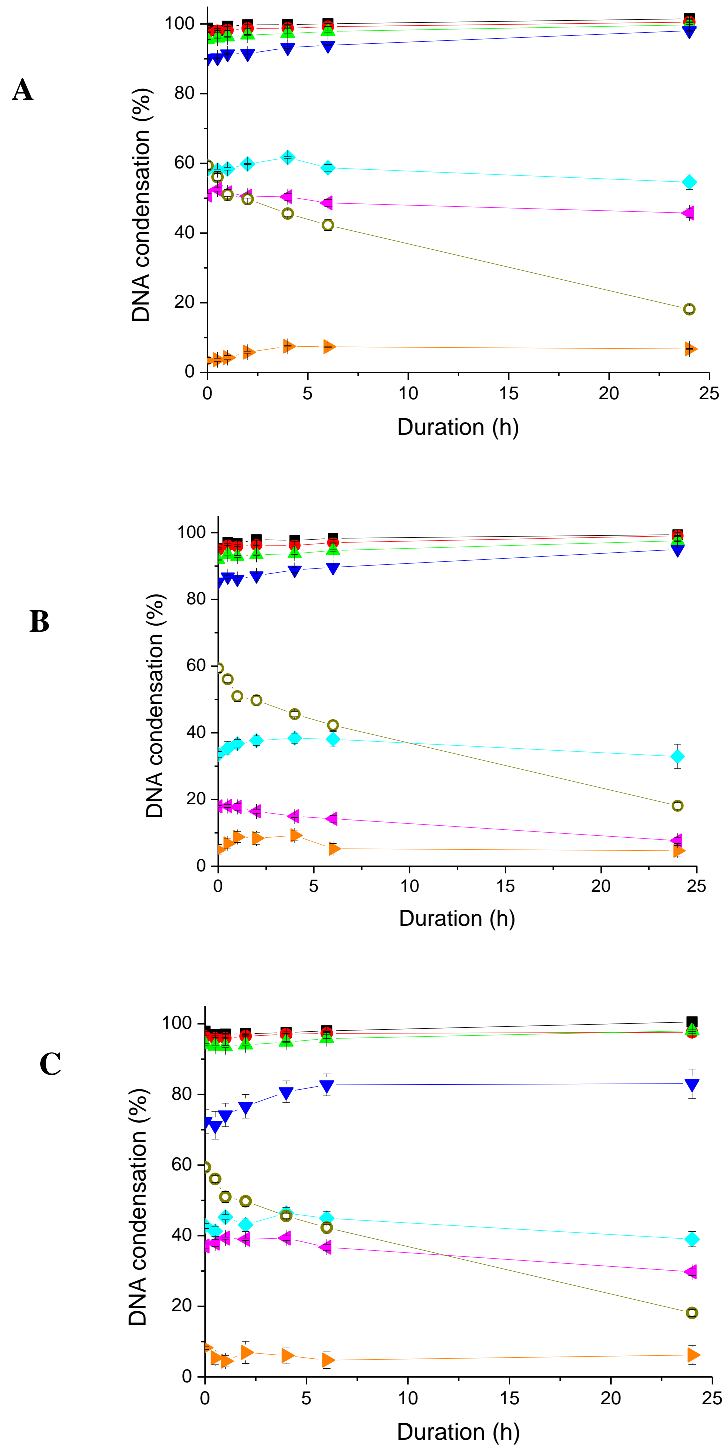


Figure 2.8: DNA condensation of DAB-Arg (A), DAB-Lys (B) and DAB-Leu (C) polyplexes using PicoGreen® reagent at various durations and dendrimer: DNA weight ratios : 20:1(■, black), 10:1 (●, red), 5:1 (▲, green), 2:1 (▼, blue), 1:1 (♦, cyan), 0.5:1 (◄, pink), DNA only (►, orange) (empty symbol, dark yellow: DAB-DNA, dendrimer: DNA weight ratio: 5:1). Results are expressed as mean ± SEM (*n*= *4*).

2.3.3 Transmission electron microscopy (TEM)

The formation of amino acid-bearing PEI (Figure 2.9) and DAB (Figure 2.10) polyplexes was visualized using transmission electron microscopy.

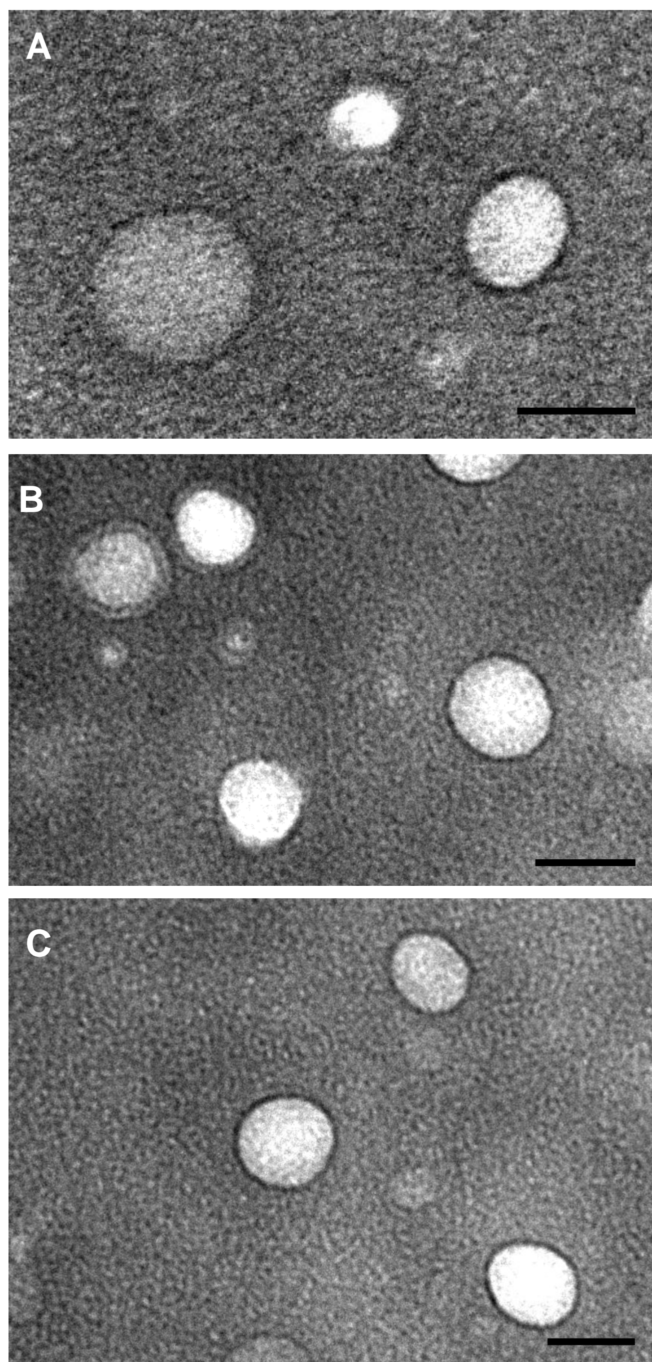


Figure 2.9: Transmission electron micrographs of (A) arginine, (B) lysine and (C) leucine-bearing PEI polyplexes (20:1 weight ratio) (Bar: 200 nm). These nanoparticles were visualized by transmission electron microscopy on a LEO 912 energy filtering electron microscope.

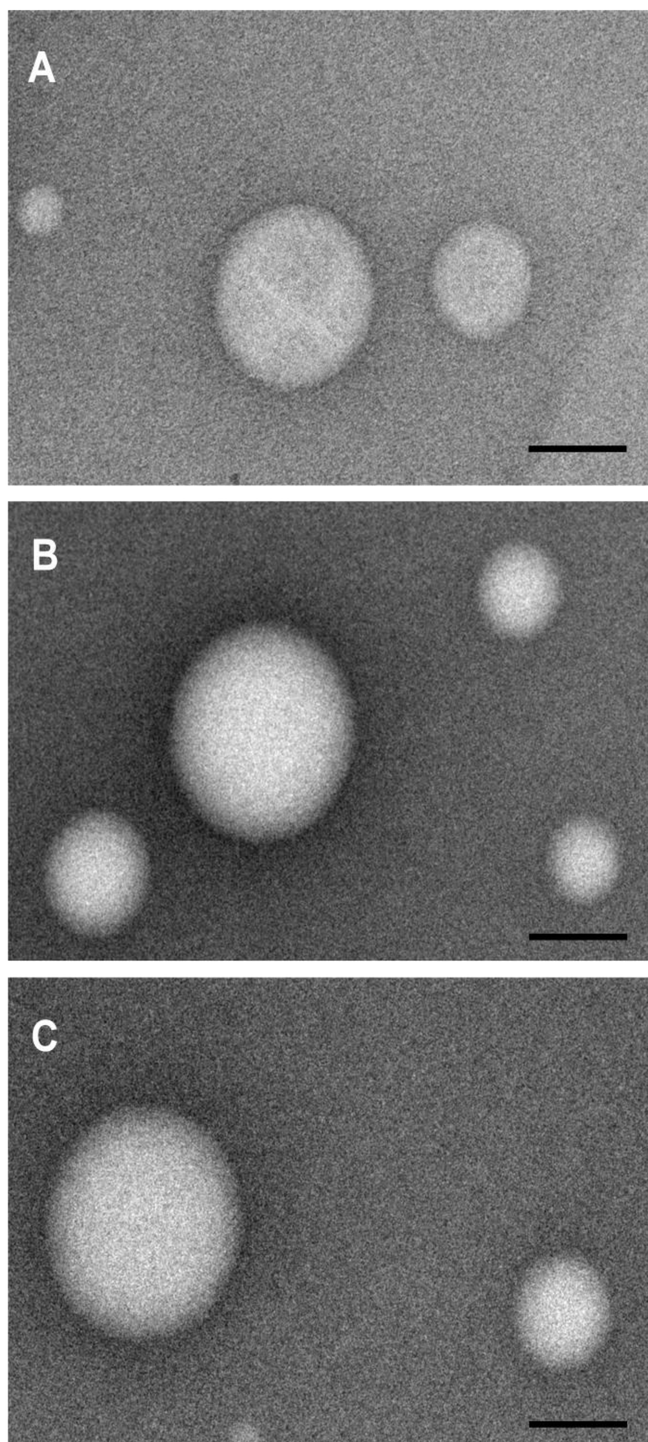


Figure 2.10: Transmission electron micrographs of (A) arginine, (B) lysine and (C) leucine-bearing DAB polyplexes (10:1 weight ratio) (Bar: 100 nm). These nanoparticles were visualized by transmission electron microscopy on a LEO 912 energy filtering electron microscope.

2.3.4 Size and zeta potential

Photon correlation spectroscopy is the most common and accurate technique to measure size and zeta potential of colloidal delivery systems to date. The sizes and zeta potentials of PEI polyplexes were examined at various polymer: DNA weight ratios ranging from 0.5 to 20. The charge screening by counter ions and resultant complex aggregation were minimized by performing the experiment in 5% w/v dextrose (Zinselmeyer *et al.,* 2002).

All of the amino acids-bearing PEI polyplexes displayed average sizes less than 305 nm at all weight ratios used. A smaller particle size were found at higher weight ratios with higher zeta potentials, suggesting that the higher charges polymer could more efficiently condense DNA and results in smaller size complexes (Figure 2.11 A). All of the polyplexes were found to have a narrow size distribution at all weight ratios examined. These results showed that the size of polyplexes is proper for gene delivery and conjugation of amino acids (arginine, lysine and leucine) does not affect the size of polyplexes significantly.

For zeta potential measurements, the polyplexes prepared showed positive zeta potential values at weight ratios above 0.5, which is due to an excess of the polycation. Zeta potential values increased gradually according to the increase of polyplex weight ratios, reaching its higher values at 20:1 weight ratios for all amino acids tested (Figure 2.11 B). These results showed that amino acids bearing PEI formed positively charged polyplexes at high weight ratios which can mediate efficient transfection, and that Arg, Lys and Leu grafting onto PEI do not affect their sizes and have advantageous favourable zeta potentials characteristics.

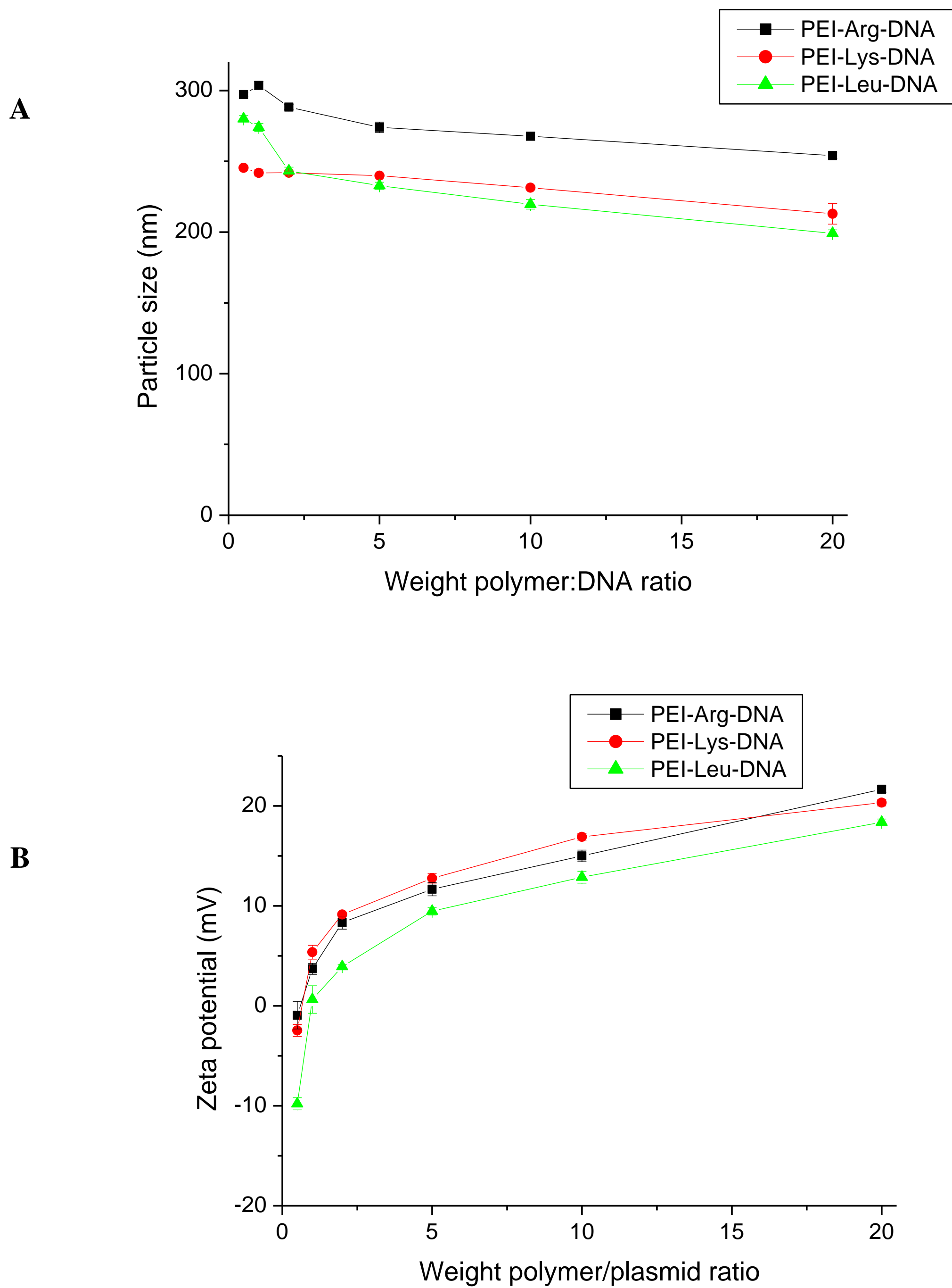


Figure 2.11: Size (A) and zeta potential (B) measurement of the polyplexes formed by polymer and plasmid DNA at different weight ratios (*n=3*).

For amino acids bearing DAB polyplexes, the sizes and zeta potentials of DAB polyplexes were examined in the same manners as for PEI polyplexes.

All of the amino acids-bearing DAB polyplexes displayed average sizes less than 300 nm at all weight ratios tested. Polyplex size decreased with increasing weight ratios, independently of the amino acid conjugated to the dendrimers (Figure 2.12 A), suggesting that the higher charges dendrimer could more efficiently condense DNA and results in smaller size complexes. Among the 3 amino acid bearing polyplexes tested, DAB-Lys polyplex at a polymer: weight ratio of 0.5:1 was found to be the largest, with an average size of 267 nm (polydispersity: 0.659), while DAB-Leu polyplex at a polymer: weight ratio of 20:1 was the smallest, with an average size of 214 nm (polydispersity: 0.363). The conjugation of amino acids to the periphery of DAB led to a slight increase of the polyplex size compared to the unmodified DAB polyplex, which had an average size of 196 nm (polydispersity index: 0.683). As the cut-off size for extravasation has been found to be 400 nm for most tumours (Yuan *et al.*, 1995), amino acid-bearing DAB polyplexes have therefore the required size to access the tumour cells.

Zeta-potential experiments demonstrated that the three amino acid-bearing DAB samples were bearing a negative surface charge (-3 mV for DAB-Arg and DAB-Lys polyplexes, -5 mV for DAB-Leu polyplex) at a weight ratio of 0.5, indicating that negatively charged DNA was not condensed yet with amino acid-bearing DAB at this ratio (Figure 2.12 B). This result was consistent with the DNA condensation and the agarose gel electrophoresis results. The zeta-potential values of the polyplexes increased with increasing weight ratios, finally reaching their maximum (24 mV, 19 mV and 16 mV respectively for DAB-Arg, DAB-Lys and DAB-Leu polyplexes) at a weight ratio of 20. These results were in accordance with the zeta potentials previously obtained when grafting Arg, Lys and Leu to PEI (Aldawsari *et al.*, 2011). The conjugation of amino acids to DAB increased the overall positive charge of the complexes compared to unmodified DAB-DNA (6 mV) for weight ratios over 2:1. The particle size decreased and the zeta potential increased with increasing polymer: DNA weight ratios, thus indicating that more positively charged polymers could more efficiently condense DNA.

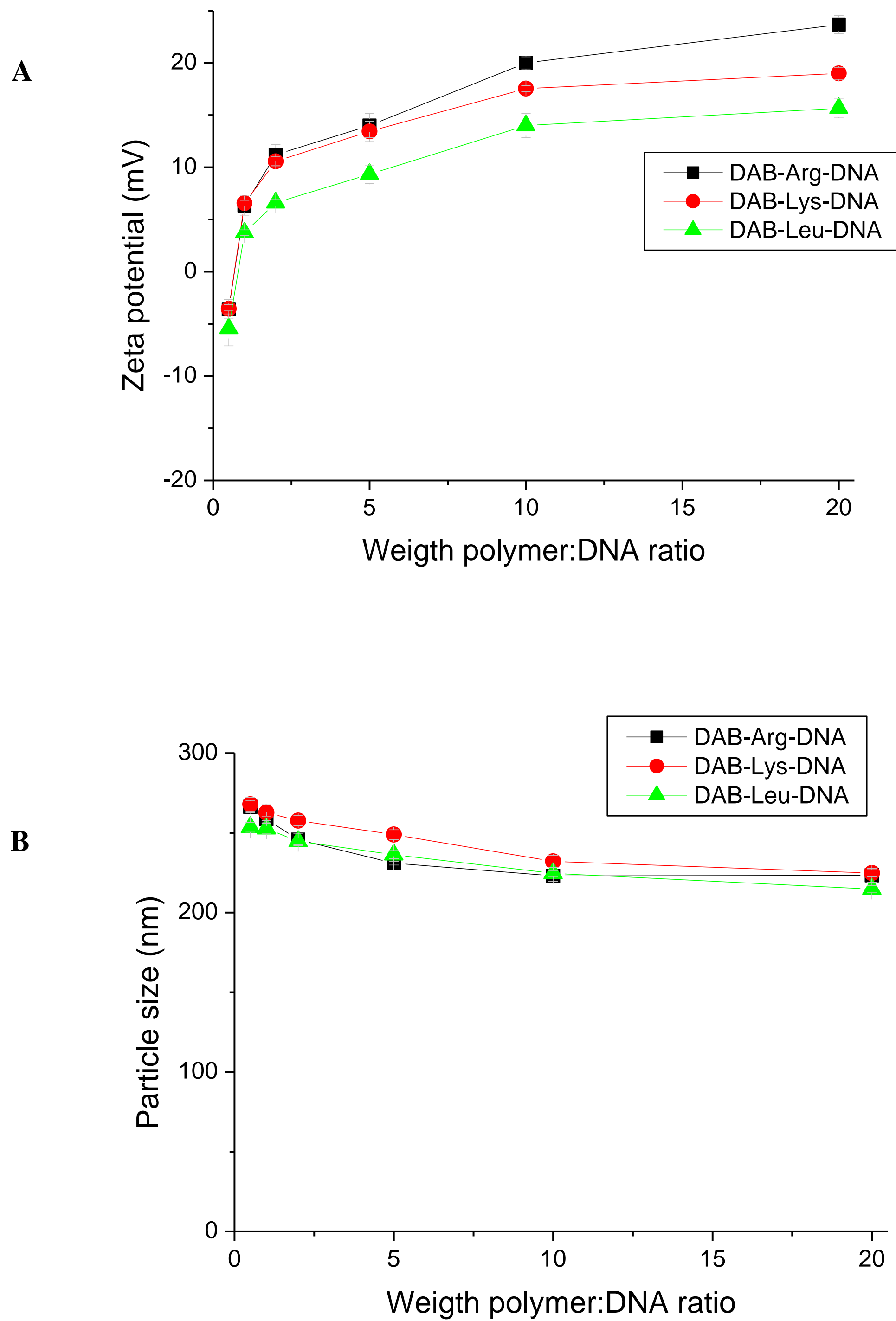


Figure 2.12: Size (A) and zeta potential (B) measurement of the polyplexes formed by dendrimer and plasmid DNA at different weight ratios (*n=3*).

2.4 DISCUSSION

Non-viral gene delivery vectors are receiving more attention for a broad variety of gene mediated therapies, particularly in cancer application. Synthetic vectors are attractive approach for pharmaceutical industry as alternatives to viral ones, because of compound stability and easy chemical modification (Kundu and Sharma, 2008). In addition, the low cost and ease of production (compared to the lengthy process of growing viruses in bioreactors followed by purification), safer profile (less immunogenic as compared to viruses) and higher flexibility make these synthetic vectors very attractive (Lollo *et al*., 2000).

For effective polyplex-mediated gene delivery, the cationic carrier has to fulfil a number of predefined criteria. The polymer has to compact and condense DNA into small size particles that can migrate through the blood circulation into the target tissues. In addition, it has to protect the DNA from degradation or undesired interaction with the biological environment, to facilitate internalization and target cell binding (Goncalves *et al*., 2002; Kundu and Sharma, 2008). The carrier should as well facilitate the progress of endosomal escape, trafficking via cytoplasmic environment and localizing into the nucleus (Kundu and Sharma, 2008). Unfortunately, no polymer is able to carry out all the extracellular and intracellular criteria. Therefore, additional functional moieties have to be included into the polyplex formulation.

Currently, many polymers have been specifically designed for use as gene delivery vectors. In many cases, the polymers were designed to address one of the perceived gene delivery obstacles. For example, DNA packaging and stability *in vivo,* biocompatibility and endosomal escape have been suggested as design criteria (Kundu and Sharma, 2008).

On the basis that amino acid residues such as Arg, Lys, and Leu were involved in enhancing DNA transport into cells, we hypothesized that the grafting of these amino acids to PEI and DAB polymers would improve their transfection efficacy in cancer cells. Previous studies have reported the use of amino acids with different non-viral cationic carriers with the aim of improving their transfection

efficacy and minimizing their toxicity (Choi *et al.*, 2004, Dong *et al.*, 2006, Kim *et al.*, 2007, Ahn *et al.*, 2008).

In our study the conjugation of amino acids to the PEI and DAB polymers was done with cross-linker dimethylsuberimidate (DMSI) as previously reported by Koppu *et al.* (2010). DMSI belongs to imidoester crosslinker which is a membrane-permeable that contains an amine-reactive imidoester group at each end (Figure 2.13). It reacts with primary amines to form amidine bonds (Wang and Moore, 1977).

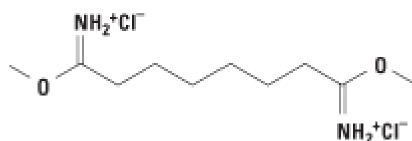


Figure 2.13: Dimethylsuberimidate (DMSI).

In comparison to a similar studies done by Kim *et al*. (2007), who grafted arginine to polypropyleneimine (PPI) dendrimer using N,N-diisopropylethylamine (DIPEA) as linking agent, the technique used in their study was complex, time consuming and involved using many organic solvents. The dialysis time described in this publication (4h) would not be long enough to thoroughly purify the final products which may take up to 24 hours. In our study, we followed a one-step simple technique, less time-consuming and produced high yields of over 90% for all formulations. The synthesis of PEI / DAB-amino acids polyplexes was analyzed by ^{1}H NMR spectroscopy and the spectrum of both polymers after the reaction exhibit signals corresponding to PEI / DAB polymers and signals corresponding to Arg, Lys and Leu, indicating that the amino acids residues were conjugated to the polymers surface successfully. Our results were in line with Kim *et al.* (2007) and Koppu *et al.* (2010).

After confirming the conjugation of amino acids to our polymers, their binding affinity to DNA were investigated using gel retardation and PicoGreen® assay. There are many factors influencing DNA binding affinity which are directly related to the intrinsic properties of the polymer, such as the number of charge

groups / single polymer, the type of charge groups (primary, secondary, quaternary amino groups, or amidine groups), the spacing of polymer's charge groups, the degree of polymer branching and finally, the hydrophobicity of the cationic vector (Kundu and Sharma, 2008). Furthermore, there are many external factors that may strongly influence the polyplex formation such as, the concentration and ionic strength of the formed polyplex solution, positive / negative charge ratios of polymer and DNA and finally the technical process followed in polyplex preparation (kinetically or thermodynamically controlled process). These extrinsic factors offer some degree of flexibility in choosing the optimum conditions for polyplex formation. However, upon administration the physiological condition will rule the stability and outcome of polyplexes behaviour. Therefore the selection of the intrinsic properties of the polymer combined with carefully optimized extrinsic conditions, are very important for the formation of polyplexes (Kundu and Sharma, 2008).

Gel retardation assay was used in our study to determine the polymer: DNA weight ratios required to compact DNA into polyplex structures suitable for gene delivery. PEI and DAB can form complexes efficiently with plasmid DNA through electrostatic interaction between the protonated primary amine of the polymer and the negatively charged phosphodiester groups of DNA (Tang and Szoka, 1997). The conjugation of amino acids to the primary amine may affect the polymer ability to form the complex with DNA. For this reason, gel retardation assay was used to investigate the influence of attachment of amino acids residues to the polymer complexing ability. The result from our study showed that the free DNA band was not seen at polymer: DNA weigh ratios higher than 5:1 for PEI conjugated to Lys and Leu, indicating that all plasmid DNA molecules were associated with the polymer. However, for PEI-Arg, the free DNA band can only be observed at weight ratio 20:1. The same pattern was observed when amino acids were conjugated to DAB dendrimer. At polymer: DNA weight ratios of 20:1, DNA was fully condensed by DAB-Arg, thus preventing ethidium bromide to intercalate with DNA. In case of DAB-Lys and DAB-Leu, DNA was fully condensed at ratio 5: and higher.

For more precise analysis and confirmation of complex formation, a PicoGreen® reagent was used to assess polyplexes formation at various weight ratios.

Previously, a commonly used method of analyzing the formation of polymers/ amino acids/ DNA complexes is the ethidium bromide exclusion assay. However, this method possesses some problems in that it is critically dependent on the concentration of the highly toxic ethidium bromide, and has a low sensitivity giving only 10-15-fold enhancement of fluorescence compared to the positive control. In our study, we used PicoGreen® reagent as a more sensitive probe for the characterization of polyplexes formation. This method was found to be highly reproducible and very sensitive for monitoring complex formation. Prior to the addition of buffer containing appropriate amount of PicoGreen® reagent, the polymer/amino acids was allowed to self-assemble with plasmid DNA. After incubation of the prepared polyplexes at different weight ratios for 30 minutes, the PicoGreen® reagent was added and the uncomplexed part of the plasmid DNA is exposed to the PicoGreen® reagent. The resultant fluorescence increases according to the degree of uncomplexation. A DNA condensation experiment was performed to choose the best amino acids modified PEI and DAB: DNA weight ratio for further experiments. In the case of PEI polymer, inhibition of fluorescence resulting from precomplexation with the polymer increased slightly with increasing weight ratios. DNA condensation increased with increasing weight ratios and was almost complete at a polymer: DNA weight ratio of 20:1 for the three polymers. Lysine and leucine were able to condense more than 80 % of DNA at less weight ratios compared to Arg. This result is in line with our gel retardation assay data. Arginine conjugation seemed to reduce the DNA condensing ability of the polymer compared to Lys and Leu conjugation.

When DAB polymer was used, the three amino acids conjugated to DAB followed the same manner as with PEI polymer. DNA condensation increased with increasing weight ratios and was almost complete at a polymer: DNA weight ratio of 20 and 10:1 for the three amino acids. DNA condensation occurring with generation 3 DAB-Arg seemed to follow the same pattern as the one described by Kim *et al.* (2007) when using generation 2 polypropyleneimine dendrimer after 2 min incubation. Although DNA condensation was similar for generation 2 DAB-Arg and DAB polyplexes at higher weight ratios, generation 3 DAB did not behave the same way: the grafting of Arg, as well as Lys and Leu, led to a much improved DNA

condensation compared with native generation 3 DAB polyplexes for weight ratios higher than 2:1. The formation of stable polyplexes at high polymer: DNA weight ratios could be explained by the fact that the pKa value of α-amine of arginine in DAB-Arg was lower than that of primary amines in DAB. Consequently, the number of moles of DAB-Arg was smaller than that of DAB at the same charge ratio, thus resulting in the formation of less stable DAB-Arg polyplexes for smaller weight ratios. However, this still need to be elucidated in the case of PEI-Arg. DAB-Leu presented the weakest DNA condensation ability at low polymer: DNA weight ratios, as already observed previously with PEI-Leu (Aldawsari *et al*., 2011). This could be explained by the fact that the conjugation of the dendrimer with cationic Arg and Lys exceeded the loss of positive charges due to the coupling, unlike what happened with hydrophobic Leu.

This was supported by the higher zeta potential values of Arg and Lys compared to Leu. A lower DNA condensation ability was also similarly described with the conjugation of the hydrophobic phenylalanine to a dendrimers (Kono *et al.,* 2005). When combined to the dendrimer chain terminal, phenylalanine residues were able to form a hydrophobic environment in the periphery of the dendrimer, reducing protonation of their amino groups. Previous studies showed that pDNA binding of hydrophobically modified polymers may decrease after substitution due to the decreased number of protonated amines (Neamnark *et al*., 2009; Forrest *et al*., 2004). This was also reported by Doody *et al*. (2006), who concluded that chemical modification of PEI may alter the pDNA binding capacity of polymers due to steric hindrance by the substituents and/or consumption of primary amines on the polymer backbone. The results obtained with DNA condensation experiment indicates that PEI and DAB could condense plasmid DNA into particles so efficiently that no DNA could interact with the added PicoGreen® reagent at low weight ratios. Moreover, it has been reported that primary amines are required to form complexes with DNA (Tang and Szoka, 1997), so it might be expected that tendency to form polyplexes would be decreased in highly modified polymers. In general, it can be concluded that the conjugation of amino acid residues on the primary amines of PEI and DAB did not affect the polymer's ability to form a complex to DNA. Transmission electron microscopy showed that the conjugation of amino acids to PEI and DAB did not

affect the polyplexes formation. The particles were dense and formed spherical particles with no sign to aggregation.

Considering the significant importance gained by the cationic polymers as non-viral carriers for gene delivery, there is a great need for a better understanding of the factors that affect their interaction with DNA for improving their efficacy. The size and surface charge of the cationic polymer-DNA complexes not only affect their colloidal stability *in vitro*, but also influence their stability *in vivo* and consequently their efficiency (Goparaju *et al.*, 2009). Polyplexes need to have a suitable size for efficient uptake and internalization into cells, and positive charges of polyplexes are thought to be helpful for their interaction with the negatively charged cell membrane and efficient intracellular trafficking (Pack *et al.*, 2005). It is important to investigate the size of DNA carriers in order to assess whether they can be used for intravenous administration. Particles intended for intravenous administration have to be smaller than 1 (µm) in diameter (Shibata *et al.*, 1997). Furthermore, the size of polyplexes can shed light on complex formation. To investigate the physicochemical characterizations of the prepared polyplexes, the size and zeta potentials were examined at various weight ratios that were desirable for their formation.

Generally, structural changes in tumours vascular pathophysiology could provide opportunities for using long-circulating particulate carrier systems. The ability of vascular endothelium to present open fenestrations was described for the sinus endothelium of the liver (Roerdink *et al.*, 1984), when the endothelium is disturbed by inflammatory process, hypoxic areas of infracted myocardium (Palmer *et al.*, 1984) or in tumours (Jain, 1989). In particular, tumour blood vessels are generally characterized by abnormalities such as high proportion of proliferating endothelial cells, pericyte deficiency and aberrant basement membrane formation leading to an enhanced vascular permeability.

In tumour microenvironment, the cut-off size for extravasation was found to be at the range of 400-600 nm (Yuan *et al.*, 1995). Although other reports suggested that sizes between 200 nm up to 2 µm were acceptable in certain conditions (Dufès *et al.*, 2004b). Danhier *et al.* (2010) reported that endothelial pores have sizes varying from 10 to 1000 nm. However, it should be noted that particles greater than 1 µm can be indicator of physical instability, and particles above 5 µm might be lethal due to

the risk of embolism (Heurtault *et al*., 2003). Ishida *et al.* (1999) reported that the optimum size for selective tumour delivery with colloidal system is ideally between 60-500 nm. It is worth mentioning that lymphatic drainage is absent or non-functional in the tumour tissue. Thus nanocarriers entered into the tumour are not removed efficiently and are retained in the tumour. This passive phenomenon has been called the “Enhanced Permeability and Retention (EPR) effect,” discovered by Matsumura and Maeda (Matsumura and Maeda, 1986; Maeda *et al*., 2001). The EPR effect is now becoming the gold standard in designing cancer-targeting strategies or what is called “passive targeting” (Figure 2.14). As shown in this Figure, nanocarriers reach tumours through the leaky vasculature surrounding the tumours. The influence of size is demonstrated by the small size molecules which diffuse freely in and out of tumour blood vessels because of their small size, and thus their effective concentration in the tumours decrease rapidly. This is not the case with the nanocarriers that cannot diffuse back into the blood stream because of their large size. All nanocarriers use the EPR effect as a guiding principle, especially in all rapidly growing solid tumours the EPR effect is applicable (Maeda *et al*., 2009).

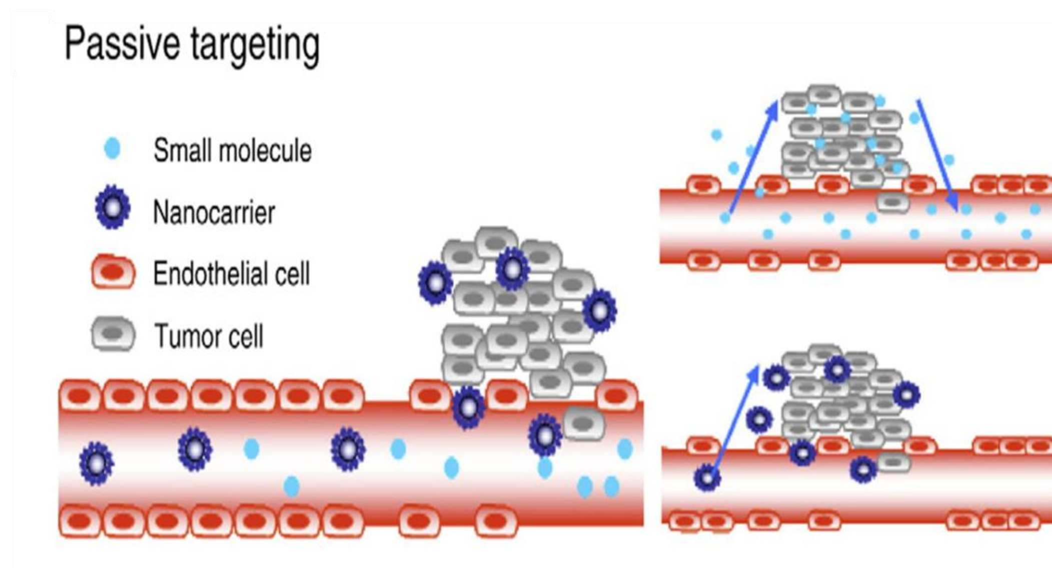


Figure 2.14: Passive targeting of nanocarriers (*After Danhier et al., 2010*).

The vascular abnormalities of tumour tissues associated with the poor lymphatic drainage explain the size relationship with the EPR effect: larger and long-circulating nanocarriers are more retained in the tumour, whereas smaller molecules easily diffuse (Pirollo and Chang, 2008). In this study, the size measurement of all of the amino acids bearing PEI (less than 305 nm) and DAB (less than 300 nm) polyplexes at all weight ratios tested were both within the reasonable size range for tumour targeting and gene delivery. A smaller particle size were found at higher weight ratios with higher zeta potentials, suggesting that the higher charges polymer could more efficiently condense DNA and results in smaller size complexes. The unmodified PEI polyplexes displayed a slightly larger size than the amino acids modified PEI polyplexes. This maybe attributed to the improved DNA binding capacity of the modified polyplexes which is confirmed by the DNA condensation data and gel retardation studies. The conjugation of amino acids to the periphery of DAB led to a slight increase of the polyplex size compared to the unmodified DAB polyplex. As the cut-off size for extravasation has been found to be 400 nm for most tumours (Yuan *et al*., 1995) amino acid-bearing DAB polyplexes have therefore the required size to access the tumour cells. In summary, polyplexes prepared with PEI and DAB polymers were found to have a narrow size distribution at all weight ratios examined, and that Arg, Leu, and Lys conjugated to them does not affect their size significantly.

In general, the zeta potentials of carrier / DNA complexes are closely connected with cellular uptake. This is because the cell surface carries an overall negative charge due to the high number of glycophospholipids in cell membranes (Petersen *et al*., 2002). Improved cellular uptake may be attributed to the interaction between the cationic complexes and the negatively charged cell membrane. Another additional factor representing the importance of zeta potential measurements is that the functional groups present on a modified polymer surface can introduce a high surface charge density that is typically not present on untreated polymer surfaces (Goddard and Hotchkiss. 2007). Most importantly, the surface charge of the polyplexes in our study helped to explain their colloidal stability, as particles with neutral surface charges do not repel each other and tend to aggregate and have low colloidal stability (Shaw, 1991).

The zeta potentials of PEI and DAB /DNA complexes were consistent with expectation showing increased zeta potential values at higher polyplexes weight ratios. This is due to the charge neutralization of the anionic DNA by the positive charge of PEI and DAB giving the complexes an overall positive charge, a feature that is favourable with gene delivery vectors. At very low weight ratios (0.5:1), polyplexes displayed negative values, suggesting that stable polyplex is not formed yet at this ratio. This was consistent with the DNA condensation and the agarose gel electrophoresis results.

Generally complexes prepared at low weight ratios which display neutral surface charges tend to aggregate. Furthermore, also complexes prepared with excess polymer displaying a highly positive surface charge exhibit enhanced aggregation tendency (Trubetskoy *et al.,* 1999). This point represents the importance of the zeta potential and its role in facilitating gene transfer in cell. The literature is ambivalent on how surface charges of DNA polymer complexes influence their transfection efficiency. Several studies (Tian *et al*., 2007; Kim *et al*., 2009; Nam *et al*., 2009) reported the importance of a positive surface charge of the complexes on improving the cellular uptake and transfection. On the other hand, numerous other publications reported that as the endocytotic uptake of particles increases with increasing zeta potentials, it also enhances toxic side effects due to unspecific interactions between the gene delivery systems and cell membranes (Putnam *et al*., 2001; Kundu and Sharma, 2008).

Comparing the zeta potential of the investigated amino acids bearing PEI and DAB polyplexes prepared in our study, desirable positive zeta-potential and PDI values were obtained at weight ratios greater than 5, providing an enhanced stability and lower cytotoxicity. Our finding was in line with Choi *et al*. (2004) who reported zeta potential values of 20-25 mV with PAMAM conjugated to Arg and Lys at weight ratios of 4 or greater. Similar zeta potential results were reported by Kim *et al*., 2009) upon conjugating Arg to poly(disulfide amine) polymer for gene delivery. Among the three amino acids investigated in our study, leucine displayed the lowest zeta potential values compared to arginine and lysine. This maybe explained by the hydrophobicity nature of leucine which may cause some degree of aggregation. Out of all the amino acids, leucine and valine are the most hydrophobic with

hydrophobicity values of 4.2 and 3.8, respectively. They can interact with other hydrophobic areas on the DNA, and thus decrease the zeta potential value (Goparaju *et al*., 2009). The fact that all investigated polyplexes have much higher surface charge than plain plasmid DNA (- 33 mV) may suggest that some area of the polyplex surface may not be shielded by DNA. If a complete coating of DNA was achieved on polyplex surface, polyplexes would not display such a positive zeta potential, as the positive charge maybe shielded by the negatively charged DNA layer.

In this Chapter, we investigated the physicochemical properties of both polymers at different weight ratios, which may give a preliminary insight about their possible behaviour at the cellular level. Both polymers were able to form complexes with DNA and their DNA binding capacity increase with higher weight ratios. It is also shown that the amino acids modified polyplexes display a size range suitable for intravenous administration, with favourable zeta potential and polydispersity values. The following Chapter involves detailed studies of their gene transfer potential, their anti-proliferative activity as well as their cellular uptake *in vitro*.

Chapter 3

In vitro evaluation of amino acid-bearing polyethyleneimine and polypropyleneimine polymers

3.1 Introduction

A major breakthrough in the study of human and animal behaviour was achieved with the introduction of cell culture more than a century ago (Freshney, 2000). The recent development of cell culture has made most cellular studies accessible such as protein synthesis, intracellular activity, membrane trafficking, receptors interactions, cell adhesion, genetic recombination techniques and immunology (Freshney, 2000). The main advantage of the cell culture techniques is the good control of the physicochemical environment such as pH, temperature and carbon dioxide, which is essential for accurate and consistent experimental studies. In the field of cancer therapeutics, cell transfection and proliferation, carrier-cell interaction, mechanism of action and screening for new therapeutics have well progressed under the knowledge of *in vitro* cell culture techniques.

The formation of cell line is usually derived from primary explants, which disperse into cell suspensions upon mechanical or enzymatic digestion. Cell suspension is then propagated into adherent monolayer cultures or as suspensions under controlled physicochemical environment. When confluence is reached (a state where all available growth area is utilized and cells make close contact with one another), cells are subcultured, or “passaged”, to ensure continuity of growth. The pattern of cell growth is usually standard in cell culture. After seeding, a lag phase (cells are maturing but not able to divide yet) followed by the log phase (a period of exponential growth) is observed. When the cell density reaches a stage where all the available substrate is occupied, or when the cell concentration exceeds the capacity of the medium, cell growth is greatly reduced. In this case, dividing of the cell culture (i.e. subculture) is recommended. For an adherent cell line, the standard procedure usually involves removal of medium and dissociation of cells with trypsin. Cell suspension, diluted to an appropriate split ratios, are re-seeded into new vessels for propagation. Subcultures are usually performed when the density of culture reaches confluences as cells often withdraw from cell cycle progression without adequate re-seeding (Freshney, 2000).

3.1.1 Cell culture

For *in vitro* studies, two cell lines were used to investigate the effect of amino acids bearing PEI and DAB polyplexes on enhancing the gene expression. Human squamous carcinoma, A431 cell line, is an epithelial carcinoma derived from the skin tissue of an 85 year old female (ECACC). A431 cells were previously reported to be used in polymer or dendrimers-mediated transfection studies *in vitro* and *in vivo* (Zinselmeyer *et al.,* 2002; Schätzlein *et al.,* 2005; Koppu *et al.,* 2010). Thus, this cell line can be exploited as an indicator cell for the transfection efficacy of the systems used in this study. On the other hand, a strain of human glioblastoma, T98G cell line, was included in our study as system L-amino acid transporters are over expressed in glioma cells. Because of its broad substrate selectivity, system L-amino acid transporter is regarded as a drug transporter that transports not only naturally occurring amino acids but also amino acid-related drugs (Kim *et al.,* 2004). We hypothesize that the use of this cell line in our study may therefore be advantageous for recognizing our delivery systems and facilitate their uptake. T98G are derived from glioblastoma multiform tumour from a 61 year old Caucasian male (ECACC). Finally, groups of mice bearing subcutaneously implanted A431 tumours were used for *in vivo* evaluation of the gene expression biodistribution of our delivery systems.

3.1.2 Cell transfection

Transfection refers to the process whereby the genetic material is introduced to host cells, taken up, transported to the nucleus an expressed as the desired protein (Lemoine, 1999). This process utilizes the properties of a delivery system with which the genetic material is complexed, as naked DNA has low transfection efficiency (Liu *et al.,* 1995). Genetic reporter assay are commonly used in cell biology as they enable gene expression and related events to be studied. Among the most commonly genetic reporters used to study gene expression are β-galactosidase and firefly luciferase (Ogris *et al.,* 1999; Brown *et al.,* 2003; Shcharbin *et al.,* 2010). They indirectly allow gene delivery efficiency to be measured via the expression level of a known reporter protein being investigated using different delivery vectors. These systems are usually sensitive, easy to use and yield rapid results. As stated in Chapter

1, the carrier: DNA complex will face many barriers that have to be overcome for successful transfection to occur, such as inefficient cellular uptake, poor endosomal escape and low nuclear uptake (Nishikawa and Huang, 2001).

It is worth mentioning, that some of the properties of a tested delivery system that produce good *in vitro* results may differ from those required for successful *in vivo* delivery (Ledley, 1995). *In vitro* transfection experiment is carried out in a less complex environment than that encountered *in vivo*. Media components and volume can be managed in an *in vitro* environment as well as the cell type being investigated (Freshney, 2000). Such a controlled environment is not encountered if systems are delivered *in vivo* and hence this adds to the lack of predictability that *in vitro* transfection results have on *in vivo* efficiency. In addition, cellular properties are often modified upon removal from their normal *in vivo* environment (Freshney, 2000). Moreover, the *in vitro* systems often involve using dividing cells and mitosis that may present beneficial effectiveness results for many non-viral systems by enhancing nuclear uptake (Mortimer *et al.,* 1999).

However, *in vitro* transfection experiments can be a useful tool for studying specific aspects of gene delivery systems and as a crude test to establish if transfection can occur, thus reducing the number of animal experiments conducted.

3.1.2.1 β-galactosidase transfection assay

In 1972, Jeffrey Miller published a protocol for determining the amount of β-gal with o-nitrophenyl- β-D-galactosidase (ONPG) (Miller, 1972). Because of this, the ONPG/ β-gal assays are referred to as "Miller" assay, and a standardized amount of β-gal activity is a "Miller Unit".

The β-galactosidase reporter system is commonly used in transfection studies and provides a convenient method for β-galactosidase (a prokaryotic enzyme) expression to be detected (Shcharbin *et al.,* 2010). Briefly, at 72 h incubation after transfection with β-galactosidase reporter vector, such as pCMV.SPORTβ-gal plasmid, the cells were lysed with 1X passive lysis buffer (PLB) (50 µl/well) for 20

min. The cell lysates were subsequently analyzed for β-galactosidase activity in the supernatant and measured in a buffer containing ONPG (1.33 $mg.mL^{-1}$). The β-galactosidase enzyme cleaves the β-bond of ONPG to form o-nitrophenol, which is yellow in alkaline solution. Samples were analyzed by measuring the absorbance photometrically at 405 nm with a microplate reader.

3.1.3 Confocal Laser Scanning Microscopy (CLSM)

A conventional light microscope is limited in biological applications because of its relatively low resolution (up to 0.2 μm). Although transmission electron microscopy offers outstanding high resolution, high electron energy beam is often damaging to biological specimens (Wright and Wright, 2002). The concept of confocal microscopy was first patented in 1975 by Marvin Minsky, suggesting a better performance using point-by-point image construction while focusing a point of light sequentially across a specimen and then collecting the returning rays out of a pinhole aperture. Modern confocal microscopy provides improvements on speed, image quality and data storage (Semwgerere and Weeks, 2005). The most widely used design in biological studies is fluorescence confocal microscope. In brief, fluorescence confocal imaging works by exciting the fluorescence compounds by a point source of laser beam. This light source is reflected by the dichroic mirror and directed to the objective lens, creating an incident beam on the focal plane of the specimen. Fluorescence emission from the specimen returns via the objective lens whereby lights with longer wavelengths pass through the dichoric mirror and a pinhole-like filter. Light that make it through the pinhole is measured by a photomultiplier detector (Figure 3.1) (Wright and Wright, 2002; Semwogerere and Weeks, 2005).

There are many advantages for utilizing confocal microscopy in biological analysis. Successful elimination of out-of-focus lights remains one of the major advantages of CLSM over conventional light microscope, for sharper and thinner optical section images can now be achieved (Inoue, 1995).

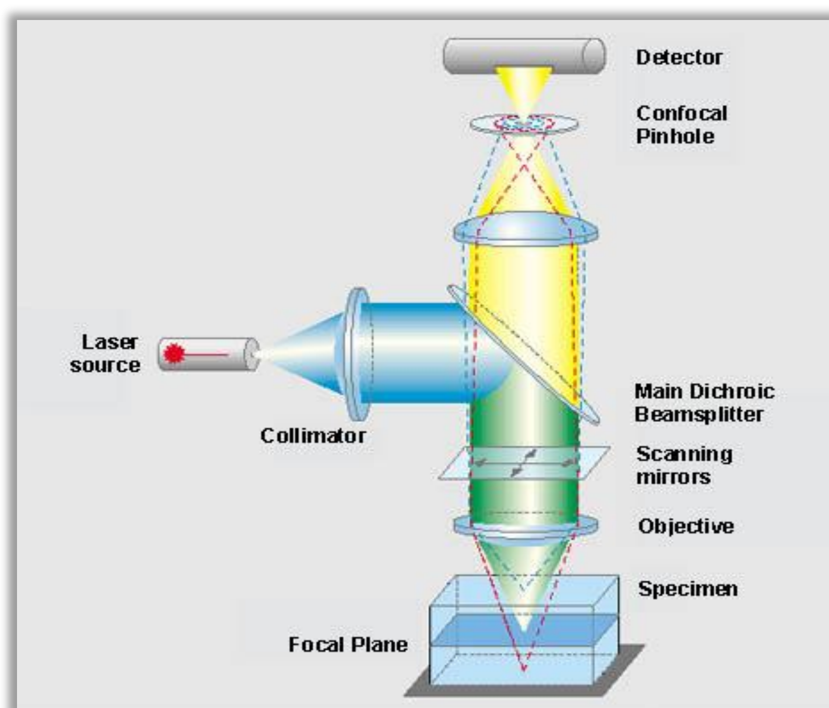


Figure 3.1: Schematic illustration of Confocal Laser Scanning Microscope (CLSM) (*After Carl Zeiss, Inc*.).

Moreover, different optical sections (i.e. the xy plane, xz plane and yz plane) can now be obtained. This is of a particular interest as the z-axis gives substantial information along the depth of a cell or tissue (Wright and Wright, 2002). This feature can be used to build a three dimensional rendition of the specimen (Semwogerere and Weeks, 2005). In addition, CLSM offers the feature of multiple-labelled imaging with the technology of detecting more than one fluorophore in a specimen where images can be merged to show relationship between different cell structures (Wright and Wright, 2002).

3.1.4 Anti-proliferative assay: MTT assay

Cytotoxicity assay [MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is one of the most widely used methods to measure the therapeutic efficacy of anti-cancer therapeutics. It is also used to quantify the cytotoxicity of novel polymeric delivery systems (Zinselmeyer *et al.,* 2002; Fischer

et al., 2003; Shcharbin *et al.,* 2010). It was first described as a technique by Mosmann in 1983, and used it as indirect measure of cell viability in immunological studies. MTT reagent is a water soluble yellow dye containing a tetrazolium ring which is cleaved by mitochondrial dehydrogenase in the living cells (but not dead ones) to produce a purple formazan product insoluble in aqueous solutions (Plumb, 2004). The amount of formazan can be dissolved in a suitable organic solvent, in this work dimethylsulfoxide (DMSO), to give a peak intensity at 570 nm that can be colorimetrically determined, which reflect the viable (metabolically active) cell number within the sample. Under optimized conditions, the MTT formazan production demonstrates a linear relationship with viable cell number, on the assumption that only viable cells are able to reduce tetrazolium salts to formazan derivatives (Plumb *et al.,* 1989, Vistica *et al.,* 1991). The reduction of MTT was taking place presumably in the mitochondria by active mitochondrial reductase enzymes. However, it was shown by Liu *et al.* (1997a) that many cellular components such as endosomes and lysosomes participate also in formazan production via MTT reduction. In a study comparing the two different cytotoxicity assays, i.e. the MTT assay and Alamar Blue assay, it was found that MTT assay was more robust technique with better correlation against protein analysis (Holst and Oredsson, 2005). Another important factor investigated by Plump *et al.* (1989) is the pH environment of the dissolved formazan product, which changes with the metabolically active cell density within each well. The authors recommended the addition of a glycine buffer to solubilised formazan products producing a final solution pH of 10.5. At this pH, only a single absorption maximum is produced at 570 nm, unlike at lower pH values where the peak may split into two components, which again may result in underestimation of the extent of dye reduction.

3.1.5 Aims and Objectives

From Chapter 2, amino acids bearing PEI and DAB polyplexes were successfully prepared and characterized. In this Chapter, the cellular uptake of the two systems was investigated and compared to unmodified delivery carriers or naked DNA in two cell lines, namely skin epithelial carcinoma A431 cells and glioblastoma

T98G cells using confocal microscopy. It was hypothesized that conjugating amino acids to our delivery systems would improve the cellular uptake facilitated by improved delivery to cancer cells. *In vitro* transfection and anti-proliferative studies form the basis for evaluating these carriers in biological system, as they provide vital characteristic information before can be further investigated for *in vivo* applications.

3.2 Materials and Methods

3.2.1 Materials

Materials	Supplier
Branched polyethyleneimine (PEI) (25 kDa)	Sigma-Aldrich, UK
Polypropyleneimine (DAB)	Sigma-Aldrich, UK
D-Arginine (Arg)	Sigma-Aldrich, UK
D-Lysine (Lys)	Sigma-Aldrich, UK
D-Leucine (Leu)	Sigma-Aldrich, UK
Dimethylsuberimidate dihydrochloride (DMSI)	Sigma-Aldrich, UK
Phosphate buffered saline tablet	Sigma-Aldrich, UK
Vectashield® mounting medium with 4',6-diamidino-2-phenylindole (DAPI)	Vector Laboratories, UK
Label IT® Cy3 Nucleic Acid Labelling kit	Cambridge Biosciences, UK
pCMVsport β-Galactosidase	Invitrogen, UK
Ampicillin	Sigma-Aldrich, UK
Tumour necrosis factor (TNF)α (pORF9-mTNFα)	InvivoGen, CA, USA
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen, UK
Foetal Bovine Serum (FBS)	Invitrogen, UK
L-Glutamine	Invitrogen, UK
Penicillin-Streptomycin	Invitrogen, UK
Trypsin	Invitrogen, UK
Triton-X	Sigma-Aldrich, UK
FluoroNunc® fluorescence 96-well Plates	Sigma-Aldrich, UK
o--nitrophenyl- β-D-galactosidase (ONPG)	Sigma-Aldrich, UK
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)	Sigma-Aldrich, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, UK
Glycine	Sigma-Aldrich, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, UK
Passive lysis buffer 5x (PLB)	Promega, UK

Sodium chloride	Sigma-Aldrich, UK
Magnesium chloride	Sigma-Aldrich, UK
Sodium hydroxide	Sigma-Aldrich, UK
β-mercaptoethanol (thioglycol)	Sigma-Aldrich, UK
Sodium phosphate (dibasic)	Sigma-Aldrich, UK
A431 human epidermoid carcinoma	The European Collection of Cell Cultures (Salisbury, UK)
T98G mouse glioblastoma	The European Collection of Cell Cultures (Salisbury, UK)

3.2.2 Cell culture

A431 and T98G cell lines were grown as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) L-glutamine and 0.5% (v/v) penicillin-streptomycin. Cells were cultured at 37 ^{o}C in a 5% carbon dioxide atmosphere.

3.2.3 pDNA synthesis and purification

Plasmids (pCMVsport β-gal and plasmid DNA encoding TNFα) were prepared and purified as previously described in section 2.1.2.

3.2.4 Preparation of complexes

All complexes were prepared and diluted in DMEM for cell viability (MTT), transfection studies and cellular uptake.

3.2.5 *In vitro* transfection

Transfection experiments were done to investigate the best ratio of amino acid bearing PEI/DAB and DNA for optimum transfection efficacy in both cell lines. PEI and DAB were used as positive controls.

Transfection efficacy of the DNA carried by the amino acid-bearing PEI or DAB carriers was assessed with a plasmid coding for β-galactosidase (pCMV βgal), using a β-galactosidase transfection assay. A431 and T98G cells were seeded in quintuplicate at a density of 2 000 cells/well in 96-well plates. After 72h incubation, the cells were treated with PEI/DAB-Arg, PEI/DAB-Leu and PEI/DAB-Lys complexed to plasmid DNA encoding β-galactosidase, at the polymer/dendrimer: DNA weight ratios used for the DNA condensation experiment. Naked DNA served as a negative control, carrier: DNA weight ratio 5:1 served as a positive control. DNA concentration (10 μg/mL and 1 μg/mL) was kept constant for all the PEI and

DAB formulations tested, respectively. After 72h incubation, cells were lysed with 1X passive lysis buffer (PLB) (50 μL/well) for 20 min. The cell lysates were subsequently analyzed for β-galactosidase expression (Zinselmeyer *et al*., 2002) by adding 50 μL ONPG (1.33 mg mL^{-1}) in 2X assay buffer (sodium dibasic phosphate 60nM; magnesium chloride 1mM; β-mercaptoethanol 50mM; pH 7.3) to each well. Plates were then incubated for 2h in the dark at 37 ^{o}C before reading the absorbance of each well at 405 nm using a plate reader (Thermo Lab Systems, Multiscan Ascent, UK). Results were compared with a standard curve (0-5 mU recombinant β-galactosidase.

3.2.6 Confocal Laser Scanning Microscopy (CLSM)

Imaging of the cellular uptake of the DNA carried by amino acid-bearing PEI and DAB polymers was carried out by confocal microscopy. Labelling of plasmid DNA with the fluorescent probe Cy3 was performed using a Label IT® Cy3 Nucleic Acid Labelling kit, as described by the manufacturer. A431 and T98G cells were seeded on cover slip in 6-well plates (1 x 10^5 cells/ well; equivalent to 0.6 x 10^6 cells / 90-mm Petri dish) at 37° C for 24 hours. The cells were then incubated for 24 h with Cy3-labelled DNA (6 μg / dish) complexed to PEI-Arg, PEI-Lys, PEI-Leu and PEI at the polymer: DNA weight ratios giving the highest transfection efficacy (20:1 for amino acid-bearing PEI, 5:1 for PEI). For DAB systems, the same was done at the dendrimers: DNA weight ratios giving the highest transfection efficacy (10:1 for amino acid-bearing DAB, 5:1 for DAB). Control slides were treated with naked DNA or remained untreated. The slides were then washed with PBS for three times at the end of incubation and fixed in methanol for 30 min. Cover slips were then rested on microscope slides with a drop of Vectashield® mounting medium containing nuclei stain propidium iodide (DAPI). Upon staining of the nuclei with DAPI, the cells were examined using a Leica TCS SP5 confocal microscope to generate the images from the slides. DAPI was excited with the 405 nm laser line (bandwidth: 415-491nm), whereas Cy3 was excited with the 543 nm laser line (bandwidth: 550-620 nm).

3.2.7 Anti-proliferative assay: MTT assay

As a measure of cytotoxicity, the 50% inhibitory concentration is well established in determining the ability of delivery systems to inhibit cancer cell proliferation. This is a value to be determined for each carrier, alone or complexed with pDNA, using the MTT reduction assay.

Anti-proliferative activity of amino acid-bearing PEI complexed with plasmid DNA encoding TNFα was assessed in A431 and T98G cell lines. Cells (2 000 cells per well in 96-well plates seeded 72 hours prior treatment) were incubated for 72 hours with the DNA formulations at final concentrations ranging from 6.4×10^{-4} to 50 µg/mL prepared by serial dilution into DMEM. Naked DNA served as a negative control, carrier: DNA weight ratio 5:1 served as a positive control. At the end of incubation, all treatments wells were emptied and replaced with fresh medium. MTT solution at 0.5 % w/v (prepared in PBS) was added to the wells at 50 µL/well. Upon 4 hours exposure, wells were emptied and replaced with DMSO at 200 µL/well. Glycin buffer was added (25 µL/well). Anti-proliferative activity was evaluated by measurement of the growth inhibitory concentration for 50% of the cell population (IC_{50}) in a standard MTT assay. Absorbance was measured at 570 nm using a plate reader (Thermo Labsystems, Multiscan Ascent). Dose-response curves were fitted to percentage absorbance values to obtain IC_{50} values (three independent experiments with n=5 for each concentration level).

Anti-proliferative activity of amino acid-bearing DAB complexed with plasmid DNA encoding TNFα was assessed in A431 and T98G cell lines in the same manner.

Results were expressed as percentage cell viability for each sample concentration according to the following equation:

$$\% \text{ cell viability} = [(A_{sample} - A_{TritonX\text{-}100}) / (A_{DMEM} - A_{tritonX\text{-}100})].100$$

IC_{50} were obtained from sigmoidal fitting of Log (base 10) plots of concentration versus % cell viability using Microcal Origin® version 7.

3.2.8 Statistical analysis

All values were expressed as means ± standard error of the mean (S.E.M). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison post-test (GraphPad Prism software). Differences were considered as significant when $P < 0.05$.

3.3 Results

In this Chapter, extensive *in vitro* studies were carried out in order to investigate the gene delivery efficiency of the two delivery systems after conjugating to amino acids. Before the therapeutic efficacy can be evaluated, transfection experiments were done to investigate the best carrier: DNA ratio which will exhibit the optimum transfection efficacy compared to controls. Cellular uptake using confocal microscopy was performed to investigate the uptake efficiency of the two systems in two cell lines compared to the unmodified carriers. This is of a particular importance as it provides insight information regarding the intracellular behaviours of the two systems used when accumulated in cancer cells. Finally, anti-proliferative activity of the optimum formulations was investigated using MTT assay.

3.3.1 *In vitro* transfection

Transfection efficacy was determined by a β-galactosidase transfection assay. For amino acids bearing PEI delivery systems, transfection mainly increased with increasing polymer: DNA ratios. The highest transfection levels for the three amino acid-bearing PEI polyplexes were observed at a polymer: DNA ratio of 20:1 on A431 and T98G cell lines. In A431 cells (Figure 3.2 A), treatment with PEI-Lys polyplex led to the highest transfection ($6.24 \times 10^{-3} \pm 0.34 \times 10^{-3}$ U/mL). Its transfection efficacy was about 1.3 times higher than that of PEI-Arg polyplex ($4.46 \times 10^{-3} \pm 0.34 \times 10^{-3}$ U/mL) and PEI-Leu polyplex ($4.30 \times 10^{-3} \pm 1.02 \times 10^{-3}$ U/mL). In T98G cells (Figure 3.2 B), transfection levels were almost similar for PEI-Lys ($7.91 \times 10^{-3} \pm 1.18 \times 10^{-3}$ U/mL) and PEI-Arg polyplexes ($8.49 \times 10^{-3} \pm 0.43 \times 10^{-3}$U/mL). They were 1.7 times higher than that obtained with PEI-Leu polyplex ($4.68 \times 10^{-3} \pm 0.59 \times 10^{-3}$ U/mL). On A431 cells, gene expression following treatment with PEI-Lys, PEI-Arg and PEI-Leu polyplexes was respectively 2.2 times, 1.6 times and 1.5 times higher than after treatment with PEI polyplex ($2.72 \times 10^{-3} \pm 0.18 \times 10^{-3}$ U/mL). On T98G cells, gene expression following treatment with PEI-Lys and PEI-Arg polyplexes was 3 times higher than that of PEI polyplex ($2.52 \times 10^{-3} \pm 0.18 \times 10^{-3}$ U/mL), and 1.8 times higher for PEI-Leu. No gene expression was observed after treatment with control DNA, as

expected. The conjugation of Arg, Lys and Leu to PEI at their optimal polymer: DNA ratio led to an improved transfection compared to PEI on both the tested cell lines (with the exception of PEI-Leu in A431 cell line. Based on these findings, along with the DNA condensation results; 20:1 ratio was selected for further therapeutic efficacy and cellular uptake experiments.

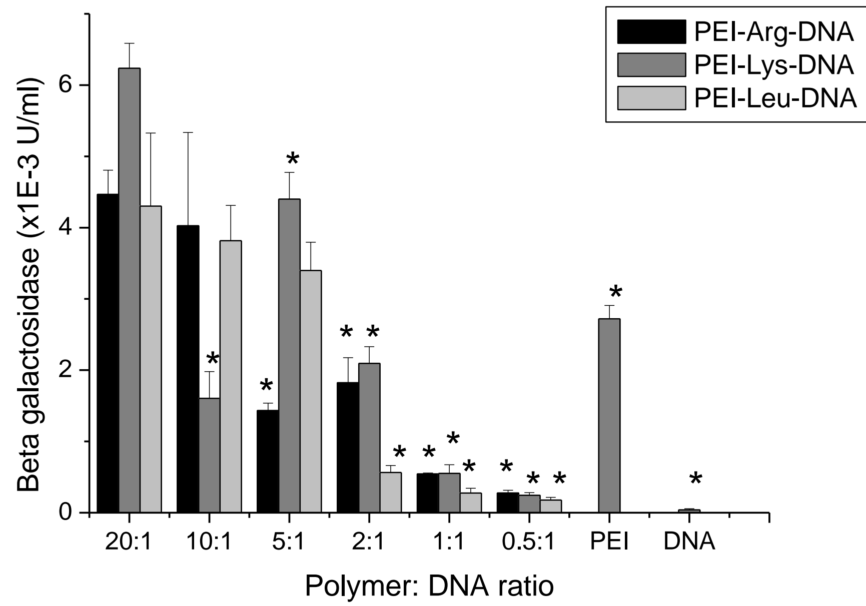
The highest transfection level after treatment with the 3 amino acid-bearing DAB polyplexes was obtained at a polymer: DNA weight ratio of 10:1 on A431 (Figure 3.3 A) and T98G cells (Figure 3.3 B). At this ratio, in A431 cells, treatment with DAB-Lys polyplex led to the highest transfection ($10.89 \times 10^{-3} \pm 0.27 \times 10^{-3}$ U/mL). Its transfection efficacy was about 1.1 and 1.2 fold higher than that of DAB-Arg polyplex ($9.79 \times 10^{-3} \pm 0.24 \times 10^{-3}$ U/mL) and DAB-Leu polyplex ($9.11 \times 10^{-3} \pm 0.18 \times 10^{-3}$ U/mL), respectively. In T98G cells, the highest transfection was observed after treatment with DAB-Lys polyplex ($11.96 \times 10^{-3} \pm 0.22 \times 10^{-3}$ U/mL), closely followed by DAB-Arg polyplex ($11.24 \times 10^{-3} \pm 0.23 \times 10^{-3}$ U/mL). It was 1.2-fold higher than that of DAB-Leu polyplex ($9.82 \times 10^{-3} \pm 0.16 \times 10^{-3}$ U/mL).

The conjugation of Arg, Lys and Leu to DAB at their optimal polymer: DNA ratio of 10:1 led to an improved transfection compared to unmodified DAB on both the tested cell lines. On A431 cells, gene expression following treatment with DAB-Lys, DAB-Arg and DAB-Leu polyplexes was respectively 2 times, 1.8 times and 1.7 times higher than after treatment with DAB polyplex ($5.49 \times 10^{-3} \pm 0.10 \times 10^{-3}$ U/mL). On T98G cells, gene expression following treatment with DAB-Lys and DAB-Arg polyplexes was 2 times and 1.8 times higher than that of DAB polyplex ($6.09 \times 10^{-3} \pm 0.11 \times 10^{-3}$ U/mL), respectively. For DAB-Leu, it was 1.6 times higher than that of the unmodified DAB. The enhancement in gene expression for both A431 and T98G cell lines was as follow: DAB-Lys > DAB-Arg > DAB-Leu > DAB-DNA.

The conjugation of Arg, Lys and Leu to DAB at their optimal polymer: DNA ratio led to an improved transfection compared to DAB on both the tested cell lines. No gene expression was observed after treatment with control DNA, as expected.

Based on these findings, along with the DNA condensation results; 10:1 ratio was selected for further transfection and therapeutic efficacy experiments.

A



B

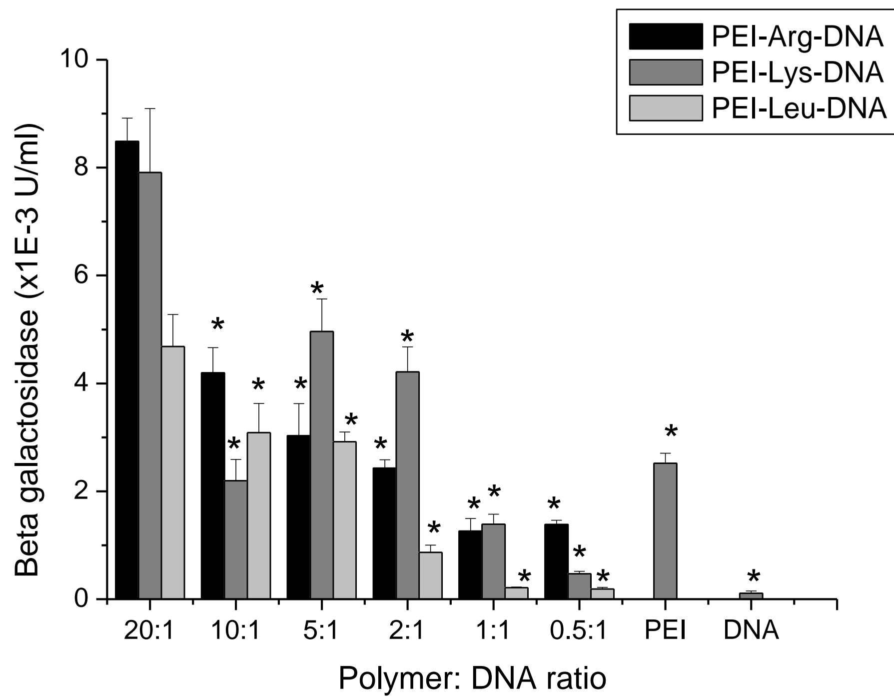
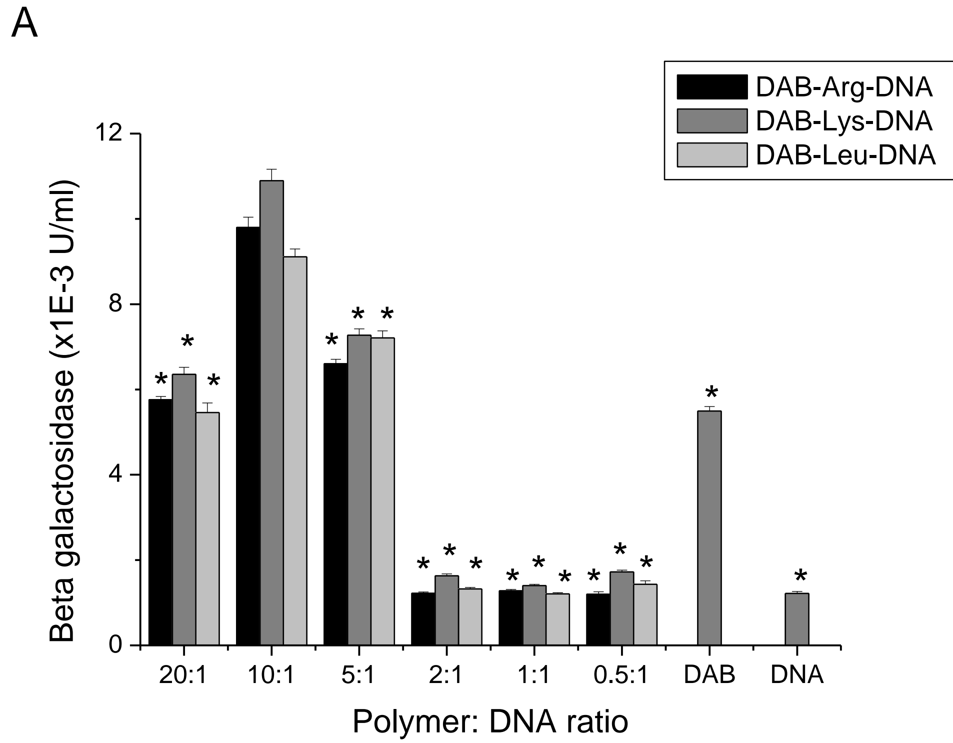


Figure 3.2: Transfection efficacy of PEI-Arg, PEI-Lys and PEI-Leu polyplexes at various polymer: DNA weight ratios relative to native PEI in A431 (A) and T98G cells (B). PEI-DNA was dosed at its optimal carrier: DNA ratio of 5:1. Results are expressed as the mean ± SEM of three replicates (*n=15*). *: P <0.05 *versus* the highest transfection ratio.

A



B

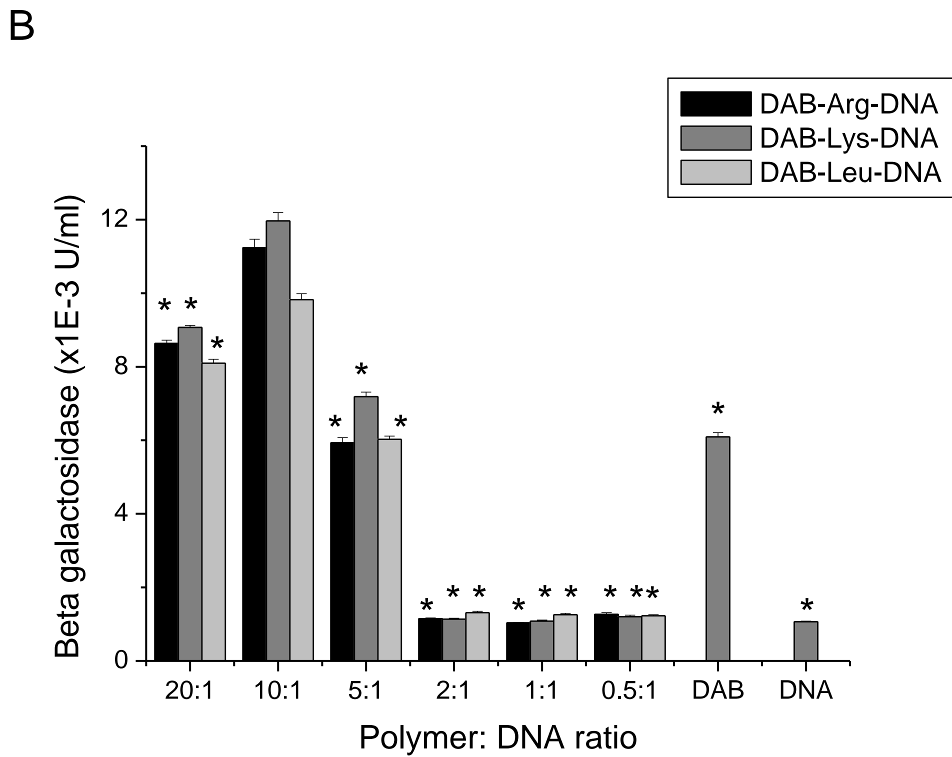


Figure 3.3: Transfection efficacy of DAB-Arg, DAB-Lys and DAB-Leu polyplexes at various polymer: DNA weight ratios relative to native DAB in A431 (A) and T98G cells (B). DAB-DNA was dosed at its optimal carrier: DNA ratio of 5:1. Results are expressed as the mean ± SEM of three replicates (*n=15*). *: P <0.05 *versus* the highest transfection ratio.

3.3.2 Confocal Laser Scanning Microscopy

The uptake of Cy3-labelled DNA by A431 and T98G cells was qualitatively confirmed using confocal microscopy. For PEI polyplexes (Figure 3.4), the complexation of plasmid DNA to PEI improved DNA uptake by both cell lines compared to DNA solution, as expected. Co-localization of DNA in the nuclei was clearly visible in both cell lines treated with PEI-Leu polyplex in A431 cells and PEI-Arg polyplex in T98G cells. It was less pronounced in the case of the other treatments. In both cell lines, Cy3-labeled DNA was also disseminated in the cytoplasm after treatment with all PEI formulations. By contrast, cells treated with naked DNA did not show any Cy3-derived fluorescence. The conjugation of Arg and Leu to PEI therefore improved DNA uptake by the nuclei of the tested cell lines, but not the cellular uptake within the cytoplasm. Although PEI-Lys was efficacious for transfection, it did not improve DNA uptake by the cells compared to PEI after 24h incubation, leading to the hypothesis that the DNA uptake following this treatment occurred at a later time.

The cellular uptake of Cy3-labeled DNA carried by amino acid-bearing DAB was qualitatively confirmed in both cell lines by confocal microscopy (Figure 3.5). Fluorescence was observed in the cytoplasm but not in nucleus. Cy3-labeled DNA was disseminated in the cytoplasm after treatment with all DAB formulations in both cell lines. The DNA uptake appeared to be more pronounced in A431 cells treated with DAB-Lys polyplex and in T98G cells treated with both DAB-Lys and DAB-Leu polyplexes. By contrast, cells treated with naked DNA did not show any Cy3-derived fluorescence. No co-localization of DNA in the nuclei was visible in any cell lines after 24h incubation, leading to the hypothesis that the nuclear uptake of DNA occurred at a latter time.

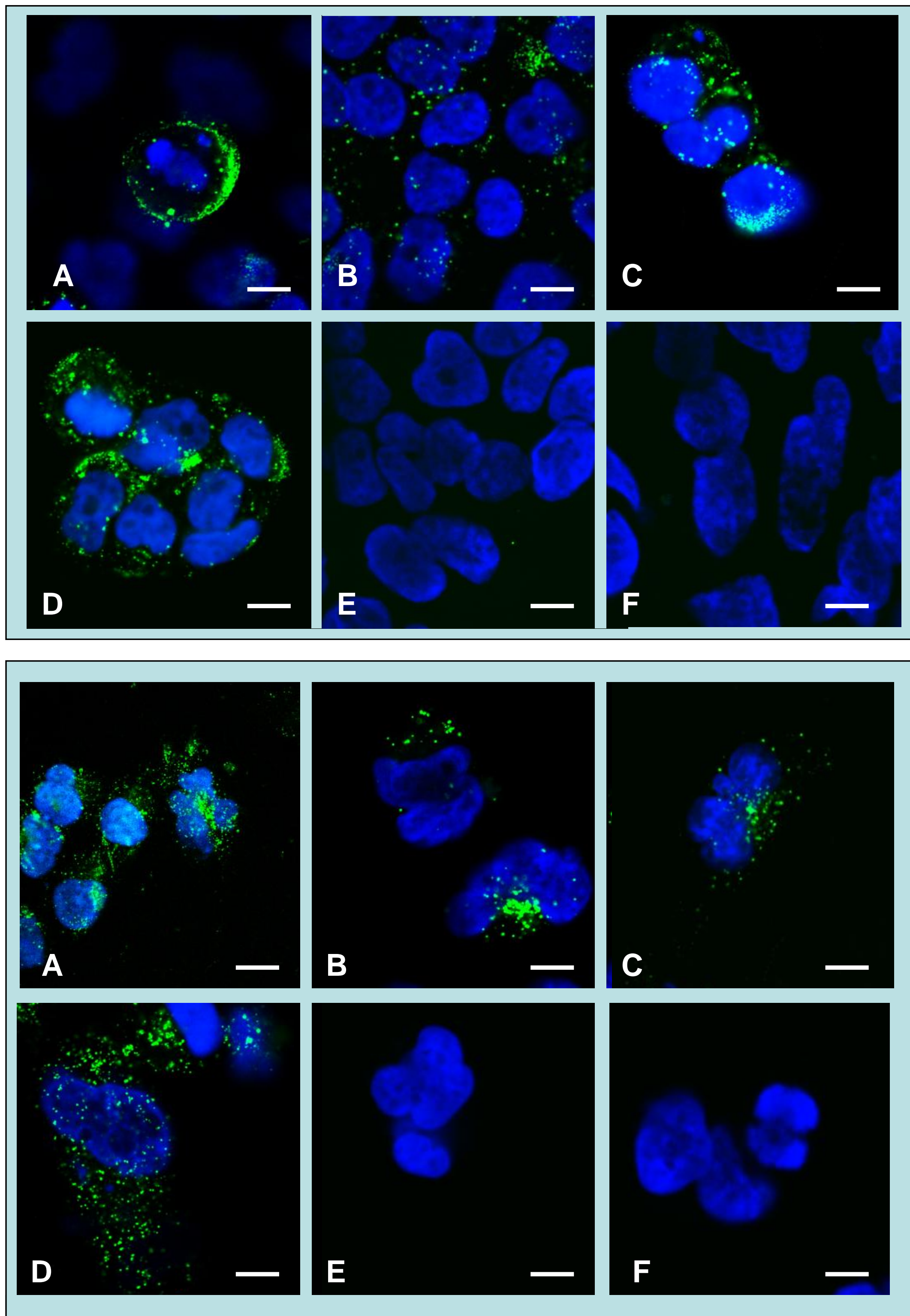


Figure 3.4: Confocal microscopy imaging of the cellular uptake of Cy3-labeled DNA (6 µg/ dish) either complexed with PEI-Arg (A), PEI-Lys (B), PEI-Leu (C), PEI (D) or free in solution (E) after incubation for 24 hours with A431 (top) and T98G cells (bottom) (control: untreated cells (F)) (Blue: nuclei stained with DAPI (excitation: 405 nm laser line, bandwidth: 415-491nm), green: Cy3-labelled DNA (excitation: 543 nm laser line. bandwidth: 550-620 nm) (Bar: 10 µm).

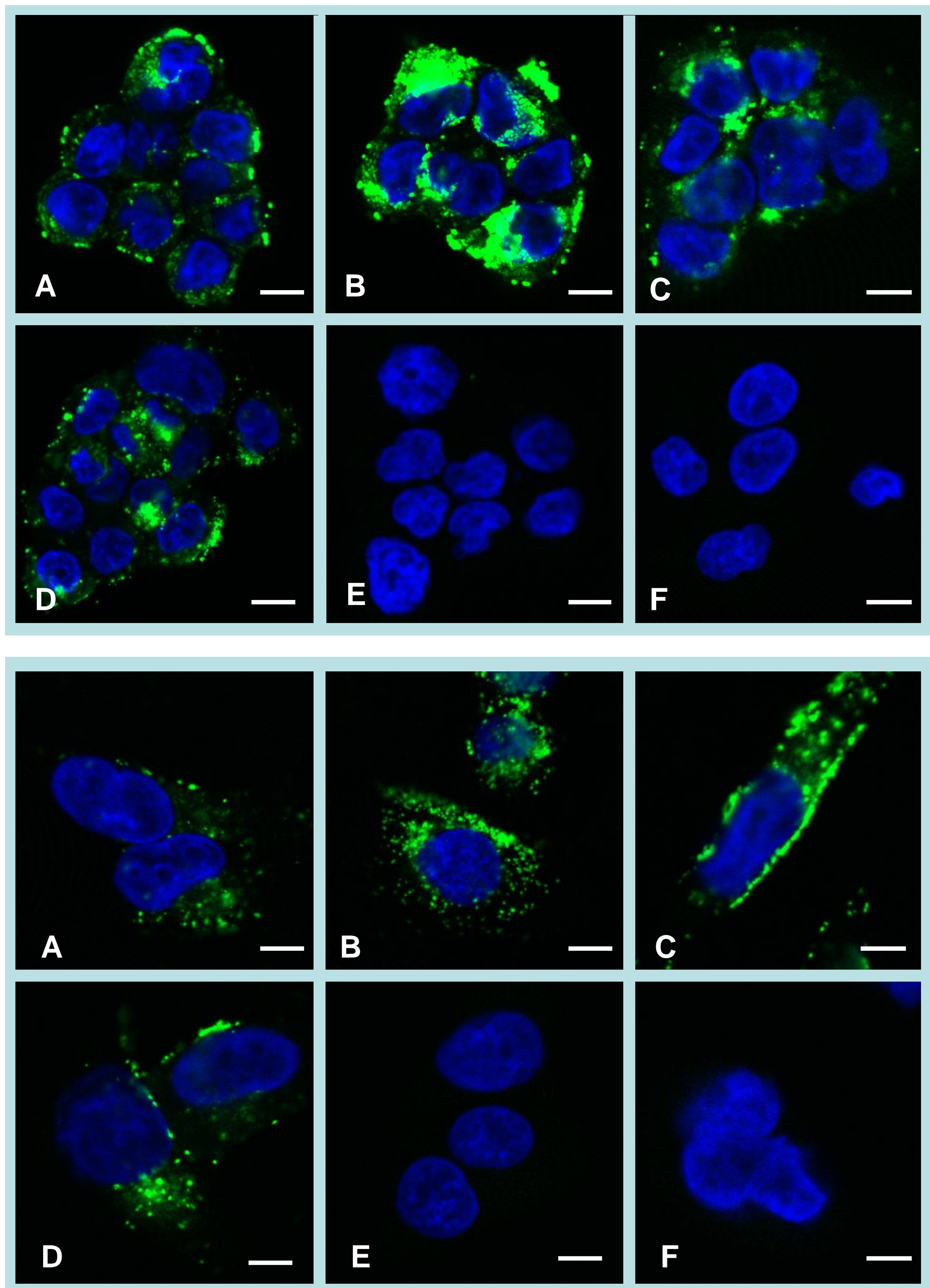


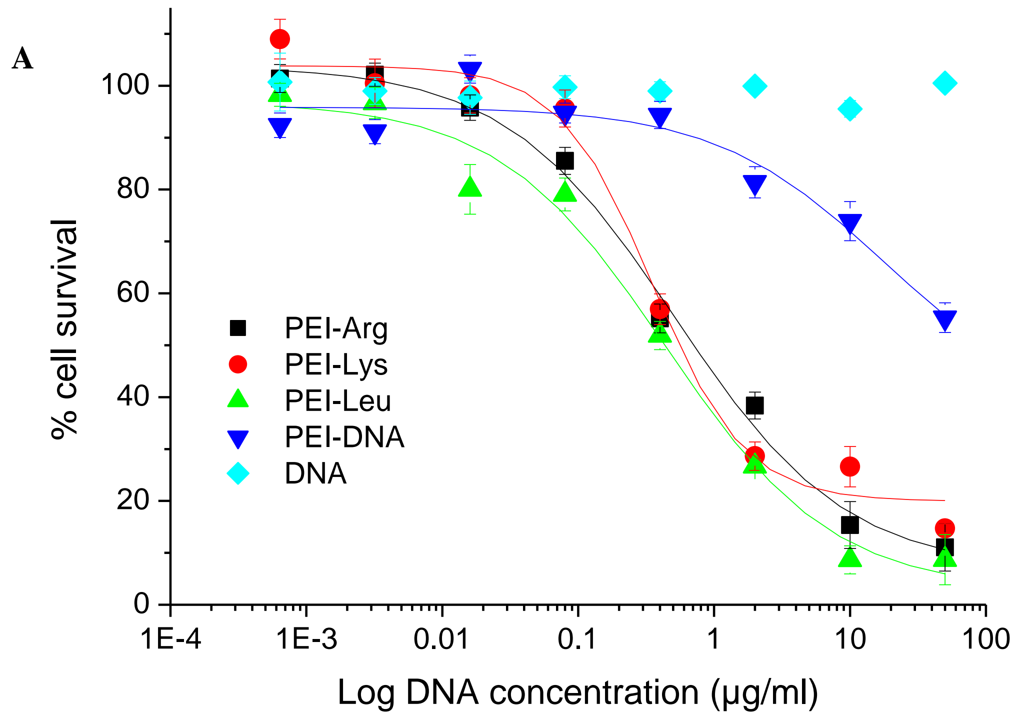
Figure 3.5: Confocal microscopy imaging of the cellular uptake of Cy3-labeled DNA (6 µg/ dish) either complexed with DAB-Arg (A), DAB-Lys (B), DAB-Leu (C), DAB (D) or free in solution (E) after incubation for 24 hours with A431 (top) and T98G cells (bottom) (control: untreated cells (F)) (Blue: nuclei stained with DAPI (excitation: 405 nm laser line, bandwidth: 415-491nm), green: Cy3-labelled DNA (excitation: 453 nm laser line. bandwidth: 550-620 nm) (Bar: 10 µm).

3.3.3 Anti-proliferative assay: MTT assay

The conjugation of Arg, Lys and Leu to PEI led to an increase of the *in vitro* anti-proliferative activity in A431 cells (Figure 3.6 A), respectively by 34-fold, 51-fold and 40-fold compared to the unmodified polyplex (IC_{50} of 0.52 ± 0.13 µg/mL, 0.35 ± 0.07 µg/mL and 0.44 ± 0.16 µg/mL respectively for PEI-Arg, PEI-Lys and PEI-Leu polyplexes, 17.86 ± 5.34 µg/mL for unmodified PEI polyplex) (Table 3.1).

In T98G cells (Figure 3.6 B), the conjugation of the amino acids to PEI improved the anti-proliferative activity of the polyplex, by 2-fold for PEI-Arg (IC_{50}: 5.08 ± 0.51 µg/mL), by 89-fold for PEI-Lys (IC_{50}: 0.12 ± 0.01 µg/mL) and by 269-fold for PEI-Leu (IC_{50}: 0.04 ± 0.04 µg/mL) compared to the unmodified PEI polyplexes (10.78 ± 1.66 µg/ mL). Uncomplexed amino acid-bearing PEI and naked DNA did not exert any cytotoxicity to the cells.

The conjugation of Arg, Lys and Leu to DAB led to a significant increase of *in vitro* anti-proliferative activity in A431 cells (Figure 3.7 A), respectively by 30-fold, 22-fold and 20-fold compared to the unmodified polyplex (IC_{50} of 0.32 ± 0.04 µg/mL, 0.43 ± 0.09 µg/mL and 0.48 ± 0.16 µg/mL respectively for DAB-Arg, DAB-Lys and DAB-Leu polyplexes, 9.47 ± 1.15 µg/mL for unmodified DAB polyplex) (Table 3.2). In T98G cells (Figure 3.7 B), the conjugation of the amino acids to DAB improved the anti-proliferative activity of the polyplex, by 43-fold for DAB-Arg (IC_{50}: 0.23 ± 0.01 µg/mL), by 47-fold for DAB-Lys (IC_{50}: 0.21 ± 0.01 µg/mL) and by 35-fold for DAB-Leu (IC_{50}: 0.28 ± 0.02 µg/mL) compared to the unmodified DAB polyplex (IC_{50}: 9.84 ± 2.79 µg/mL). Uncomplexed amino acid-bearing DAB and naked DNA did not exert any cytotoxicity to the cells. These results may be attributed to the improved transfection efficacy when treated with Arg-, Lys- and Leu-bearing DAB polyplex. DAB-Lys polyplex was the most efficacious treatment on T98G cells, probably as a result of its highest transfection efficacy on the same cell line.



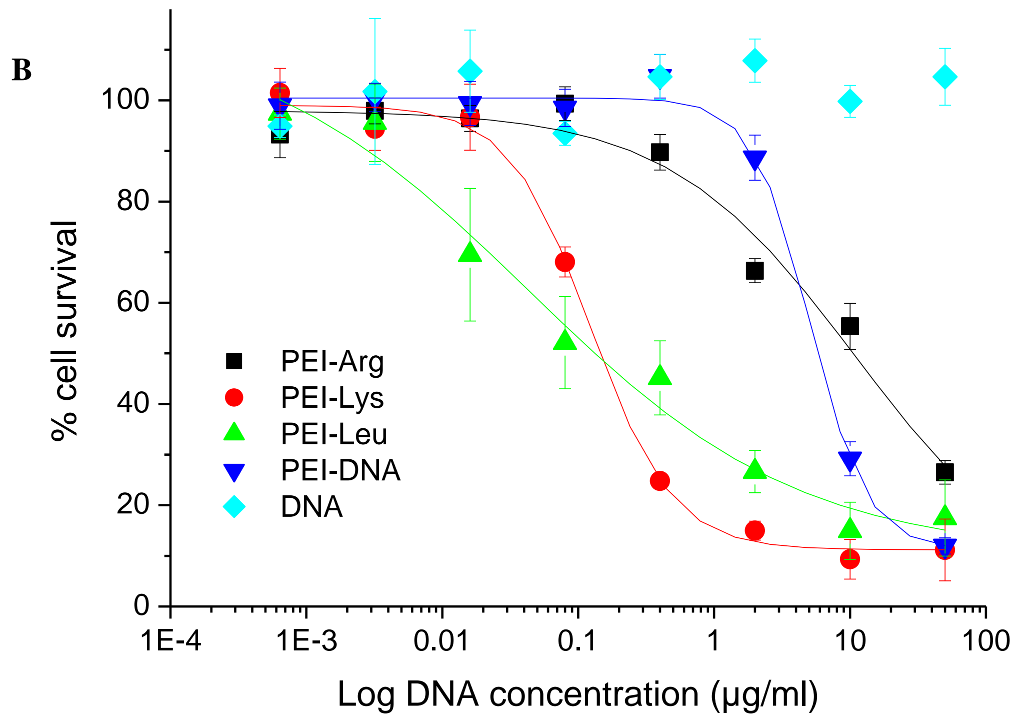


Figure 3.6: Anti-proliferative activity of amino acids bearing PEI polyplexes in A431 (A) and T98G (B) cell lines (*n=15*).

Table 3.1: Anti-proliferative activity of different PEI polyplexes formulations in A431 and T98G cells, expressed as IC_{50} values (*n=15*)

Formulation	***IC_{50} (µg/mL) (mean ± S.E.M)***	
	A431	**T98G**
PEI-Arg-DNA	0.52 ± 0.13	5.08 ± 0.51
PEI-Lys-DNA	0.35 ± 0.07	0.12 ± 0.01
PEI-Leu-DNA	0.44 ± 0.16	0.04 ± 0.04
PEI-DNA	17.86 ± 5.34	10.78 ± 1.66
PEI-Arg	> 50	> 50
PEI-Lys	> 50	> 50
PEI-Leu	> 50	> 50
DNA	> 50	> 50

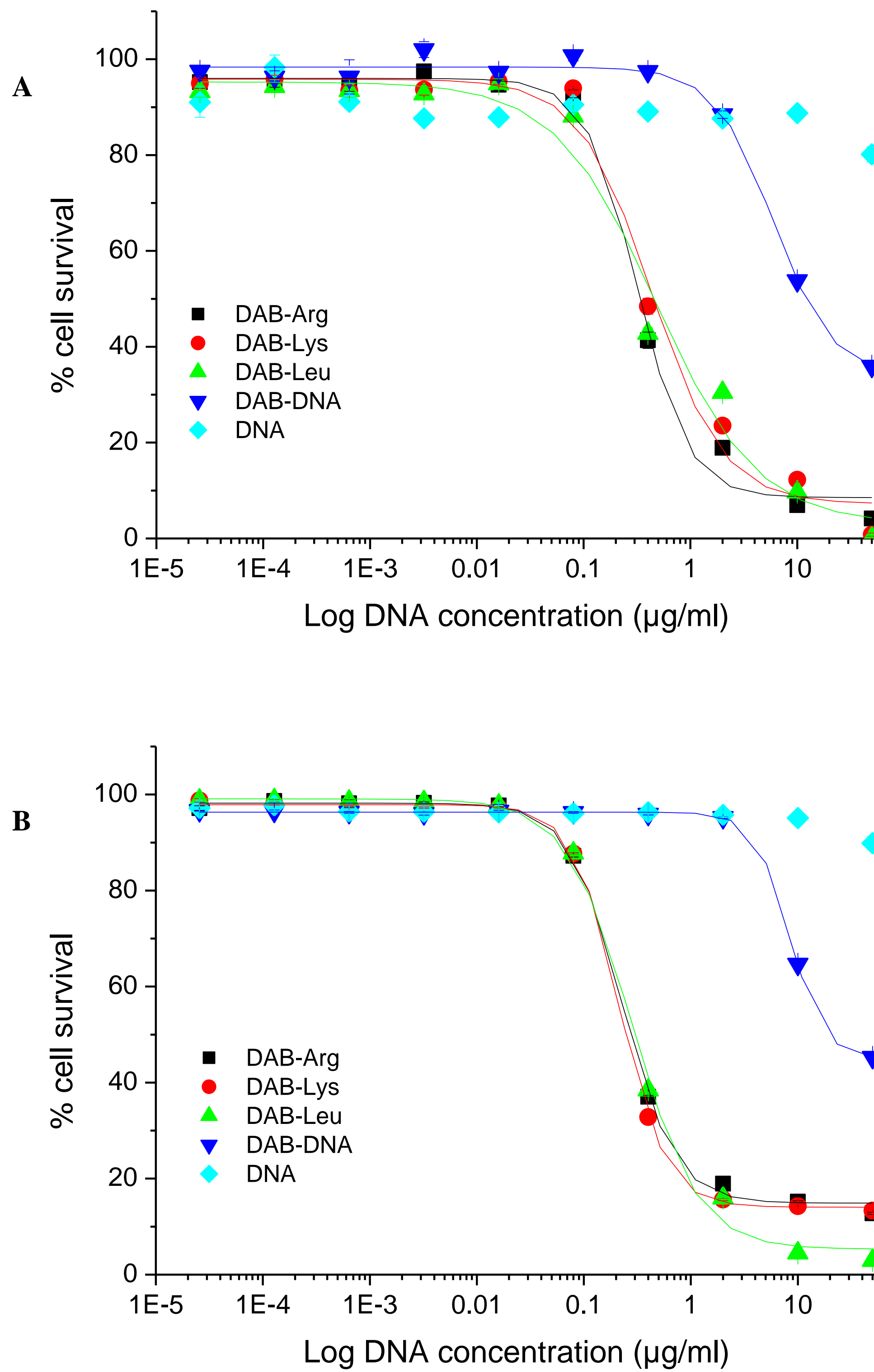


Figure 3.7: Anti-proliferative activity of amino acids bearing DAB polyplexes in A431 (A) and T98G (B) cell lines (*n=15*).

Table 3.2: Anti-proliferative activity of different DAB polyplexes formulations in A431 and T98G cells, expressed as IC_{50} values (*n=15*)

Formulation	***IC_{50} (µg/mL) (mean ± S.E.M)***	
	A431	**T98G**
DAB-Arg-DNA	0.32 ± 0.04	0.23 ± 0.01
DAB-Lys-DNA	0.43 ± 0.09	0.21 ± 0.01
DAB-Leu-DNA	0.48 ± 0.16	0.28 ± 0.02
DAB-DNA	9.47 ± 1.15	9.84 ± 2.79
DAB-Arg	> 50	> 50
DAB-Lys	> 50	> 50
DAB-Leu	> 50	> 50
DNA	> 50	> 50

3.4 Discussion

Despite the efforts given in vector technology, designing an ideal vector which efficiently delivers therapeutic agents to the target cells in a safe way still remains a major issue (Kay *et al*., 2001). Despite the potent immunogenicity concerns of the viral vehicles, their advanced cell entry mechanism and high transfection efficiency in both dividing and non-dividing cells is highly desirable (Liu *et al*., 2010). Nowadays, non-viral vectors with minimal toxicity have been investigated to mimic the cell entry mechanism of viruses. However, the efficacy of these non-viral vectors for gene delivery is still less than desired, because of the low transfection efficiency and high toxicity. This has been attributed to a number of interactions that exist between the cells and the transfection complexes (Chen *et al*., 2001). These interactions include binding to the cell membrane, uptake of the complex by endocytosis, ability to escape the endosomal pathway which is considered a major obstacle in efficient gene delivery, and finally nuclear transport of DNA (Chen *et al*., 2001).

The cell membrane plays a crucial role in both the structure and function of all cells. It defines the margins, and also determines the type and nature of all communication which occurs between the inside and outside of cells (Chen *et al*., 2001). The membrane lipid bilayer represents a highly impermeable barrier to most polar molecules because of its hydrophobic interior nature, thus preventing water-soluble contents of the cells from moving out. In order to carry molecules into or out of cells, plasma membrane has specific transmembrane transport proteins, in which many of them are selective for a specific molecule or group of closely related substances. In case of small size molecules and ions, gated ion channels and pumps control import and export of substances for the cells. However, large substances and particles will gain access by another mechanism, known as endocytosis. As explained previously in Chapter 1, endocytosis occurs via three different mechanisms. The first two types are pinocytosis and phagocytosis, which are distinguished based on the size. Pinocytosis involves the ingestion of fluids/ solute via small vesicles, while phagocytosis refers to the ingestion of larger particles via larger vesicles. The uptake of these larger particles occurs mainly in specialized

phagocytic cells, such as macrophage or rapidly proliferating tumour cells. The third type of endocytosis occurs via receptor-mediated pathway. In this type of endocytosis, detection and recognition occur via a surface binding receptor and considered as a highly efficient mechanism of internalization.

Many biologically active substances, such as proteins, plasmids and oligonucleotides are polar and hydrophilic in nature. These molecules have low cellular membrane permeability, which in turn results in a considerable reduced therapeutic efficacy in cell culture *in vivo* (Chen *et al*., 2001).

Another obstacle to the efficient gene delivery is the inability of the complex to escape the endosomal pathway after it gain an access to the cells (Fominaya and Wels, 1996). The endocytic pathway (Figure 3.8) is the cellular uptake mechanism of therapeutic materials which consists of endosomal vesicles with an internal pH around 5. This endosomes mature in a unidirectional manner from early endosomes to late ones before fusing with the intracellular lysosomes which contain certain digestive enzymes (Gruenberg and van der Goot, 2006). Therefore, the endosomal release of the therapeutics is necessary before lysosome mediated digestion of the therapeutics.

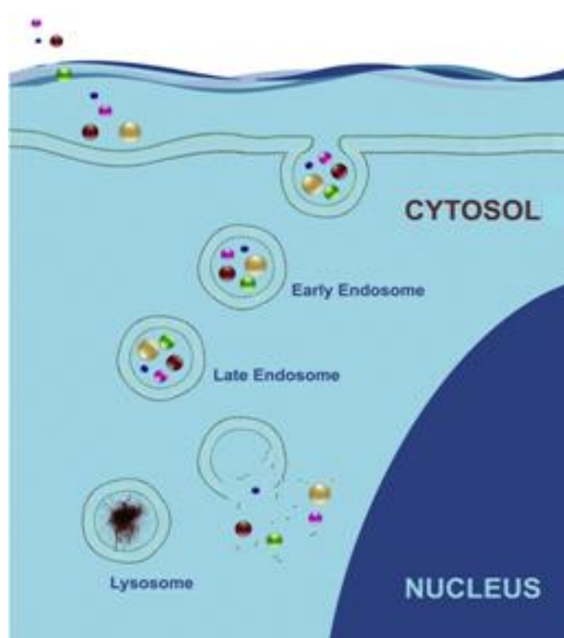


Figure 3.8: The internalization of therapeutics into the cell through endocytic pathway *(After Varkouhi et al., 2010).*

To address the endosomal entrapment problem, several strategies have been proposed to improve the delivery of therapeutic agents to the intracellular targets. These strategies were based on identified mechanisms for endosomal escape, like pore formation in the endosomal membrane and the pH buffering effect synthetic polymers. These include the use of viral and bacterial proteins and some synthetic polymers. In our study we employed one of the commonly investigated synthetic polymers (PEI) which contain pH-sensitive chemical functionalities and investigated as new endosomal releasing components (Varkouhi *et al*., 2010). The pH buffering effect or what's known as proton sponge effect is mediated by some molecules with high buffering capacity to swell upon protonation. Protonation is a process occurs with an extensive inflow of ions and water into the endosomal environment which eventually leads to rupture of the endosomal membrane and release of its entrapped components (Figure 3.9).

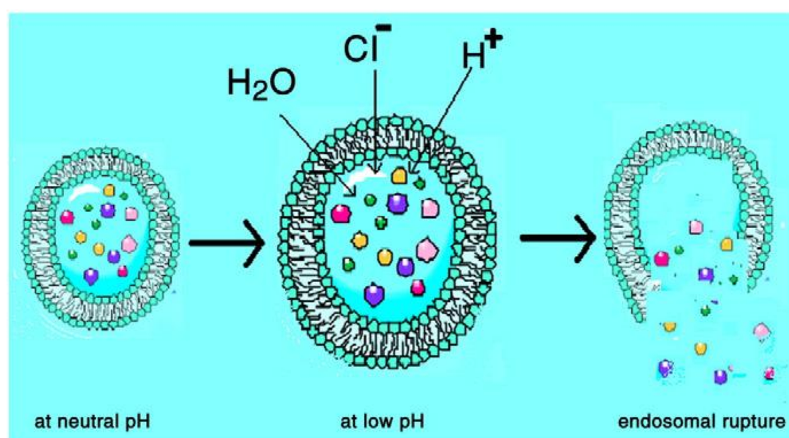


Figure 3.9: The proton sponge hypothesis *(After Varkouhi et al., 2010).*

Tertiary amine groups with a hydrophobic chain have been reported to be accumulated in endosomes with acidic pH and become detergents upon protonation resulting in membrane disruption (Miller *et al*., 1983). As an example, PEI (Liu *et al*., 2010) and poly(amido amine) (Varkouhi *et al*., 2010) polymers have a high buffering effect due to the presence of protonated amine groups in their structure which directs the endosomal osmotic pressure to increase with subsequent disruption of the endosomal membrane (Lin and Engbersen, 2008). Other agents having the

same buffering effect is the histidine-rich molecules which exhibit the buffering effect upon protonation of the imidazole ring of histidine and eventually the disruption of the endosomal membrane (Moreira *et al*., 2009).

More recently, some peptide sequences known as protein transduction domains (PTD) or membrane translocalization signals (MTS) were recognized and introduced into cationic vectors for DNA delivery (Tung and Weissleder, 2003; Choi *et al*., 2004; Brooks *et al*., 2005; Nam *et al*., 2008). Cell-penetrating peptides containing positively charged arginine and lysine are well known of having excellent cell penetration features. These amino acids residues were applied to polymeric gene delivery carriers such as poly(amido amine) (PAMAM) dendrimers (Choi *et al*., 2004), polypropyleneimine (PPI) dendrimers (Kim *et al*., 2007) and polyethyleneimine (PEI) polymers (Morris and Sharma, 2011) and they showed significantly enhanced transfection efficiency compared to the unmodified carrier.

Hatefi *et al*. (2006) investigated the use of lysine residues for their pDNA condensing abilities and histidine residues to promote endosomal escape for improving the transfection efficiency of a polymeric gene carrier. Others reported enhanced gene transfection efficiency using conjugated arginine even though they do not exist as oligo-peptide form (Gao *et al*., 2008; Yamanouchi *et al*., 2008). Furuhata *et al.* (2006) reported that oligo-arginine conjugates demonstrated similar characteristics to cell penetrating-peptides (CPPs) in cell translocation and the transfection efficiency in Hela cells. They concluded that the transfection efficiency of PEGlyted lipids could be highly improved by conjugating them to oligo-arginine.

Gao *et al*. (2008) investigated the potential of arginine-chitosan (Arg-Cs)/DNA self-assemble nanoparticles (ACSNs) as a safe and effective non-viral vector for gene delivery. The *in vitro* transfection efficiency against epithelial cell line HEK 293 (Human embryonic kidney) and COS-7 (African green monkey kidney) cells was demonstrated higher transfection efficacy of the modified nanoparticles compared to the unomdified nanoparticles.

Moreover, many studies have reported that hydrophobic moieties affect the transfection activity of cationic polymers (Kurisawa *et al*., 2000; Han *et al*., 2001; Wang *et al*., 2002; Kono *et al*., 2005; Tian *et al*., 2007). It was reported by Kurisawa

et al. (2000) that hydrophobic units are expected to improve transfection efficiency by modulating complex interactions with cells, such as adsorption on cell surfaces and cellular uptake. They hypothesized that hydrophobic interactions between the hydrophobic units are expected to inhibit dissociation of polymer/DNA to a much lesser degree than ionic interactions between cationic units and phosphates of DNA, since DNA does not participate in hydrophobic interactions. Authors concluded that adjustment of the hydrophobicity of DNA/carrier complex can be achieved more easily for synthetic polymeric gene carriers than lipid/DNA systems by using copolymerization of a desirable amount of hydrophobic monomers. These encouraging characteristics of the hydrophobic units may lead to better transfection efficiency than polymer systems using only ionic interactions (Kurisawa *et al*., 2000).

On the basis that amino acids such as arginine, lysine and leucine were involved in enhancing DNA transportation into cells, we hypothesized that the grafting of these amino acids to cationic non-viral carriers, namely, PEI and DAB would improve transfection efficacy in cancer cells.

Results from the transfection efficacy studies demonstrated that the conjugation of Arg, Lys and Leu to PEI led to an improved transfection compared to the unmodified PEI on both the tested cell lines, with the exception of PEI-Leu in A431 cells. The improved β-gal expression induced by amino acid-bearing PEI compared to PEI most likely resulted from the higher zeta-potential of their DNA complexes, due to the strong correlation between cellular uptake and positive charge density of polyplexes (Futaki *et al*., 2001). These results suggest that the grafting of basic amino acids on a polymer enhances the transfection activity compared to unmodified polymer. To the best of our knowledge, it is the first time that the grafting of Lys residues to gene delivery systems gave such improvement in gene expression.

On the other hand, Arg, Lys and Leu conjugated to DAB polyplexes at their respective optimal weigh ratios (10:1) improved the transfection efficiency compared to the unmodified DAB. The grafting of arginine residues to DAB which gave rise to improved transfection efficacy in our experiments has previously been shown to

improve gene delivery with mixed success depending of the polymers. For example, Choi *et al*. (2004) demonstrated that PAMAM-Arg improved gene expression in comparison with unmodified PAMAM, with results comparable to those obtained with PEI. In the same study, the transfection results obtained with PAMAM-Lys were slightly improved compared with those observed with unmodified PAMAM.

Kim and colleagues (2007) previously demonstrated that the transfection efficacy of Arg-bearing generation 2 PPI was increased compared to native PPI, with maximum efficacies reaching 27-54% of that of PEI 25 kDa. In our previous experiments, we showed that gene expression following treatment with PEI-Arg and PEI-Lys polyplexes was 3-fold higher than following treatment with PEI polyplex on T98G cells (Aldawsari *et al*., 2011). The use of amino acid-bearing DAB now further increases the gene expression level, to reach a maximum increase of 4.7-fold after treatment with DAB-Lys polyplex compared with native PEI. This favourable transfection profile could also be due to the ability of Arg and Lys to enable endosomal escape of the polyplex to the cytoplasm (Nam *et al*., 2009).

Cell transfection process is as follow: cellular membrane interaction, cellular uptake and endosomal escape, followed by gene expression. To enter the cytoplasm and nucleus, it is very crucial to escape from the endosome, which is generally achieved by the proton sponge effect (Nam *et al*., 2008). Newkome and Shreiner (2008) reported that the G3 polypropyleneimine (DAB 16) dendrimers, at low proton concentration, exhibit some protonation. This in a part may explain the enhanced transfection efficiency of DAB. The grafting of hydrophobic moieties has previously been shown to improve the transfection activity of cationic polymers, probably as a result of destabilization of cell membrane (Kurisawa *et al*., 2000; Kono *et al*., 2005).

In our study, the conjugation of Leu to DAB enhanced the transfection efficiency on both cell lines tested compared to the unmodified DAB. This may be attributed to the possibility that the hydrophobic moiety on DAB surface interacted strongly with the hydrophobic region of the cell membranes causing their destabilization, thereby enhancing translocation of the gene through the membrane (Kono *et al*., 2005). Many studies have established that hydrophobic functionalities affect transfection activity of cationic polymers (Kurisawa *et al*., 2000; Han *et al*.,

2001; Kono *et al*., 2005). Takahashi *et al*. (2003) reported that PAMAM dendrimers with hydrophobic moieties showed much higher transfection activity than unmodified PAMAM dendrimers of the same generation. They concluded in their study that introduction of appropriate amount of hydrophobic moieties to the dendrimers can elevate their transfection performance.

Many physicochemical and environmental factors can affect the cellular uptake of nanocarriers. In general, particle size, charge and surface modifications of non-viral gene delivery nanocarriers are the main determinants. In the case of particle size, macromolecules between 200 nm - 1 µm are most likely transported via caveolae-mediated endocytosis, whereas particles below 200 nm in size are mainly transported via clathrin-mediated endocytosis (Decuzzi and Ferrari, 2008; Hillaireau and Couvreur, 2009; Kelf *et al*., 2010). Particles over 1 µm in size, are internalize via macropinocytosis, although the uptake rate starts to drop dramatically for particles above 1 µm due to the difficulty of forming large vesicles (Kelf *et al*., 2010).

The role of surface charge is another important factor in the cellular uptake of nanocarriers, which controls to a large extent their interaction with the cell membrane. As mentioned earlier, positively charged nanocarriers show better association and internalization rate as a result of favourable interaction with negatively charged cell membrane (Hillaireau and Couvreur, 2009).

Surface modification of nanocarriers with different moieties that influence the efficiency of their internalization is an attractive strategy to promote the cellular uptake and ensure a better transfection performance. Such specific surface moieties were exploited in our study using amino acids for their excellent cell penetrating properties. The grafting of these amino acids to PEI and DAB systems significantly improved the internalization of the polyplexes/ polyplexes and DNA uptake by A431 and T98G cells as qualitatively confirmed by confocal microscopy. Co-localization of DNA in the nuclei was clearly visible in both cell lines treated with amino acids bearing PEI polyplexes in both cells. The conjugation of amino acids to DAB polyplexes improved DNA uptake in the cytoplasm in both cell lines. It was suggested that nuclear uptake of the DNA following this treatment may occur at a later time.

Assuming that therapeutic efficacy depends mainly on the amount of DNA being delivered to the cells, anti-proliferative studies (MTT assays) were done to confirm this point.

The grafting of these amino acids to PEI significantly improved the therapeutic efficacy of the system on the two cancer cell lines studied. PEI-Lys polyplex was the most efficacious treatment on A431 cell line with 51-fold improvement compared to PEI alone, probably as a result of its highest transfection efficacy on this cell line. Although PEI-Leu was the least efficacious of the three amino acid-bearing polymers for transfection, it led to the highest anti-proliferative activity on T98G cell line (269-fold compared to unmodified PEI). As this result cannot be attributed to its intrinsic toxicity nor to the enhanced nuclear uptake of DNA following this treatment, it is hypothesized that PEI-Leu polyplex may damage the cells due to the hydrophobic nature of leucine, in conjunction with the efficacy of the therapeutic plasmid.

In case of amino acids bearing-DAB polyplexes, IC_{50} was improved by 30-fold for DAB-Lys in A431 cells and 47-fold in T98G cells compared to the unmodified DAB. For so long, limited success of non-viral carriers has been associated to low transfection efficiency compared to the viral ones. Here, a positive correlation between cellular uptake and enhanced transfection efficiency was clearly shown. The results demonstrate that with the efficient delivery of therapeutic plasmid to cancer cells, potent anti-proliferative effect was observed.

In summary, enhanced gene transfection efficiency and anti-proliferative effect of amino acids conjugated to PEI and DAB carriers were in agreement with our hypothesis. Despite the presence of some cell line variation, the positive outcome achieved was probably due to the following factors. As a start, the choice of cationic vectors which have the ability to condense DNA into small size complexes and provide high stability against degradation. Complexes comprising cationic carriers and DNA are formed by ionic interactions that lead to increased cellular uptake and protect DNA from lysosomal enzyme degradation. However, most cationic polymeric vectors show lower transfection efficiency due to difficulty in dissociating to free DNA within cells. This difficulty was attributed to the stable ionic

interactions formed between amino group of the carriers and phosphate group of DNA that strongly resist dissociation (Kurisawa *et al*., 2000). As PEI and DAB are known for having high buffering capacity or what's known as "proton sponge" effect which enable the escape of carrier/DNA complexes from the endosome to the cytoplasm after entering inside the cells (Nam *et al*., 2009), improved gene expression was expected based on utilizing these two carriers in our study.

Moreover, the use of amino acids that are known for their excellent cell-penetrating features facilitate the cellular uptake and penetration into the cell membrane and nucleus significantly which led to higher transfection levels compared with the unmodified carriers. A hydrophobic amino acid (leucine) was used in this study, based on many studies reported that hydrophobic functionalities may influence the formation and stability of complexes between carriers and DNA, as well as enhancing interactions between the carriers and cells (Kono *et al*., 2005; Tian *et al*., 2007). The results obtained in our study showed enhanced transfection profile and improved cellular uptake of our modified systems, with highly improved anti-proliferative activity.

As the results from this Chapter provided an overall representation of the *in vitro* behaviour of these amino acids modified systems compared to the unmodified vectors, their promising potential have encouraged us to move forward for further *in vivo* investigational experiments.

Chapter 4

In vivo evaluation of amino acid-bearing polyethyleneimine and polypropyleneimine polymers

4.1 Introduction

The future of non-viral gene therapy depends largely on a better understanding of the delivery barriers. These include physicochemical properties that limit the design of delivery systems and influence the self-assembly of colloidal particulate formulations. However, biological barriers that compromise delivery of the DNA to its target site is another obstacle to the overall successful gene delivery process.

As the development of new cancer therapeutics is a very expensive and time consuming process, it is important that realistic strategies are adopted for early clinical trials. Typically, from concept to the end of Phase III clinical trials and acquiring the regulatory approvals, this may take more than 10 years and cost over 500 million dollars (Kelland, 2004). Generally, it should be possible to improve the efficiency of non-viral gene delivery systems by learning from the attributes of their *in vitro* transfection data that enable predict their performance across biological membranes. Although cell culture techniques and *in vitro* characterization studies have contributed to many recent advances in molecular physiology and gene therapy, *in vivo* study remains an essential tool in developing a successful gene delivery system due to the complexity of the biological system (Kelland, 2004). It is well known that *in vitro* transfection is typically far more efficient than the corresponding *in vivo* gene delivery, since the whole organism imposes the extracellular matrix, inhibitory biological fluids and many other barriers (Brown *et al*., 2001).

Accordingly, *in vivo* study is considered to be one of the most crucial studies prior to clinical trials in a drug evaluation cascade. As scientific research has its ultimate goal in improving human life, the use of animal in these researches has brought remarkable benefits not realised in any other way.

4.1.1 Animal studies

In laboratory animal studies, the moral balance is supported by researcher’s duties and the Animal (Scientific Procedure) Act 1986. Moreover, in accordance with the Cruelty to Animal Act, 1876, researchers have a responsibility to use the minimum number of animals whenever possible while minimizing any distress or

pain that might be caused (Smyth, 1978). Experimental aims should be well defined with the lowest possible procedure severity in a way that weights the harms against the benefits, thus proceeding only when there is a favourable balance. Finally, the Animals (Scientific Procedure Act) 1986 regulates the designated procedures where animal welfare and health must constantly be maintained and monitored by authorized personal, and project licences are appropriately justified, trained and supervised (Wolfensohn and Lloyd 1998).

Transplantation of human cancer cells or tumour biopsies into immunodeficient rodents (xenograft models) has constituted the main preclinical screen for developing novel cancer therapeutics (Morton and Houghton, 2007). In the past, cancer therapeutics screening relied on inoculation of murine cells on mouse models (Sausville and Burger, 2006). In the mid-1980s, the discovery of nude anythmic (nu/nu) mice which are T-cell deficient and subsequently B-cell and T-cell-deficient severe combined immunodeficient (scid/scid) mice allowed propagation of established *in vitro* human cancer cells in mouse model (Morton and Houghton, 2007). After this discovery, the formation of solid tumours in mouse model has become practical, providing a platform for studies on tumour biology, disease progression and evaluation of therapeutic formulations. Subcutaneous xenograft models are commonly selected due to the ease of tumour assessment (Sausville and Burger, 2006) compared to orthotopic transplantation which is often limited by difficulties in technical skills to produce vascularised tumours, not to mention other associated limitations such as cost and time consumption (Bibby, 2004). On the other hand, subcutaneous xenografts readily produce vascularised palpable tumours and are straightforward technically where substantial data can be obtained within limited time frame (Bibby, 2004).

It is well known that the most reliable results are most likely obtained when healthy animals are used in the study. Animal housing and environmental conditions are essential in conducting any studies (ScotPIL Training Manual, 2006). All mice were kept in animal unit for one week following their arrival, before starting any experiment. For maximum adaptation, mice are housed in groups of 5-6 with temperatures ranging between 19- 23°C, 40-70% humidity, 12 to 15 air changes per

h, and 12 h daylight cycle for circadian rhythm regulation (Wolfensohn and Lloyd 1998). Light intensity should be between 350-400 1x, while noise level should be kept minimal as mice are sensitive to ultrasounds. The normal fed for adult mice is about 3-5 g of pelleted mouse diet/day and 6-7 mL water/day (Wolfensohn and Lloyd 1998).

4.1.2 Biodistribution of gene expression

Accurate measurement of reporter gene activity is important in the process of developing novel gene delivery systems. It allows accurate evaluation of delivery vectors and in the same time it is important in the process of comparison of transgene expression by different promoters (Zinselmeyer *et al.,* 2003). In an ideal transfection study, the readouts from reporter gene assays should offer convenience and ease of use, and most importantly should be a true reflection of cellular transcription/translation processes. Another important point is the biological inertness of the reporter gene: the gene or gene product should not itself modulate expression, interfere with cellular signalling or modify the cells metabolism (Torbett, 2002). Ideally a different expression cassette/gene cloned into the same expression vector would give the same or very similar expression profile.

It is common to use plasmids with inserted reporter genes in transfection studies. One such reporter gene plasmid is the pCMVsportβ-galactosidase that encodes the β-galactosidase gene. The pCMVsportβ-galactosidase encoded lacZ (β-galactosidase) gene act as reporter gene markers *in vitro* and *in vivo* transfection studies.

In the past, one of the first assays to be employed for the measurement of transgene expression *in vivo* was the chloramphenicol acetyltransferase (CAT) assay. It involves the cleavage of the polar 3H-acetyl-Coenzyme A (CoA) and the release of 3H-chloramphenicol. The assay initially required chromatographic separation (Young *et al.,* 1985) or extraction (Sleigh, 1986) of substrate and product. A mixed phase procedure taking advantage of the selective diffusion of the product into the organic scintillation fluid simplifies the assay (Nielsen *et al.,* 1989). The assay

involves many steps to simplify it and raise environmental issues. Moreover, inconvenience in handling radioactivity makes the assay less convenient (Young *et al.,* 1985; Nielsen *et al.,* 1989; De Maio and Buchman, 1990).

The green fluorescent protein (GFP) of the bioluminescent jellyfish *Aequoria* is another method used in cellular imaging applications due to its great potential for use as a cellular marker (Hoffman, 2002). However, the quantification of gene expression using the fluorescence GFP and its derivatives depends on the biochemistry of the molecule and the environmental factors (Coralli *et al.,* 2001). Most frequently flow cytometric assays have been employed to quantify the amount of GFP *in vitro* (Tseng *et al.,* 1997), but this approach is not very practical for *in vivo* measurement. Autofluorescence and the modulation of cellular fluorescence by transfection reagents (Guo *et al.,* 2001) also limit assay specificity. Bio- and chemiluminescence have the advantage of being very specific, in particular when enzymes which lack an endogenous counterpart (e.g. luciferase) are being used (Schenborn and Groskreutz, 1999). While a number of complex bio-molecules will contribute to tissue autofluorescence the background is extremely low for luminescence. These properties make luciferase-based assays very useful tools for the sensitive measurement of reporter gene activity (Zinselmeyer *et al.,* 2003).

Cell transfected with pCMVsportβ-galactosidase plasmid DNA will express the β-galactosidase enzyme. β-galactosidase is an example of glycosidase enzymes which are known to be highly sensitive in hydrolytic cleavage of the bonds linking their favoured sugar (Zinselmeyer *et al.,* 2003).

In our *in vivo* transfection studies, the activity of β-galactosidase was quantified by measuring the enzymatic cleavage of *9H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)-D-galactopyranoside (DDAO-G, Molecular Probes) to 7-hydroxy-*9H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) moiety as the substrate for β-galactosidase enzyme expressed in successfully transfected cell. The product, DDAO, absorbs and emits light at much longer wavelength ($\lambda_{excitation}$ = 630 nm/ $\lambda_{emission}$ = 650 nm) than the substrate DDAO-G ($\lambda_{excitation}$ = 460 nm/ $\lambda_{emission}$ = 610 nm).

There are several factors contribute to a successful β-galactosidase *in vivo* transfection study. Briefly, the longer excitation wavelength of DDAO ($\lambda_{excitation}$ = 630 nm/ $\lambda_{emission}$ = 650 nm) compared to the substrate DDAO-galactoside ($\lambda_{excitation}$ = 460 nm/ $\lambda_{emission}$ = 610 nm) enables the DDAO to be excited without interference with its substrates. The fluorescence excitation and emission of DDAO above 600 nm is detected at wavelengths far beyond those emitted by most biological samples (at a wavelength of 360-600 nm) (Chance *et al.,* 1962; Aubin, 1979).

Another important factor for the β-galactosidase activity measured in a gene expression study is that it should be free from background activity resulting from non-glycoside hydrolases. In our study, we reduced this background activity by the addition of maltose to the DDAO-galactosidase reaction mix. The added maltose can compete with DDAO-galactoside for hydrolysis cleavage to glucose products by non-glycoside hydrolases (Zinselmeyer *et al.,* 2003). Besides that, the transgene product in the tissue lysate must be stable to withstand the effect of degrading enzymes released during homogenization and cellular lysis. Finally, protease inhibitor cocktail is added to enhance the stability of β-galactosidase activity as β-galactosidase enzyme is a liable protein to the attack of protease degradation (Zinselmeyer *et al.,* 2003).

4.1.3 Bioluminescence imaging

Progress in molecular biology has made available new bio-analytical tools that take advantage of the high detection ability and the simple analytical format of bioluminescence. Bioluminescence imaging provides a highly sensitive method for non-invasive, cost-effective, and real-time monitoring of sophisticated biological processes in intact animals (Zhang *et al.,* 2001).

In vivo bioluminescent imaging is a method to non-invasively measure luciferase expression in animals. This concept is based on the expression of luciferase in the living animal followed by an intraperitoneal (i.p.) dose of D-luciferin, and subsequent imaging of the bioluminescence in anesthetized animals by a cooled, charge-coupled device (CCD) camera (Figure 4.1). This imaging modality

has been broadly used in the examination of molecular processes due to its technical simplicity in quantifying photons emitted by the enzyme-catalyzed, chemiluminescent reaction. In particular, bioluminescent has been used to study tumour metastasis (van der Pluijm *et al.,* 2005) and monitor potential cancer therapeutics (Hoffman, 2005). Bioluminescence imaging is also widely used in gene therapy and accepted as the most definitive method to detect transgene expression *in vivo*. This is primarily because it is fast, sensitive, and free of false positives (Sato *et al.,* 2004). Compared to other methods for detecting *in vivo* transgene expression, bioluminescence imaging eliminates tissue harvesting. As a result, the same animals can be monitored over a time course, and the total number of animals used is greatly reduced. Gene transfer studies of both viral and non-viral vectors have used bioluminescence imaging to measure relative transfection efficiencies *in vivo* (Banerjee *et al.,* 2004; Rettig *et al.,* 2006). In our study, the biodistribution of gene expression was visualized by bioluminescence imaging, using an IVIS Spectrum. Production of luciferase enzyme is able to catalyze the oxidation of substrate D-luciferin to non-reactive oxyluciferin, emitting photons of light at 562 nm (Sato *et al.,* 2004). The depth of light emission is able to penetrate tissues of several millimeters-centimeters, thus allowing organ-level resolution (Sato *et al.,* 2004). The intensity of emitted light can be converted into a pseudo-colour graphic which provides a visual interpretation of tumour growth, regression in addition to monitor the efficacy of potential cancer therapeutics (Sato *et al.,* 2004; Welsh and Kay, 2005).

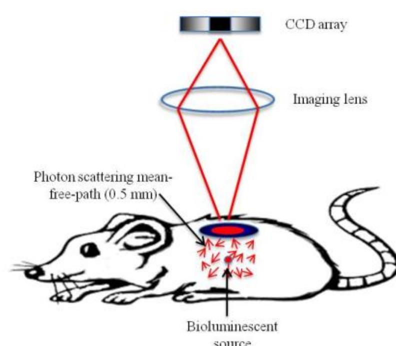


Figure 4.1: Illustration of photon transport of light from an internal source to the visible position on the surface of the animal.

4.1.4 Aims and Objectives

In Chapter 3, the *in vitro* transfection and therapeutic efficacies of PEI and DAB systems on A431 human epidermoid carcinoma and T98G mouse glioblastoma cell lines were investigated. The results showed promising potentials for evaluating the performance of these systems *in vivo*.

In the present Chapter, we investigated if the conjugation of arginine, lysine and leucine residues onto the surface of PEI and DAB delivery systems, could lead to an improved gene expression *in vivo* in tumours after intravenous administration in mice bearing A431 tumours.

Moreover, the biodistribution of gene expression was also visualized by bioluminescence imaging, using an IVIS Spectrum 200 on mice bearing subcutaneous A431 tumours.

4.2 Materials and Methods

4.2.1 Materials

Materials	Supplier
Amino acids bearing polyethyleneimine (PEI) polyplexes	Prepared as described in Chapter 2
Amino acids bearing polypropyleneimine (DAB) polyplexes	Prepared as described in Chapter 2
Phosphate buffered saline tablet	Sigma-Aldrich, UK
pCMVsport β-Galactosidase	Invitrogen, UK
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen, UK
Foetal Bovine Serum (FBS)	Invitrogen, UK
L-Glutamine	Invitrogen, UK
Penicillin-Streptomycin	Invitrogen, UK
Trypsin	Invitrogen, UK
Ampicillin	Sigma-Aldrich, UK
A431 human epidermoid carcinoma	The European Collection of Cell Cultures (Salisbury, UK)
Luciferase Assay Reagent	Promega, UK.
Protease Inhibitor Cocktail (PIC)	Sigma-Aldrich, UK
Passive Lysis Buffer (5x)	Promega, UK.
DDAO-Galactoside	Invitrogen, UK
Maltose	Fischer Scientific, UK
Dextrose	Fischer Scientific, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, UK
Organic solvents	Fischer Scientific, UK
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, UK

4.2.2 Cell culture

A431 cell line was grown as monolayers in DMEM medium. Growth medium was supplemented with 10% v/v foetal calf serum, 1% v/v L-glutamine and 0.5% v/v penicillin-streptomycin. Cells were cultured at 37°C in a humid atmosphere of 5% CO2.

4.2.3 Animals

Female immunodeficient BALB/c mice were housed in groups of five at 19°C to 23°C with a 12-hour light-dark cycle. They were fed a conventional diet (Rat and Mouse Standard Expanded, B&K Universal, Grimston, United Kingdom) with mains water *ad libitum*. Experimental work was carried out in accordance with UK Home Office regulations and approved by the local ethics committee.

4.2.4 Biodistribution of gene expression

Groups of mice (n=4, initial mean weight 20 g) bearing subcutaneously implanted A431 tumours were used in this study. Tumours were palpable (typical diameter 5 mm) 6 days after subcutaneous implantation of A431 cancer cells in exponential growth (1 x 10^6 cells/ flank). Mice were treated with a single injection of amino acid-bearing and control PEI and DAB carrying β-galactosidase expression plasmid (50 µg of DNA) via tail vein injection. Mice were sacrificed 24 h after injection. Their organs were removed, immediately frozen in liquid nitrogen and analyzed for their β-galactosidase levels. A homogenization / lysis buffer was prepared. Each organ was weighed, and 1 mL of the freshly prepared mash buffer was added to each organ (except for Liver= 2 mL). Passive lysis buffer (25 mL) was prepared as follow:

- 500 µl protease inhibitor cocktail
- 1000 µl PMSF 50 mM in methanol
- 5 mL PLB 5X
- 18.5 mL distilled water

Organs in homogenization/lysis buffer were homogenized using a tissue homogenizer (PowerGen 125, Fischer Scientific), and the resultant tissue homogenates were incubated on ice.

Quantification of β-galactosidase enzymatic activity were performed by measurement of β-galactosidase enzymatic cleavage of its substrate 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β-D-galactopyranoside (DDAO-Galactoside) to 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) product.

A DDAO-Galactoside Reaction Mix was prepared for each tissue homogenate, as follow: (For 1 sample: 15 µl DDAO gal in DMSO 5 mg/mL, 20 µl PMSF, 100 µl maltose in PBS: 20 g for 100 mL, 15 µl protease inhibitor cocktail, 150 µl PBS).

To 100 µl tissue homogenates, 300 µl of DDAO-Galactoside reaction mix is added and incubated at 37° C, with occasional mixing for the appropriate incubation time optimized for each organ (45 minutes for liver, 90 minutes for the other organs). A volume of 200 µl of the incubated reaction mixture was then transferred into another container and incubated in a heating block at 90° C for 2 minutes. The heated incubation was performed to stop the enzymatic cleavage of β-galactosidase on the DDAO-galactoside substrate and precipitate a large proportion of proteins. To extract the DDAO product, 800 µl isopropanol were added to dissolve the DDAO and the mixture incubated for 20 minutes at 5° C. Subsequently, the mixtures were centrifuged (7 minutes at 22 000 rpm). 500 µl of the supernatant were mixed with 500 µl of 80% (v/v) water-isopropranol mixture and measured in a fluorescence spectrophotometer (Varian Cary Eclipse Fluorescence spectrophotometer, λ_{exc} = 630nm, λ_{em}= 650 nm, slit 5 nm). The fluorescence units were used to calculate the β-galactosidase activity based on a linear regression ($f(x) = a + b.x$) fitted to a β-galactosidase standard curve. The concentration of β-galactosidase in the organs was determined by using the following equations, given by a β-galactosidase standard curve:

Concentration of β-galactosidase in 100 µL sample C1 (in mU):

$$C1 = (\text{fluorescence intensity} + 7.0067) / 5.36$$

Concentration of β-galactosidase per organ C2 (in mU):

$$C2 = C1 \times (1 + \text{weight sample (in g)} / 0.1$$

4.2.5 Bioluminescence imaging

The biodistribution of gene expression was also visualized by bioluminescence imaging, using an IVIS Spectrum (Caliper Life Sciences, USA). Mice bearing subcutaneous A431 tumours were treated intravenously with a single injection of amino acid-bearing and control PEI and DAB carrying luciferase expression plasmid (50 µg of DNA). Twenty-four hours after treatment, they were intraperitoneally injected with the luciferase substrate D-luciferin (150 mg/kg body weight) and anaesthetized by isoflurane inhalation. Light emission was measured 10 min after injection of the D-luciferin solution, for 2 min, using Living Image software. The resulting pseudo-colour images represent the spatial distribution of photon counts within the animal. Identical illumination settings were used for acquiring all images.

4.3 Results

4.3.1 Biodistribution of gene expression

For PEI polyplexes, the intravenous administration of PEI-Arg, PEI-Lys and PEI-Leu polyplexes led to a significant increase of gene expression in the tumour, with a β-galactosidase amount at least 3-fold higher than that obtained after treatment with unmodified PEI polyplex (18.2 ± 1.2, 20.4 ± 3.7, 19.2 ± 3.1mU β-galactosidase per tumour respectively for PEI-Arg, PEI-Lys and PEI-Leu polyplexes, compared to 6 ± 1.8 mU β-galactosidase per tumour for PEI polyplex) (Figure 4.2). The three amino acid-bearing PEI led to similar levels of gene expression in the tumour. The treatments were well tolerated by the mice at the administered doses. Gene expression was also increased in the kidneys (by at least 4-fold) and in the liver (by about 5-fold for PEI-Arg and PEI-Leu, 2-fold for PEI-Lys) after administration of amino acid-bearing PEI polyplexes. These results highlight the need of tumour-targeting moieties conjugated to the polyplexes in future experiments for increasing tumour gene expression specificity. PEI-Arg polyplex also increased gene expression level in the spleen by about 2-fold compared to the other amino acid-bearing and unmodified PEI polyplexes. Gene expression within the heart and lung was weak following all treatments, reaching a maximum of 3.1 ± 0.5 mU β-galactosidase per heart after treatment with PEI-Leu polyplex and 3 ± 1.1 mU β-galactosidase per lung after treatment with PEI-Arg polyplex.

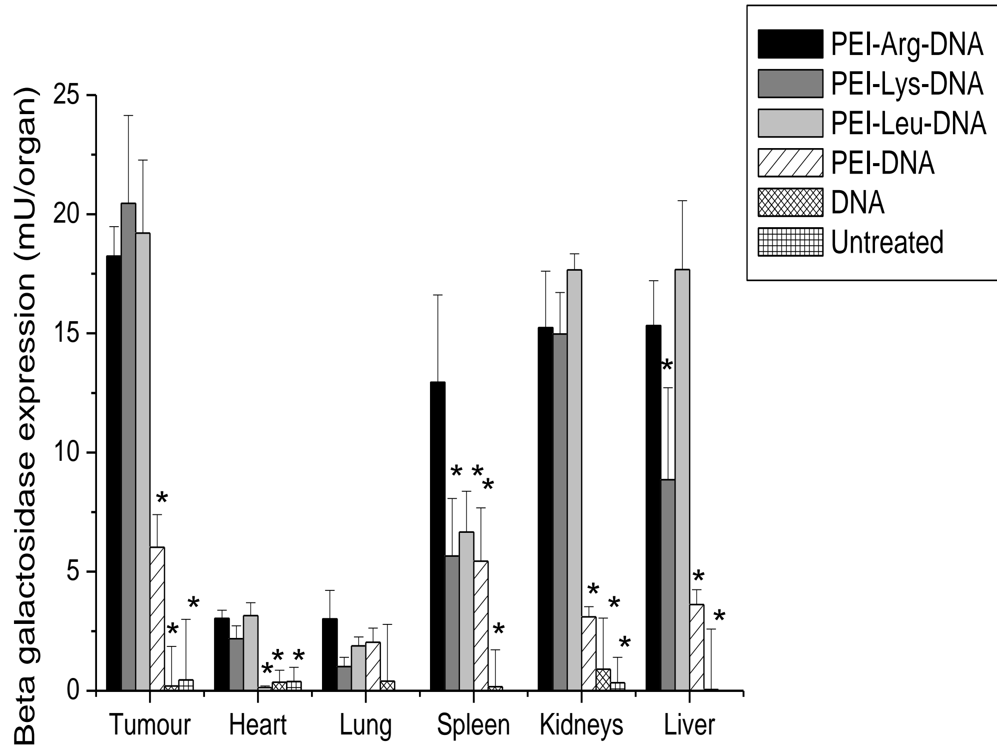


Figure 4.2: Biodistribution of gene expression after a single intravenous administration of Arg-, Lys- and Leu-bearing PEI polyplexes (50 µg DNA administered) and unmodified PEI polyplexes (50 µg DNA administered). Results were expressed as milliunits β- galactosidase per organ (*n=5*). *: $P < 0.05$: highest gene expression treatment *vs*. other treatments for each organ.

The intravenous administration of DAB-Arg, DAB-Lys and DAB-Leu polyplexes led to a significant increase of gene expression in the tumour compared to that obtained after treatment with unmodified DAB polyplex (34.9 ± 0.4, 39.9 ± 0.8, 36.7 ± 0.2 mU β-galactosidase per tumour respectively for DAB-Arg, DAB-Lys and DAB-Leu polyplexes, compared to 25.8 ± 5.6 mU β-galactosidase per tumour for DAB polyplex) (Figure 4.3). Among the 3 amino acid-bearing polymers tested, DAB-Lys led to the highest level of gene expression in the tumour. The treatments were well tolerated by the mice at the administered doses. Gene expression was also decreased in the heart (by about 15-fold for DAB-Arg, 7-fold for DAB-Lys and 5-fold for DAB-Leu) and in the spleen (by about 3-fold for DAB-Arg, 2-fold for DAB-Lys and 1.5-fold for DAB-Leu) compared with that of DAB polyplex. The results obtained in the lung and the kidneys were more amino-acid dependent: while the grafting of Arg led to a 5-fold decrease of gene expression compared to that of the unmodified polymer, the grafting of Lys did not modify it, and the grafting of Leu even increased it by more than 9-fold. A similar pattern was observed in the kidneys, where only the grafting of Arg led to a 2-fold decrease in the level of gene expression. In the liver, the grafting of Arg seemed to lead to an increase of gene expression compared to the other treatments. This increase was however not significantly different. The predominant gene expression in the liver compared to the other organs has been previously reported following intravenous administration of DAB polyplex, due to their removal by macrophages (Schätzlein *et al*., 2005). It is unusual, as particulate cationic gene transfer agents are generally reported to preferentially transfer genes to the lung, probably due to particle aggregation and accumulation in the small lung capillaries (Barron *et al*., 1999).

In summary, it was demonstrated in murine models bearing subcutaneous A431 tumours that gene expression was significantly enhanced in tumours when both amino acids modified PEI and DAB systems were administered intravenously.

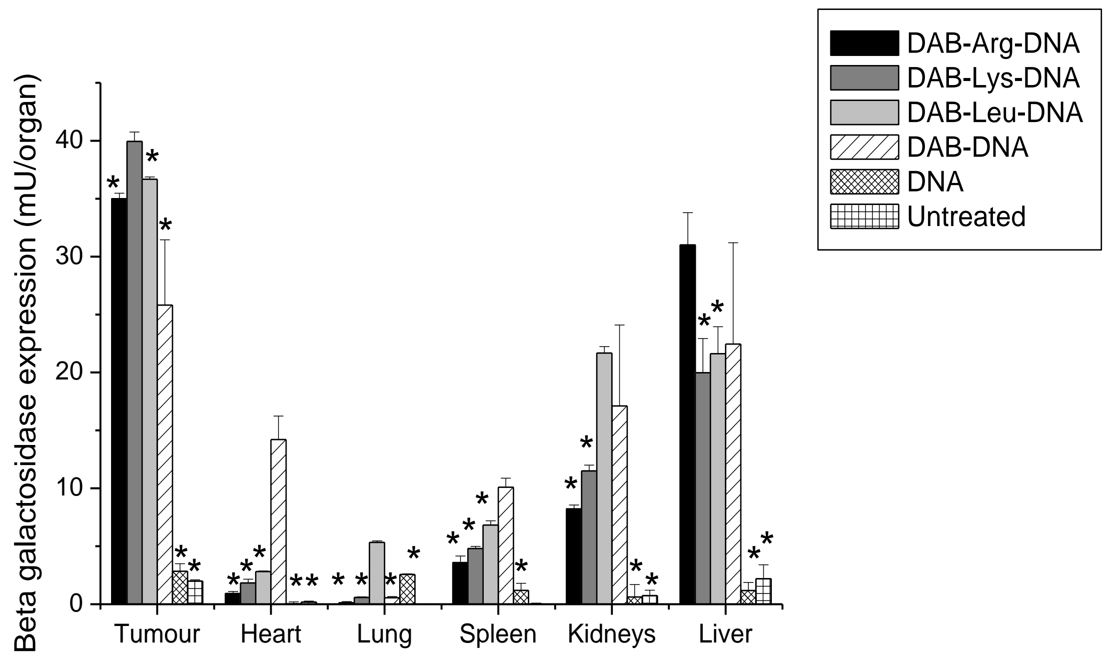


Figure 4.3: Biodistribution of gene expression after a single intravenous administration of Arg-, Lys- and Leu-bearing DAB polyplexes (50 µg DNA administered) and unmodified DAB polyplexes (50 µg DNA administered). Results were expressed as milliunits β-galactosidase per organ (*n=5*). *: P <0.05: highest gene expression treatment *vs*. other treatments for each organ.

4.3.2 Bioluminescence imaging

The biodistribution of gene expression was also confirmed using non-invasive bioluminescence imaging (Figure 4.4). For PEI systems, co-localization of gene expression in the tumours was clearly visible following treatment with the amino acid-bearing PEI polyplexes. Luciferase expression appeared to be heterogeneously distributed within the tumours, probably concentrated in highly vascularised areas within the tumours. It was however not visible in the tumours after treatment with unmodified PEI polyplex and naked DNA and appeared to be very limited in the other organs. This might be explained by the chosen parameters for image acquisition and the short imaging times, which probably allowed only the most intensely luminescent tissues to be analyzed. These data demonstrated that the conjugation of Arg, Lys and Leu to PEI increased gene expression within the tumours.

The luciferase expression of DAB systems, when conjugated to amino acids or administered as native dendrimers was also validated using bioluminescence imaging system (Figure 4.5). Co-localization of gene expression in the tumours was clearly visible following treatment with the amino acid-bearing DAB polyplexes. Luciferase expression appeared to be heterogeneously distributed within the tumours, probably concentrated in highly vascularized areas within the tumours. It was however not visible in the tumours after treatment with unmodified DAB polyplex and naked DNA and appeared to be very limited in the other organs, with the exception of the liver after treatment with DAB-Arg polyplex. These data demonstrated that the conjugation of Arg, Lys and Leu to DAB increased gene expression within the tumours. However, the significantly elevated gene expression in the liver highlights the need of tumour-targeting moieties conjugated to the polyplexes in future experiments for increasing the specificity of tumour gene expression.

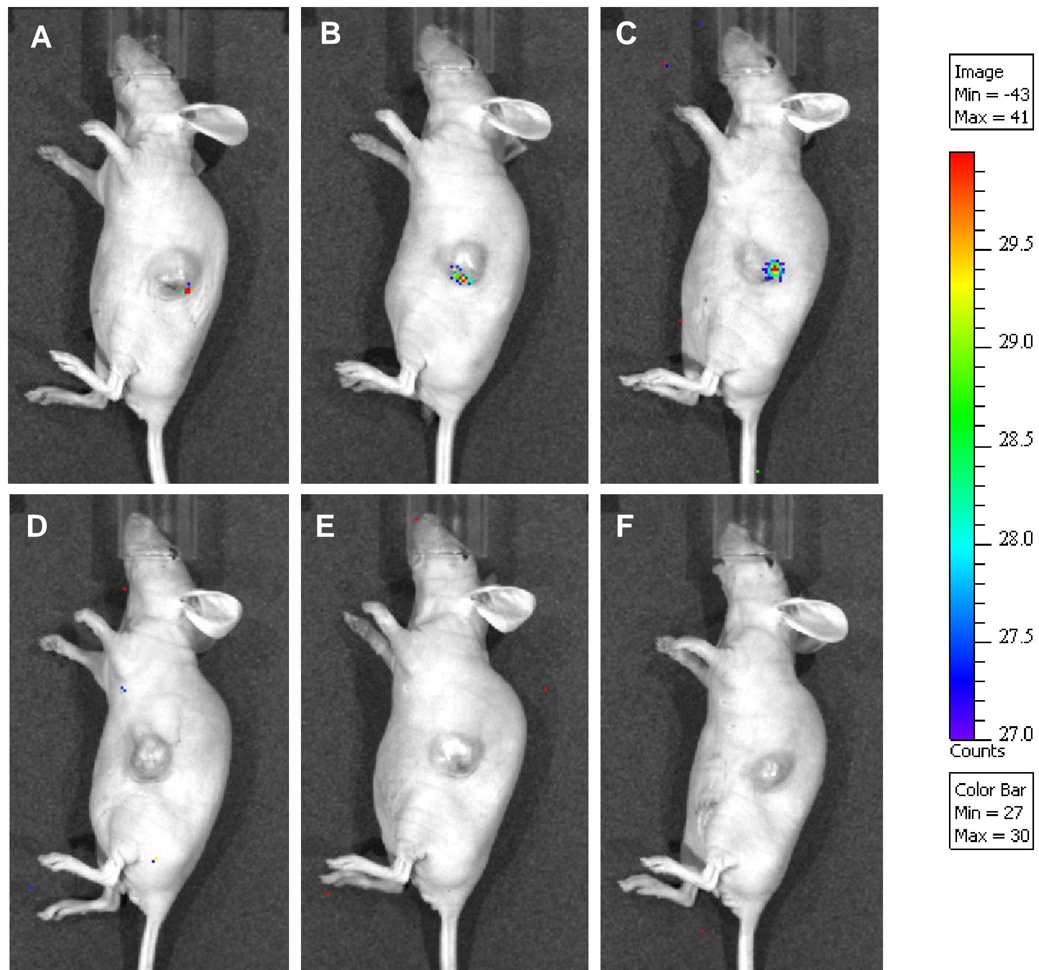


Figure 4.4: Bioluminescence imaging of gene expression after intravenous administration of luciferase-encoding plasmid DNA complexed with PEI-Arg (A), PEI-Lys (B), PEI-Leu (C), PEI (D) or free in solution (E) in living mice (50 µg DNA administered) (control: untreated cells (F). The mice were imaged using the IVIS Spectrum 24h after injection of the treatments. The scale indicates surface radiance (photons/s/cm^2/steradian).

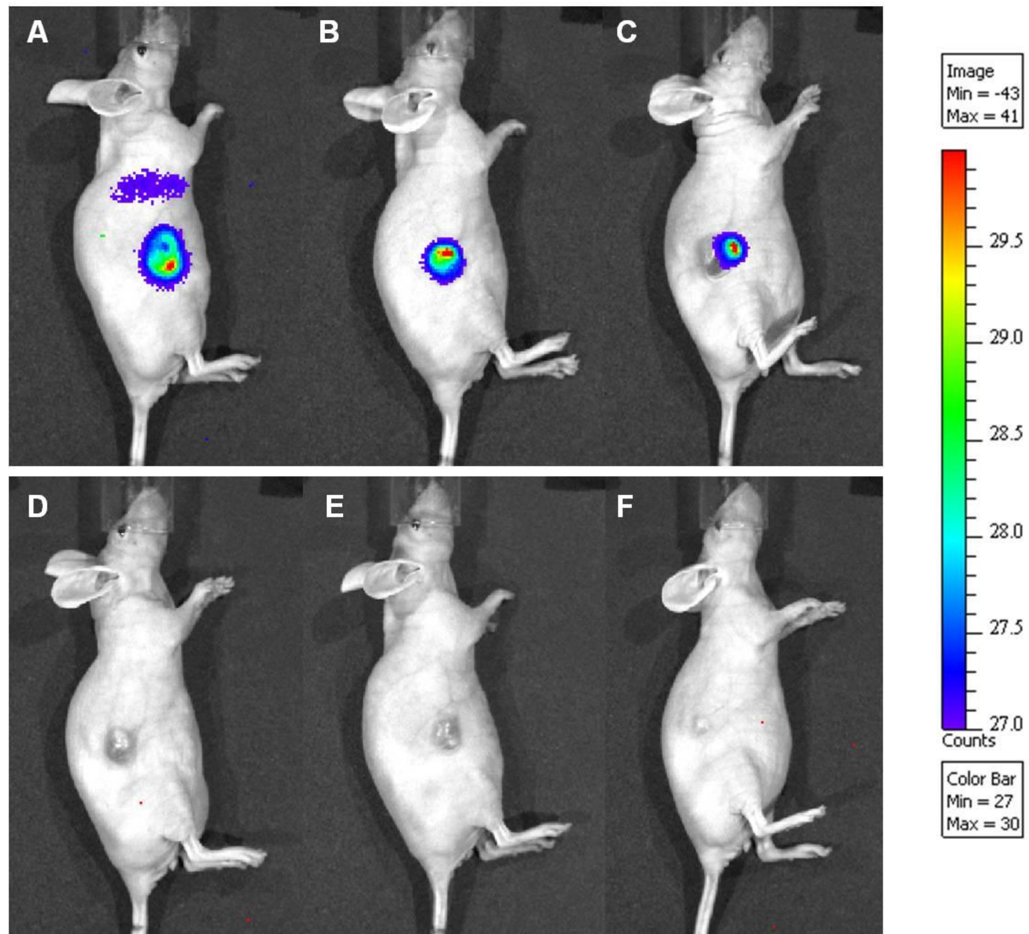


Figure 4.5: Bioluminescence imaging of gene expression after intravenous administration of luciferase-encoding plasmid DNA complexed with DAB-Arg (A), DAB-Lys (B), DAB-Leu (C), DAB (D) or free in solution (E) in living mice (50 μg DNA administered) (control: untreated cells (F). The mice were imaged using the IVIS Spectrum 24h after injection of the treatments. The scale indicates surface radiance (photons/s/cm^2/steradian).

4.4 Discussion

Gene therapy offers an exciting and novel therapeutic approach for treating cancer (Brooks, 2002). Non-viral vectors for gene delivery are receiving increasing attention for application in a broad variety of human gene mediated therapy. They represent attractive alternatives to the viral systems because they provide opportunities for improved safety and greater flexibility. In the same time, they lack the high transfection ability of the virus ones. In fact, the majority of non-viral systems are able to transfect exogenous DNA to cells *in vitro*, but only a few have been reported to transfect efficiently *in vivo* (De Smedt *et al*., 2000). However, to achieve a greater potential for gene therapy, genes must be delivered directly to the patient.

An optimal non-viral vector for gene delivery must have the ability to fulfil to some extent a number of pre-defined biological criteria depending on its specific therapeutic goal. In general, the bound, condensed carrier/DNA complex must be stable to the extracellular, as well as the intracellular environment. Once internalized, the complex must be resistant to degradative enzymes (Kim *et al.,* 2007). Finally, it must promote the nuclear translocation of plasmid DNA to efficiently exert the desired gene expression without any toxic effects (Kundu and Sharma, 2008).

As shown in the previous Chapter, both amino acids bearing-PEI /DAB systems were able to transfect A431 and T98G tumour cells *in vitro*. Their transfection efficiency as well as their anti-proliferative activity showed superior results compared to the unmodified carriers in both cell lines used.

In this Chapter, we evaluated the gene expression of amino acid bearing two cationic vectors; PEI and DAB after intravenous administration. We aimed at promoting the cellular uptake of DNA while protecting the DNA from enzymatic degradation by shielding the systems with amino acids. This result in an improved gene expression in the tumour as shown by the β-galactosidase expression of our modified systems when we use amino acids compared to the unmodified systems or DNA alone. Furthermore, the effect was visualized by bioluminescence imaging using an IVIS Spectrum on mice bearing subcutaneous A431 tumours.

The use of amino acids to enhance gene transfection in non-viral delivery systems was reported in literatures. Fujita *et al.* (2008) recognized that oligoarginine

which is one of cell-penetrating peptides (CPPs) such as HIV-1 Tat fragments, has attracted much attention because CPPs can deliver their associated molecules into cells. Since oligoarginine has similar characteristic to CPPs, it has been used in their study to deliver its associated molecules, genes and proteins into cells via intratumoural injection. Their results showed an improved gene expression in tumours following intratumoural injection of tetraarginine-PEG lipid coated protamine / DNA compared with the control delivery system. Similarly, Kim *et al.,* (2006) demonstrated that the intratumoural administration of novel glutamate-based cationic lipoplexes led to improved gene expression in the tumours compared to their aspartate-based counterparts.

In the two studies performed by Fujita *et al*. (2008) and Kim *et al*. (2006), it was shown that simple and effective transfection of subcutaneous solid tumours with strong β-galactosidase expression levels may be achieved only by direct intratumoural injection. Others had advocated the transfection abilities of PEI/DNA complexes were reported only when theses complexes were delivered at a slow rate of injection using a micropump which was believed to favour diffusion into tumour cells (Coll *et al*., 1999). Most trials in human gene therapy involve direct administration of DNA in a DNA carrier complex to a specific location in the body where gene expression is desired (Hagstrom, 2000). An intravenous administration of DNA carrier systems that could find their way to the specific location would be a much convenient option for both patients and clinicians.

In our study, Arg-, Lys- and Leu-bearing PEI and DAB systems administered intravenously led to an improved gene expression in subcutaneous tumours and should therefore have the potential to deliver and express their carried DNA to remote tumours or metastases unsuitable for intratumoural treatments. Among the three amino acids, lysine exhibits the highest gene expression in tumours followed by leucine and finally arginine in both systems. In contrast, Kono *et al*. (2005) reported the inability of leucine to improve the transfection activity of PAMAM dendrimers compared to phenylalanine amino acid. In comparison to their synthesis procedure which was time consuming and involves the use of many organic solvents, the resultant phenylalanine modified dendrimers was not water soluble and hardly form a complex with DNA at pH 7.4. The enhanced transfection efficiency observed with

arginine conjugated PEI/DAB was in accordance with previous studies (Choi *et al.,* 2004; Okuda *et al.,* 2004; Kim *et al.,* 2007; Nam *et al.,* 2008; Morris and Sharma, 2010).

There are many key factors governing the transfection efficiency of the non-viral carriers *in vivo*. First, smaller particles often believed to have a higher diffusibility through the extra cellular space and possibly also have a high chance of being endocytosed (Schwartz *et al*., 1995). It is well known that the choice of medium used to prepare the DNA formulations may influence transfection efficiency. Accordingly, 5% Glucose solution was chosen instead of saline solution because preparation of PEI/DNA complexes in such non-ionic medium has been found to form non-aggregating nano-sized structure (Coll *et al*., 1999). This phenomenon was also reported with DAB systems (Zinselmeyer *et al*., 2002). The highly resistant nature of PEI and DAB/DNA complexes towards aggregation in 5% glucose was attributed to the presence of strong repulsive forces between theses cationic complexes which preserve their colloidal stability (Coll *et al*., 1999).

Amino acids bearing PEI/DNA complexes were of a smaller size compared to unmodified PEI/DNA complex. Interestingly, this was not the case for DAB formulations in which the modified complexes showed slightly larger size compared to the DAB/DNA complex. This may be attributed to the higher DNA condensation ability of PEI compared to DAB.

Another important factor is the complex ability to escape from the endosomal compartment, or what is known as the "proton sponge" theory. PEI contains primary amines for DNA binding, and secondary as well as tertiary amines for endolysosomal escape of DNA complexes. Therefore, it can induce efficient gene transfection in many cell lines and has been used as the "gold standard" vector for gene transfection. From our finding, the expression level of β-galactosidase produced by the modified amino acids DAB/DNA complexes was higher than that obtained with PEI systems. These results were in line with those obtained in our *in vitro* transfection studies in Chapter 3 that showed superior transfection efficacy of DAB-amino acids polyplexes compared to the PEI ones.

The significantly high gene expression of both modified systems in tumours may be attributed to the fact that the permeability of the tumour vasculature is higher than that found in normal organs. This results in increased blood supply to the tumour and accumulation of transfection complexes in the tumour tissue. Moreover, the conjugation of amino acids may facilitate the cellular uptake and enhance cell penetration ability of the carriers in tumour tissues. This was achieved by the ability of the amino acids to shield the positive charge of these systems, and consequently prolonged their circulation time and facilitates their receptor mediated uptake. Kircheis *et al.* (2001) suggested that shielding PEI/DNA complexes could be achieved by coating with either polyethylene glycol (PEG) and/or by incorporating transferrin ligand at high density to target the TNF-α gene. Their results demonstrated high gene expression in tumour without detectable serum levels. Our *in vivo* results were in accordance with his finding by conjugating PEI and DAB to amino acids that result in high tumour gene expression. It was reported previously that amino acid transport across the plasma membrane is mediated via amino acid transporters located on the plasma membrane (Sang *et al.,* 1995; Kim *et al.,* 2004). Among the amino acid transport systems, the system L-amino acid transporter is highly expressed in malignant tumours presumably to support their continuous growth and proliferation (Sang *et al*., 1995; Kanai *et al*., 1998). This may as well contribute to the high gene expression obtained with our amino acids modified systems used in this study.

In the other organs, gene expression of both systems was also high in liver, kidney and spleen. This may be due to non-specific cellular uptake by the reticuloendothelial system (such as Kupffer cells in liver). Particulates on intravenous administration have been known to accumulate in the liver and spleen (Gregoriadis and Ryman, 1972) due to their removal by macrophages. Surface modification of such particulates with hydrophilic moieties minimized this liver uptake (Blume and Cevc, 1990). The uptake of DAB-Arg DNA was higher than that of DAB-Lys-DNA and DAB-Leu-DNA. There was no significant difference between DAB-DNA and any of the DAB-amino acid-DNA complexes.

In this study, amino acids bearing DAB complexes showed superior gene expression in tumours compared to liver (with the exception of DAB-Arg). This

finding was in contrast to what was previously reported that polypropyleneimine carriers are good candidates for liver targeting gene therapy (Zinselmeyer *et al.,* 2003). This may be explained by the fact that leakiness of the tumour vasculature appears to override the intrinsic targeting ability of DAB to the liver.

This study presented evidence that amino acid-bearing DAB polyplexes can lead to improved gene expression in the tumours after intravenous administration. Other groups have already demonstrated gene transfer capabilities of amino acid-bearing gene delivery systems, but following intratumoural rather than intravenous administration. Tetraarginine-PEG lipid coated protamine DNA has been previously reported to improve gene expression in tumours following intratumoural injection, compared to the control delivery system (Fujita *et al*., 2008). Similarly, the intratumoural administration of novel glutamate-based cationic lipoplexes improved the level of gene expression in the tumours compared to their aspartate-based counterparts (Kim *et al.,* 2006). Our previous study (Aldawsari *et al.,* 2011) presented first time evidence of the improved gene expression in the tumours after intravenous administration of amino acid-bearing PEI polyplexes, used as a model polymer. In the second part of the study, we wanted to evaluate if this improvement could be generalized to other types of gene delivery systems, such as dendrimers and especially generation 3 DAB dendrimer, which has been shown to be an extremely promising non-viral gene delivery system *in vitro* as well as *in vivo*. We demonstrated that this is indeed the case. Our objective will now be to improve the tumour specificity of gene expression, by conjugating a tumour-targeting ligand together with amino acids, with the hope to optimize the therapeutic effect.

In both modified systems, lower level of lung gene expression was observed, which is consider to be a good sign of the stability of our prepared systems. This finding is an indication that no particle aggregation and accumulation of the prepared complexes in the small lung capillaries occurred.

The biodistribution of gene expression was also confirmed by bioluminescence imaging, which is widely used technique to measure the localization and the relative degree of luciferase expression. The intensity of bioluminescence should be proportional to the amount of luciferase assuming that D-

luciferin is in saturation. Our results showed that luciferase expression appeared to be most intensely luminescence within the tumours, in both amino acids conjugated PEI and DAB systems. In fact, it appears more intensely luminescent in case of amino acids bearing DAB systems compared to modified PEI systems, which is in accordance with the transfection data *in vitro* and *in vivo*. It was however not visible in the tumours after treatment with unmodified systems and naked DNA and appeared to be very limited in the other organs (with the exception of Arg- DAB in liver). This might be explained by the chosen parameters for image acquisition and the short imaging times, which probably allowed only the most intensely luminescent tissues to be analyzed. These data demonstrated that the conjugation of Arg, Lys and Leu to PEI and DAB increased gene expression within the tumours.

This work corresponds to the first evaluation of gene expression of amino acid-bearing DAB delivery systems in tumours after intravenous administration. Most importantly, we did not observe any of the secondary effects generally resulting from treatment with PEI, suggesting that the grafting of Arg, Lys and Leu to PEI could be an alternative to PEG shielding with improved gene expression in the tumours. In fact between the two systems used, DAB conjugated to lysine exhibited the strongest gene expression values.

Chapter 5

Conclusion and future work

5.1 Conclusion

Cancer is one of the most wide-spread diseases of modern times, with an estimated increase in the number of patients diagnosed worldwide, from 11.3 million in 2007 to 15.5 million in 2030 (World Health Organization: Cancer. http://www.who.int/cancer/enS. Accessed February 2009). Despite the significant progress in current therapies in the past decades, they still have many limitations and are far from ideal. For effective cancer therapy, it is necessary to improve the knowledge of cancer physiopathology, discover new anti-cancer therapies and develop novel biomedical technologies (Byrne *et al.,* 2008).

Although gene therapy has recently offered great promise as a new approach in cancer treatment, it is nearly 20 years since the technique was first applied in a human clinical trial with limited success (Blaese *et al.,* 1995). The furthermost factor accountable for the slow progress is the lack of a safe and efficient method to deliver the genetic material to patient cells. While viral vectors have been the main vehicles for gene delivery due to their *in vivo* efficacy, clinical researches within the last decade have revealed that their safety is questionable (Gabrielson and Cheng, 2010). On the other hand, synthetic non-viral vectors provide opportunities for enhanced safety with better flexibility. While safer than viruses, their transfection efficiency is far lower than their viral counterparts (Kundu and Sharma, 2008).

Recently, polymer-based nanocarriers that include the use of polymer–DNA complexes (polyplexes) have received increasing attention for its ability to improve the efficacy of cancer therapeutics. Owing to their small size, these nanosized polymer therapeutic agents can circulate in the bloodstream for long periods of time, allowing them to reach the target tumour tissues (Park *et al*., 2008). However, efficient delivery systems have yet to be designed for these vector systems.

From this point, attempts have been made to improve polymeric gene delivery efficacy through rational design of vehicles capable of overcoming the various extra- and intracellular barriers that restrain their performance. Therefore, it is now possible to design delivery systems that specifically target cancer therapeutics to tumours based on the particular characteristics of the tumour microenvironment

(Gabrielson and Cheng, 2010). These nanosized carriers can reach tumours passively through the leaky vasculature surrounding the tumours by the Enhanced Permeability and Retention effect “EPR”, whereas ligands grafted at the surface of nano-carriers allow active targeting by binding to the receptors overexpressed by cancer cells (Park *et al*., 2008). Furthermore, the modification of polymeric vectors has also shown successful improvements in promoting intracellular gene transfer efficiency. Numerous systemic and cellular barriers, including serum proteins in blood stream, cell membrane, endosomal compartment and nuclear membrane, were successfully circumvented by designing polymer carriers having a smart molecular structure (Park *et al*., 2006).

On the basis that amino acids such as arginine and lysine were involved in enhancing DNA transportation into cells, we hypothesized that the grafting of these amino acids to the highly promising generation 3 diaminobutyric polypropylenimine (DAB) and polyethyleneimine (PEI) polymers would improve their transfection efficacy in cancer cells.

Leucine, a hydrophobic amino acid was also used in this study following several literature reviews about the role of hydrophobic moieties in enhancing the transfection efficacy of cationic non-viral gene delivery carriers.

Preparation and Characterization. Amino acids bearing-PEI polyplexes were successfully prepared by an easy one-step procedure producing high yields of over 95 % for all formulations. The polyplexes displayed average diameter sizes less than 305 nm and 300 nm for PEI and DAB respectively, at all weight ratios tested. The modified polyplexes displayed an overall favourable positive charge at their optimum weight ratios selected (20:1 and 10:1 for amino acids bearing-PEI and DAB polyplexes, respectively). DNA complexation studies showed that three amino acid-bearing PEI polyplexes were able to condense more than 80% of DNA, at polymer-to-DNA weight ratios higher than 2:1 for PEI-Arg, 0.5:1 for PEI-Lys, and 1:1 for PEI-Leu. On the other hand, the three amino acid-bearing DAB polyplexes were able to condense more than 70% of the DNA at polymer: weight ratios of 2:1 or higher. The DNA condensation observed for dendrimer: DNA weight ratios of 2:1 or higher was much higher than that observed for the unmodified dendrimer, which was of 60% at its best and decreasing with time. For both polymers, DNA condensation occurred almost instantaneously and was found to be stable over at least 24h.

In vitro Evaluation. In cellular studies, transfection efficacy mainly increased with increasing polymer-to-DNA ratios. The highest transfection levels for the three amino acid-bearing PEI polyplexes were observed at a polymer-to-DNA weight ratio of 20:1 on A431 and T98G cell lines. In A431 cells, treatment with PEI-Lys polyplex led to the highest transfection. Gene expression following treatment with PEI-Lys and PEI-Arg polyplexes was 2.2 times and 1.6 times higher, respectively, compared to PEI polyplex. The grafting of Leu to PEI did not bring any significant improvement in the level of transfection compared to unmodified PEI. On T98G cells, gene expression following treatment with PEI-Lys and PEI-Arg polyplexes was three times higher than that of PEI polyplex. Treatment with PEI-Leu polyplex was 1.8 times higher than that of PEI polyplex. For amino acids modified DAB polyplexes, the highest transfection level was obtained at a polymer: DNA weight ratio of 10:1 on A431 and T98G cells. At this ratio, treatment with DAB-Lys polyplex led to the highest transfection in both cell lines. The conjugation of Arg, Lys and Leu to DAB at their optimal polymer: DNA ratio of 10:1 led to an improved

transfection compared to unmodified DAB on both the tested cell lines. In A431, gene expression was improved by 1.7, 1.9 and 1.6-fold for DAB-Arg, DAB-Lys and DAB-Leu polyplexes, respectively compared to unmodified DAB polyplexes. In T98G, the improvement was 1.8, 1.9 and 1.6-fold for DAB-Arg, DAB-Lys and DAB-Leu, respectively compared to DAB polyplexes.

In vitro anti-proliferative activity was significantly increased in A431 cells after the conjugation of Arg, Lys, and Leu to PEI by 34-fold, 51-fold, and 40-fold, respectively compared to the unmodified PEI polyplex. In T98G cells, the conjugation of the amino acids to PEI improved the anti-proliferative activity of the polyplex, by 2-fold for PEI-Arg, 89-fold for PEI-Lys and by 269-fold for PEI-Leu compared to PEI polyplexes. For DAB modified polyplexes, the conjugation of Arg, Lys and Leu to DAB led to a significant increase of *in vitro* anti-proliferative activity in A431 cells, respectively by 30-fold, 22-fold and 20-fold compared to the unmodified polyplex. In T98G cells, the conjugation of the amino acids to DAB improved the anti-proliferative activity of the polyplex, by 43-fold for DAB-Arg, by 47-fold for DAB-Lys and by 35-fold for DAB-Leu compared to the unmodified DAB polyplex.

Finally, the improved gene expression of our modified amino acids polyplexes *in vitro* has encouraged us to test their potentials *in vivo* in mice bearing subcutaneous A431 tumours.

In vivo Evaluation. The intravenous administration of PEI-Arg, PEI-Lys, and PEI-Leu polyplexes led to a significant increase of gene expression in the tumour, with a β-galactosidase amount at least three-fold higher than that obtained after treatment with unmodified PEI polyplex. The three amino acid-bearing PEI polyplexes led to similar levels of gene expression in the tumour. The treatments were well tolerated by the mice at the administered doses. Bioluminescence imaging confirmed that the conjugation of Arg, Lys, and Leu to PEI increased gene expression within the tumours. Co-localization of gene expression in the tumours was clearly visible following treatment with the amino acid-bearing PEI polyplexes.

However, it was not visible in the tumours after treatment with PEI polyplex and naked DNA, and appeared to be very limited in the other organs.

The intravenous administration of DAB-Arg, DAB-Lys and DAB-Leu polyplexes led to a significant increase of gene expression in the tumour of at least 1.4-fold compared to that obtained after treatment with unmodified DAB polyplex Among the 3 amino acid-bearing polymers tested, DAB-Lys led to the highest level of gene expression in the tumour. The treatments were well tolerated by the mice at the administered doses. The biodistribution of gene expression was confirmed by bioluminescence imaging. Co-localization of gene expression in the tumours was clearly visible following treatment with the amino acid-bearing DAB polyplexes. However, luciferase expression was not visible in the tumours after treatment with DAB polyplex and naked DNA and appeared to be very limited in the other organs, with the exception of the liver after treatment with DAB-Arg polyplex.

In summary, new Arg, Lys, and Leu-bearing PEI polymers have been prepared with the aim of improving gene expression in cancer cells. *In vitro*, the administration of therapeutic DNA in complex with Arg-, Lys-, and Leu-bearing PEI resulted in an enhanced anti-proliferative efficacy compared to native polyplex on both cancer cell lines, by up to 269-fold after treatment by PEI-Leu polyplex on T98G cells. *In vivo*, the intravenous administration of PEI-Arg, PEI-Lys, and PEI-Leu polyplexes led to an improved tumour gene expression, with a β-galactosidase amount at least threefold higher than that obtained after treatment with unmodified PEI polyplex. The three amino acid-bearing PEI polyplexes led to similar levels of gene expression in the tumour. The treatments were well tolerated by the mice. Arg, Lys, and Leu-bearing PEI polymers are therefore highly promising gene delivery systems for cancer therapy.

For DAB polyplexes, the grafting of arginine, lysine and leucine to DAB dendrimer has been shown to enhance the anti-proliferative activity of the polyplexes *in vitro*, by up to 47-fold for DAB-Lys in T98G cells compared to the unmodified polyplex. *In vivo*, the intravenous administration of amino acid-bearing DAB polyplexes resulted in an improved tumour gene expression, with the highest gene expression level observed after treatment with DAB-Lys polyplex. To our

knowledge, it is the first time that the intravenous administration of an amino acid-bearing polyplex was able to induce such a pronounced gene expression in a tumour model. These transfection effects, together with the lack of toxicity, make arginine-, lysine- and leucine-bearing generation 3 polypropylenimine polymers highly promising gene delivery systems for both *in vitro* and *in vivo* tumour gene expression.

5.2 Future Work

Gene therapy is currently limited by the lack of safe and efficacious gene delivery carriers able to deliver therapeutic genes selectively to tumours by intravenous administration (Luo and Saltzman, 2000). Systemic gene delivery systems are needed for therapeutic application to cells unreachable by percutaneous injection and for tumour located in numerous sites, i.e. metastases (Morille *et al.,* 2008). In order to remediate to this problem, numerous non-viral gene delivery systems, including cationic polymers and lipids, have been developed, due to their merits in safety, ease of manufacturing, stability and high flexibility regarding the size of the transgene delivered (Li and Huang, 2007).

In our study, two novel non-viral gene carriers have been synthesized and characterized *in vitro* and *in vivo* as potential gene delivery agents. The promising results presented in this thesis propose different opportunities for research which could be pursued in the future.

In particular, chemical modification of polymer therapeutic agents with targeting ligands, such as transferrin or folate, capable of specifically binding receptors that are over-expressed in cancer cells can markedly augment their therapeutic efficiency. In our study, the *in vivo* studies showed that the gene expression was significantly increased in the tumours for both amino acid-modified systems compared to the native polymers. However, in the case of amino acids bearing-PEI polyplexes, gene expression was also increased in the kidneys, liver and in the spleen after administration of amino acid-bearing PEI polyplexes. For amino acids-bearing DAB polyplexes, the significantly elevated gene expression in the liver (with Arg-DAB polyplexes) highlight the need for tumour-targeting moieties conjugated to the polyplexes in future experiments for increasing tumour gene expression specificity.

Previously, Koppu *et al*. (2010) investigated if the conjugation of the generation 3 diaminobutyric polypropyleneimine (DAB) dendrimers to transferrin, whose receptors are overexpressed on numerous cancer cells, could achieve a selective gene delivery to tumours after intravenous administration, leading to an

increased therapeutic efficacy. The results from their study showed a rapid and sustained tumour regression over one month, with long term survival of 100% of the animals (90% complete response, 10% partial response).

Morris and Sharma (2011) investigated the possibility of creating a systemic stable tumour targeted PEGylated-PEI based vectors coupled with an arginine amino acids. They conjugated folate in their system aiming at delivering gene to specific tumour sites including brain, lung, ovary, uterus and kidney where folate receptors are overexpressed. The modified polymers showed enhanced transfection with high accumulation of targeted polymers in the tumour tissues of tumour bearing mice. The authors highlighted the potential of this modified polymer for brain targeting experiments.

Another interesting possibility would be to evaluate gene expression efficacy using different amino acids such as histidine (Okuda *et al.,* 2004) and other hydrophobic amino acids such as phenylalanine (Kono *et al.,* 2005).

In addition, other cancer cell lines may be investigated based on the L-amino acid transporter systems (LAT1 and LAT2). Dysregulated expression of LAT1 is feature of many primary human cancers and is connected to tumour invasion. LAT1 mRNA is only expressed in selected organs such as the brain, spleen, placenta and testis (Kanai *et al.,* 1998; Kim *et al.,* 2004). Based on the above information, other cancer cell lines like B16F10, prostate cancer cells and brain tumour cells may be investigated.

In particular, active targeting to brain tumours represents an interesting non-invasive approach for improving the gene delivery to the brain. Delivering therapeutics to the brain is usually limited by the presence of the blood brain barrier (BBB), which separates the blood from cerebral parenchyma, thus preventing the penetration of therapeutics to the central nervous system (Beduneau *et al.,* 2007). The concept of active targeting to the BBB is based on using various influx transport systems expressed within the cerebral endothelial such as, carrier-mediated transports, receptor-mediated endocytosis and adsorptive-mediated endocytosis systems. These transport systems have a critical physiological function in the delivery of vital materials to the brain. They also play a key role in the growth of

tumour cells and thus, they are usually overexpressed on tumours. Over 20 transporters have been identified, all highly expressed on the cerebral capillaries of the BBB. Among these transporters, large neutral amino acids (LAT1) can be utilized for future work using our prepared systems, to enhance both the selective brain targeting and the brain uptake of gene therapeutics.

The results obtained by our modified PEI and DAB polyplexes have demonstrated impressive gene expression accumulation in tumours after intravenous administration when conjugated to amino acids. Their gene expression in the tumours could be further enhanced by conjugating a tumour-targeting ligand, with the hope to optimize their overall anti-cancer therapeutic effect.

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Appendix I: Publications

1. Aldawsari, H., B.S. Raj, R. Edrada-Ebel, D.R. Blatchford, R.J. Tate, L. Tetley, and C. Dufes. 2010. Enhanced Transfection Efficacy of Polyethylenimine by Surface Modification with Arginine, Lysine, and Leucine. *CRS Newsletter*. 27 (6):10-12. (Cover of the issue).

2. Aldawsari, H., B.S. Raj, R. Edrada-Ebel, D.R. Blatchford, R.J. Tate, L. Tetley, and C. Dufes. 2011. Enhanced gene expression in tumors after intravenous administration of arginine-, lysine- and leucine-bearing polyethylenimine polyplex. *Nanomedicine*. *In Press, Corrected Proof, (http://dx.doi.org/10.1016/j.nano.2011.01.016).*

3. Aldawsari, H., R. Edrada-Ebel, D.R. Blatchford, R.J. Tate, L. Tetley, and C. Dufes. 2011. Enhanced gene expression in tumors after intravenous administration of arginine-, lysine- and leucine-bearing polypropylenimine polyplex. *Biomaterials. (http://dx.doi.org/10.1016/j.biomaterials.2011.04.079).*

Appendix II: Conference abstracts

1. Aldawsari, H., B.S. Raj, R. Edrada-Ebel, D.R. Blatchford, R.J. Tate, L. Tetley, and C. Dufes. *In vitro* evaluation of novel gene delivery systems for cancer therapy. The 3rd Saudi International Conference 2009 (Awarded the prize of the outstanding poster).
2. Aldawsari, H., B.S. Raj, R. Edrada-Ebel, D.R. Blatchford, R.J. Tate, L. Tetley, and C. Dufes. Enhanced gene expression of polyethyleneimine by surface modification with arginine, lysine and leucine. UK and Ireland Controlled Release Society Symposium 2010.
3. Aldawsari, H., B.S. Raj, R. Edrada-Ebel, D.R. Blatchford, R.J. Tate, L. Tetley, and C. Dufes. Enhanced gene expression of polyethyleneimine by surface modification with arginine, lysine and leucine. 37th Annual Meeting & Exposition of the Controlled Release Society 2010.
4. Aldawsari, H., B.S. Raj, R. Edrada-Ebel, D.R. Blatchford, R.J. Tate, L. Tetley, and C. Dufes. Enhanced gene expression of polyethylenimine by surface modification with arginine, lysine and leucine. The 4th Saudi International Conference 2010.

Others

1. *Second year PhD student's prize (poster presentation) in the Biomedical Chemistry and Drug Delivery (BCDD) Research day at University of Strathclyde. June 2009.*
2. *Outstanding Poster Award at the 3rd Saudi International conference, University of Surrey. June 2009.*
3. *Certificate of Excellence Award for contribution to Research in the Strathclyde Institute of Pharmacy and Biomedical Sciences. University of Strathclyde, August, 2010.*
4. *Runner-up for Scotland at the British Council's 2011 Shine! International Student Award.*

Newsletter

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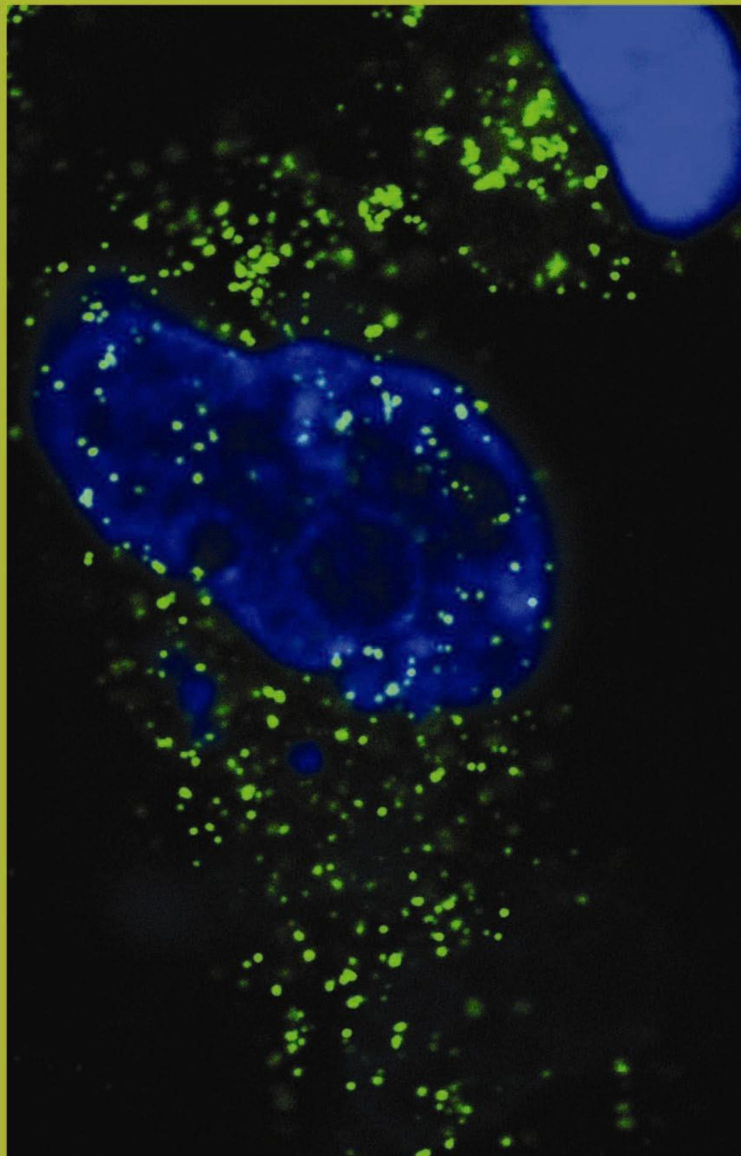

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Enhanced Transfection Efficacy of Polyethylenimine by Surface Modification with Arginine, Lysine, and Leucine

Hibah Aldawsari,[1] Behin Sundara Raj, RuAngelie Edrada-Ebel, David R. Blatchford, Rothwelle J. Tate, and Christine Dufès
Strathclyde Institute of Pharmacy and Biomedical Sciences, Glasgow, U.K.

Introduction

The potential of gene therapy is currently limited by the lack of delivery systems able to efficiently carry therapeutic DNA to their site of action. Non-viral vectors are receiving increasing attention as gene delivery vehicles due to the limitations associated with viral vectors in terms of safety and immunogenicity. Unfortunately, their use is hampered by their lower transfection efficacy compared with viral systems. The present study investigates the possibility of improving transfection by grafting amino acids onto the surface of a non-viral gene delivery system. We chose to use the amino acids arginine, lysine, and leucine because they have been reported to enhance transportation into cells (1–3). As a model delivery system, we chose to use the polymer polyethylenimine (PEI), because it has been widely used for non-viral transfection *in vitro* and *in vivo* and combines strong DNA compaction capacity with an intrinsic endosomolytic activity known as the proton sponge effect (4–5). It is hypothesized that arginine-, lysine-, and leucine-bearing polyethylenimine would lead to improved transfection efficacy through the synergistic action of the proton sponge effect and hydrophobic interactions with the cellular membranes.

The objectives of this study, therefore, are 1) to prepare and characterize arginine-, lysine-, and leucine-bearing polyethylenimine; 2) to evaluate their transfection and therapeutic efficacies *in vitro* on the A431 human epidermoid carcinoma cell line; and 3) to evaluate their transfection efficacy *in vivo* after intravenous administration in mice bearing A431 tumours.

Experimental Methods

Amino acid coupling to PEI was performed using a cross-linking agent and characterized as previously described (6). The *in vitro* cellular uptake of the amino acid-bearing polyplexes was qualitatively evaluated by confocal microscopy on A431 cells. The transfection efficiency was investigated at different polymer/DNA weight ratios and compared with unmodified PEI and DOTAP. Cytotoxicity was determined using an MTT assay. The *in vivo* biodistribution of gene expression in mice bearing subcutaneous A431 tumours was assessed using a β-galactosidase assay.

Results and Discussion

The grafting of arginine, lysine, and leucine residues on PEI was confirmed by ^{1}H NMR, and the three amino acid-bearing polymers were fully characterized. The highest *in vitro* transfection efficacy for the three amino acid-bearing PEI polyplexes was observed at a polymer/DNA weight ratio of 20:1, although it was not significantly different from that observed at a weight ratio of 10:1 for arginine-bearing polyplex and 10:1 and 5:1 for leucine-bearing polyplex (Figure 1A). The conjugation of arginine and

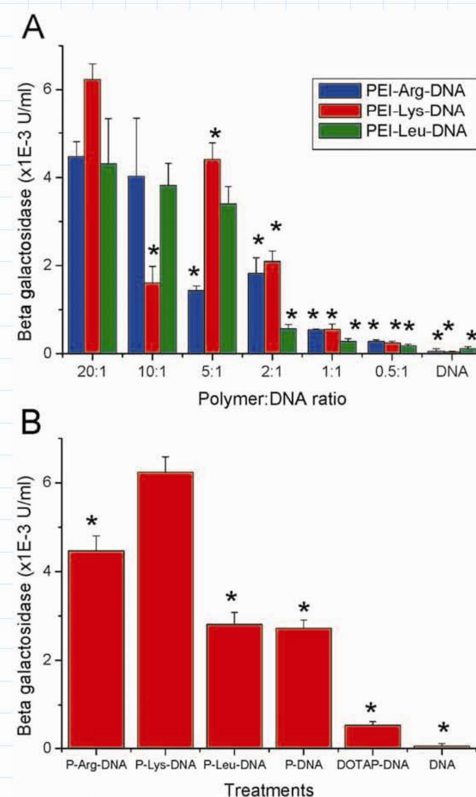

Figure 1. *Transfection efficacy of PEI-Arg, PEI-Lys, and PEI-Leu polyplexes at various polymer/DNA weight ratios (**A**) relative to DOTAP and native PEI (**B**) in A431 cells. DOTAP-DNA and PEI-DNA were dosed at their optimal carrier/DNA ratio of 5:1. Results are expressed as the mean ± SEM of three replicates (n = 15). * indicates P < 0.05 versus the highest transfection ratio (**A**) or treatment (**B**).*

[1] Corresponding author. E-mail: hibah.aldawsari@strath.ac.uk.

lysine residues to PEI led to improved transfection compared with the native PEI and DOTAP on the tested cell line, with improvements of up to 11.7 times following treatment with lysine-bearing polyplex compared with DOTAP-DNA (Figure 1B).

The administration of a therapeutic DNA complexed with arginine-, lysine-, and leucine-bearing PEI led to improved therapeutic efficacy compared with the unmodified polymer on the cancer cell line tested, by up to 51 times (Table 1).

The uptake of Cy3-labeled DNA by A431 cells was qualitatively confirmed using confocal microscopy (Figure 2). The complexation of plasmid DNA to PEI improved DNA uptake by the cells compared with DNA solution. Co-localization of DNA in the nuclei was clearly visible after treatment with PEI-Arg polyplex. It was less pronounced in the case of the other treatments.

The intravenous *in vivo* administration of PEI-Arg, PEI-Lys, and PEI-Leu polyplexes led to a significant increase in gene expression in the tumour, with a β-galactosidase amount at least

Table 1. Anti-proliferative activity of TNFα-expressing DNA complexed with PEI-Arg, PEI-Lys, PEI-Leu, and PEI in A431 cells, expressed as IC_{50} values (n = 15)

Formulation	IC_{50} (μg/mL) (mean ± SEM)
PEI-Arg-DNA	0.52 ± 0.13
PEI-Lys-DNA	0.35 ± 0.07
PEI-Leu-DNA	0.44 ± 0.16
PEI-DNA	17.86 ± 5.34
PEI-Arg	>50
PEI-Lys	>50
PEI-Leu	>50
DNA	>50

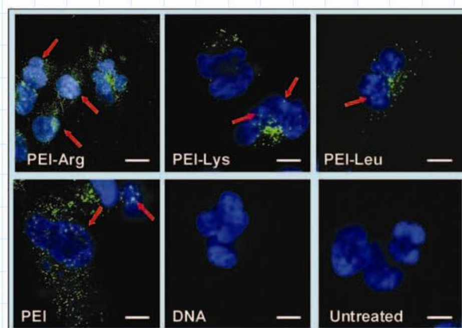

Figure 2. *Confocal microscopy imaging of the cellular uptake of Cy3-labeled DNA (6 μg/dish) either complexed with PEI-Arg, PEI-Lys, PEI-Leu, or PEI or free in solution after incubation for 24 hr with A431 cells (control: untreated cells). Blue: nuclei stained with DAPI (excitation:405-nm laser line; bandwidth: 415–491 nm); green: Cy3-labeled DNA (excitation: 453-nm laser line; bandwidth: 550–620 nm). Bar = 10 μm.*

threefold higher than that obtained after treatment with unmodified PEI polyplex (18.2 ± 1.2, 20.4 ± 3.7, and 19.2 ± 3.1 mU of β-galactosidase per tumour, respectively, for PEI-Arg, PEI-Lys, and PEI-Leu polyplexes compared with 6 ± 1.8 mU of β-galactosidase per tumour for PEI polyplex) (Figure 3).

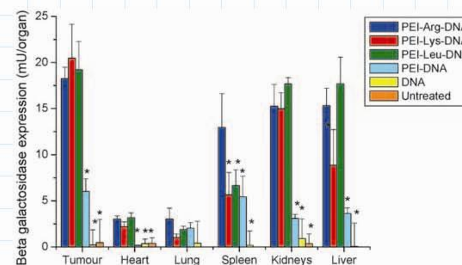

Figure 3. *Biodistribution of gene expression after a single intravenous administration of Arg-, Lys-, or Leu-bearing PEI polyplexes (50 μg of DNA administered) or unmodified PEI polyplexes (50 μg of DNA administered). Results were expressed as milliunits of β-galactosidase/organ (n = 5). * indicates P < 0.05: highest gene expression treatment versus other treatments for each organ.*

The three amino acid-bearing PEI led to similar levels of gene expression in the tumour. The treatments were well tolerated by the mice at the administered doses. Gene expression was also increased in the kidneys (by at least fourfold) and in the liver (by approximately fivefold for PEI-Arg and PEI-Leu and twofold for PEI-Lys) after administration of amino acid-bearing PEI polyplexes. PEI-Arg polyplex also increased gene expression level in the spleen by approximately twofold compared with the other amino acid-bearing and unmodified PEI polyplexes. Gene expression within the heart and lung was weak following all treatments, reaching a maximum of 3.1 ± 0.5 mU of β-galactosidase per heart after treatment with PEI-Leu polyplex and 3 ± 1.1 mU of β-galactosidase per lung after treatment with PEI-Arg polyplex.

Conclusions

New arginine-, lysine-, and leucine-bearing PEI polymers have been prepared with the aim of improving gene expression in cancer cells. The therapeutic efficacy *in vitro* of DNA encoding TNF-α was improved when delivered with amino acid-bearing PEI, by up to 51 times compared with unmodified PEI on the A431 cell line. The intravenous *in vivo* administration of PEI-Arg, PEI-Lys, and PEI-Leu polyplexes led to improved tumour gene expression. Arginine-, lysine-, and leucine-bearing PEI polymers, thus, are promising gene delivery systems for cancer therapy.

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Original Article

Enhanced gene expression in tumors after intravenous administration of arginine-, lysine- and leucine-bearing polyethylenimine polyplex

Hibah Aldawsari, PharmD[a], Behin Sundara Raj, PharmD[a], RuAngelie Edrada-Ebel, PhD[a], David R. Blatchford, CBiol FSB[a], Rothwelle J. Tate, PhD[a], Laurence Tetley, PhD[b], Christine Dufès, PhD[a,*]

[a]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, United Kingdom
[b]College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom

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Abstract

The potential of gene therapy to treat cancer is currently limited by the low expression of therapeutic genes in the tumors. Because amino acids are known to have excellent properties in cell penetration and gene expression regulation, we investigated if the conjugation of arginine (Arg), lysine (Lys) and leucine (Leu) onto the surface of the gene delivery system polyethylenimine (PEI) could lead to an improved gene expression in tumors. The intravenous administration of Arg-, Lys- and Leu-bearing PEI polyplexes led to a significant increase of gene expression in the tumor, with a *β*-galactosidase expression amount at least threefold higher than that obtained after treatment with unmodified PEI polyplex. The three amino acid-bearing PEI polyplexes led to similar levels of gene expression in the tumor. The treatments were well tolerated by the mice. Arg-, Lys- and Leu-bearing PEI polyplexes are therefore highly promising gene delivery systems for cancer therapy.
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Key words: Gene delivery; Amino acid; Gene expression; Cancer therapy; Polyethylenimine

Gene therapy has become a promising strategy for the treatment of many inheritable or acquired diseases that are currently considered incurable. Although the majority of gene delivery approaches so far have involved viral delivery systems, non-viral nanovectors are receiving increasing attention as gene delivery vehicles because of advantages such as their low toxicity, ease of manufacturing, stability and high flexibility regarding the size of the transgene delivered. Unfortunately, their use is still hampered by their lower transfection efficacy as compared with viral systems.[1]

Many techniques have been tried to overcome this problem. Among them, the conjugation of ligands to gene delivery systems seems particularly promising. It has recently been demonstrated that some peptide sequences known as transduction domains or membrane translocation signals contain positively charged amino acid residues such as arginine and lysine, which have been reported to have a high cell-penetrating ability.[2-4] Arginine (Arg) residues have previously been conjugated to polyamidoamine (PAMAM) dendrimers, polypropylenimine dendrimers, and poly(l-lysine) polymers, resulting in an enhanced transfection efficacy compared to native polymers in vitro.[5-8] Positively charged Arg and lysine (Lys) have been reported to have enhancing transfection ability; however, this also appeared to be the case for hydrophobic moieties such as leucine (Leu). Kono et al[9] demonstrated that PAMAM dendrimers conjugated with phenylalanine (Phe) or Leu residues achieved efficient gene transfection of cells. They also demonstrated that dendrons with hydrophobic moieties led to a much increased transfection activity compared to PAMAM dendrimers of the same generation. Arg, Lys and Leu could therefore improve transfection efficacy in vitro, but little is known about how the improvement achieved in transfection efficacy of cationic and hydrophobic amino acids compares among them. Similarly, little is known about their transfection

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*Corresponding author: Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, United Kingdom.
E-mail address: C.Dufes@strath.ac.uk (C. Dufès).

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efficacy in vivo after intravenous administration of an amino acid-bearing polyplex.

In the present study we investigated whether the conjugation of Arg, Lys and Leu residues onto the surface of a non-viral gene delivery system could lead to an improved gene expression in tumors after intravenous administration. As a model delivery nanosystem, we chose to use the cationic polymer polyethylenimine (PEI), because it has been widely used for non-viral transfection in vitro and in vivo, and it combines strong DNA compaction capacity with an intrinsic endosomolytic activity, also known as the proton sponge effect.[10] The use of PEI as a transfection reagent is severely limited, however, by its cytotoxic effects.[11] This problem has been overcome by shielding PEI with polyethylene glycol (PEG) moieties, to the detriment of transfection efficacy.[12] It is proposed that Arg-, Lys- and Leu-bearing PEI would lead to an improved transfection efficacy by synergistic actions of the proton sponge effect and interactions with the cellular membranes.

The objectives of this study were therefore (1) to prepare and characterize Arg-, Lys- and Leu-bearing PEI, (2) to evaluate the transfection and therapeutic efficacies of the three PEI polyplexes in vitro on A431 human epidermoid carcinoma and T98G mouse glioblastoma cell lines, and (3) to evaluate their transfection efficacy in vivo after intravenous administration in mice bearing A431 tumors.

Methods

Cell lines and reagents

Quant-iT PicoGreen double-stranded DNA reagent and tissue culture media were obtained from Invitrogen (Paisley, United Kingdom). Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Peterborough, United Kingdom). Passive lysis buffer was purchased from Promega (Southampton, United Kingdom). Label IT Cy3 Nucleic Acid Labeling kit was from Cambridge Biosciences (Cambridge, United Kingdom). Firefly d-luciferin was purchased from Caliper Life Sciences (Hopkinton, Massachusetts). Branched PEI, Arg, Lys, Leu and the other reagents were purchased from Sigma-Aldrich (Poole, United Kingdom). A431 human epidermoid carcinoma and T98G mouse glioblastoma cell lines were obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom). The expression plasmids encoding tumor necrosis factor (TNFα) (pORF9-mTNFα) and β-galactosidase (pCMVsport β-galactosidase) were obtained from InvivoGen (San Diego, California) and Invitrogen, respectively. The expression plasmid encoding luciferase (pEF1α-Luc) was constructed by subcloning the elongation factor 1 alpha (EF1α) promoter from pEF1/*Myc*-His vector (Invitrogen) into the promoterless pGL3-Basic vector (Promega). They were purified using an Endotoxin-free Giga Plasmid Kit (Qiagen, Hilden, Germany).

Synthesis and characterization of PEI-Arg, PEI-Lys and PEI-Leu

Conjugation of the amino acids to PEI

Amino acid coupling to PEI was performed by using dimethylsuberimidate as a cross-linking agent in a similar manner to that reported for transferrin-bearing dendrimers and vesicles.[13,14] PEI (8.6 mg) was added to the amino acids Arg, Leu or Lys (6 mg) and dimethylsuberimidate (12 mg) in triethanolamine HCl buffer (pH 7.4, 2 mL). The conjugation reaction was allowed to take place at 25°C for 2 hours while stirring. The final product was purified by size exclusion chromatography using a Sephadex G75 column and freeze-dried. The grafting of the amino acids to PEI was assessed by proton nuclear magnetic resonance spectroscopy.

Characterization of polyplex formation

The degree of DNA accessibility following complexation with the amino acid-bearing PEI polymers was assessed by PicoGreen (Invitrogen) assay, performed according to the protocol from the supplier. The fluorescence of PicoGreen significantly increases on intercalation with double-stranded DNA. The electrostatic interaction between the anionic DNA and cationic groups of the polymer on formation of the DNA-PEI-amino acids nanocomplex condenses the DNA and reduces the number of PicoGreen binding sites, ultimately reducing the fluorescence intensity for the PicoGreen solution.

PicoGreen reagent was diluted 200-fold in Tris-EDTA buffer (10 mmol Tris, 1 mmol EDTA, pH 7.5) on the day of the experiment. One milliliter of complex polymer-DNA at various polymer-to-DNA weight ratios (20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1, 0:1) was added to 1 mL PicoGreen solution. The DNA concentration in the cuvette (10 μg/mL) was kept constant throughout the experiment. The fluorescence intensities of the complexes in the presence of PicoGreen were analyzed at various time points with a Varian Cary Eclipse Fluorescence spectrophotometer (λ_{exc} 480 nm, λ_{em} 520 nm) (Varian, Palo Alto, California). Results were represented as a percentage of DNA condensation (=100 - % relative fluorescence to DNA control) and compared with those obtained for PEI-DNA (polymer-to-DNA weight ratio 5:1) (n = 4).

Polyplex size and zeta potential measurement

Size and zeta potential of the amino acid-bearing PEI in complex with DNA were measured by photon correlation spectroscopy and laser Doppler electrophoresis using a Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, United Kingdom).

In vitro biological characterization

Cell culture

A431 and T98G cell lines were grown as monolayers in Dulbecco's Modified Eagle Medium supplemented with 10% (vol/vol) fetal bovine serum, 1% (vol/vol) l-glutamine, and 0.5% (vol/vol) penicillin-streptomycin. Cells were cultured at 37°C in a 5% CO_2 atmosphere.

In vitro transfection

Transfection efficacy of the DNA carried by the amino acid-bearing polymers was assessed with a plasmid coding for β-galactosidase (pCMV βgal), using a β-galactosidase transfection assay. A431 and T98G cells were seeded in quintuplicate at a density of 2000 cells per well in 96-well plates. After 72 hours of incubation the cells were treated with PEI-Arg, PEI-Leu, and PEI-Lys in complex with plasmid DNA encoding β-galactosidase, at the polymer-to-DNA weight ratios used for the DNA condensation experiment. Naked DNA served as a

negative control; PEI-DNA (polymer-to-DNA weight ratio 5:1) served as a positive control. DNA concentration (10 μg/mL) was kept constant for all the formulations tested. After 72 hours of incubation, cells were lysed with 1× passive lysis buffer (50 μL/well) for 20 minutes. The cell lysates were subsequently analyzed for β-galactosidase expression.[15]

Cellular uptake

Imaging of the cellular uptake of the DNA carried by amino acid-bearing PEI was done by confocal microscopy. Labeling of plasmid DNA with the fluorescent probe Cy3 was performed using a Label IT Cy3 Nucleic Acid Labeling kit, as described by the manufacturer (Mirus Bio LLC, Madison, Wisconsin). A431 and T98G cells were grown on microscope slides (0.6×10^6 cells per 90-mm Petri dish) at 37°C for 24 hours. The cells were then incubated for 24 hours with Cy3-labeled DNA (6 μg per dish) in complex with PEI-Arg, PEI-Lys, PEI-Leu, and PEI at the polymer-to-DNA weight ratios giving the highest transfection efficacy (20:1 for amino acid-bearing PEI, 5:1 for PEI). Control slides were treated with naked DNA or remained untreated. The slides were then washed with PBS and fixed in methanol for 30 minutes. Upon staining of the nuclei with DAPI, the cells were examined using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). DAPI was excited with the 405-nm laser line (bandwidth: 415-491 nm), whereas Cy3 was excited with the 543-nm laser line (bandwidth: 550–620 nm).

In vitro antiproliferative activity

Antiproliferative activity of amino acid-bearing PEI in complex with plasmid DNA encoding TNFα was assessed in A431 and T98G cell lines. Cells (2000 cells per well in 96-well plates seeded 72 hours before treatment) were incubated for 72 hours with the DNA formulations at final concentrations of 6.4×10^{-4} to 50 μg/mL. Antiproliferative activity was evaluated by measurement of the growth-inhibitory concentration for 50% of the cell population (IC_{50}) in a standard MTT assay. Absorbance was measured at 570 nm using a plate reader. Dose-response curves were fitted to percentage absorbance values to obtain IC_{50} values (three independent experiments with $n = 5$ for each concentration level).

In vivo study

Animals

Female immunodeficient BALB/c mice were housed in groups of five at 19°C to 23°C with a 12-hour light-dark cycle. They were fed a conventional diet (Rat and Mouse Standard Expanded; B&K Universal, Grimston, United Kingdom) with water ad libitum. Experimental work was carried out in accordance with United Kingdom Home Office regulations and approved by the local ethics committee.

Biodistribution of gene expression

Groups of mice ($n = 4$, initial mean weight 20 g) bearing subcutaneously implanted A431 tumors were treated intravenously with a single injection of amino acid-bearing and control PEI carrying β-galactosidase expression plasmid (50 μg of DNA). Mice were euthanized 24 hours after injection. Their organs were removed, immediately frozen in liquid nitrogen and analyzed for their β-galactosidase levels as previously described.[16]

The biodistribution of gene expression was also visualized by bioluminescence imaging, using an IVIS Spectrum (Caliper Life Sciences). Mice bearing subcutaneous A431 tumors were treated intravenously with a single injection of amino acid-bearing and control PEI carrying luciferase expression plasmid (50 μg of DNA). Twenty-four hours after treatment they were intraperitoneally injected with the luciferase substrate d-luciferin (150 mg/kg body weight) and anesthetized by isoflurane inhalation. Light emission was measured 10 minutes after injection of the d-luciferin solution, for 2 minutes, using Living Image software (Caliper Life Sciences). The resulting pseudo-color images represent the spatial distribution of photon counts within the animal. Identical illumination settings were used for acquiring all images.

Statistical analysis

Results were expressed as means ± standard error of the mean. Statistical significance was determined by one-way analysis of variance followed by the Bonferroni multiple comparison post-test (GraphPad Prism software, San Diego, California). Differences were considered as significant when $P < 0.05$.

Results

Synthesis and characterization of PEI-Arg, PEI-Lys, and PEI-Leu

Conjugation of the amino acids to PEI

The synthesis of PEI-Arg, PEI-Lys, and PEI-Leu was achieved by an easy one-step process and was confirmed by proton nuclear magnetic resonance spectrum (Supplementary Figure S1, which can be found in the online version of this article).

Characterization of polyplex formation

The conjugation of amino acid residues on the primary amines of PEI might affect the polymer's ability to form a complex to DNA. For this reason we examined the influence of the conjugation of amino acids on the polymer's ability to form nanocomplexes with DNA, by using a PicoGreen assay. The three amino acid-bearing polymers were able to condense more than 80% of DNA, at polymer-to-DNA weight ratios higher than 2:1 for PEI-Arg, 0.5:1 for PEI-Lys, and 1:1 for PEI-Leu (Figure 1). DNA condensation increased with increasing weight ratios and was almost complete at a polymer-to-DNA weight ratio of 20:1 for the three polymers. It occurred almost instantaneously and was found to be stable for at least 24 hours. At the polymer-to-DNA ratio of 5:1, optimal for PEI polyplex, the DNA condensation was either improved for PEI-Arg and PEI-Leu, or similar for PEI-Lys compared to that observed with PEI, which was about 90% independently of the duration. The formation of nanoparticles of amino acid-bearing PEI polyplexes was also demonstrated by transmission electron microscopy (Supplementary Figure S2 online). A gel retardation assay confirmed the complete or partial DNA condensation by the

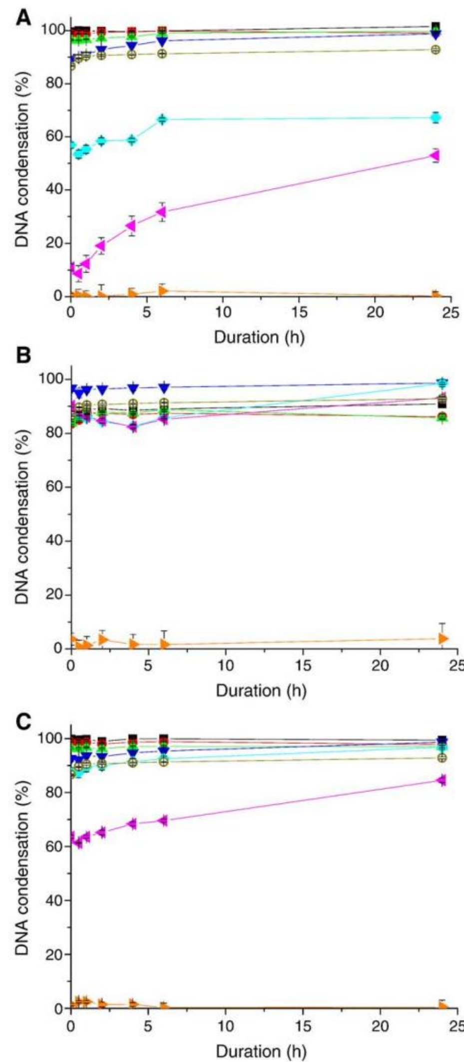

Figure 1. DNA condensation of PEI-Arg **(A)** PEI-Lys **(B)** and PEI-Leu **(C)** polyplexes using PicoGreen reagent at various durations and polymer-to-DNA weight ratios: 20:1 (■, *black*), 10:1 (●, *red*), 5:1 (▲, *green*), 2:1 (▼, *blue*), 1:1 (♦, *cyan*), 0.5:1 (◄, *pink*), DNA only (►, *orange*) (empty symbol, *dark yellow*: PEI-DNA, polymer-to-DNA weight ratio: 5:1). Results are expressed as mean ± SEM (n = 4).

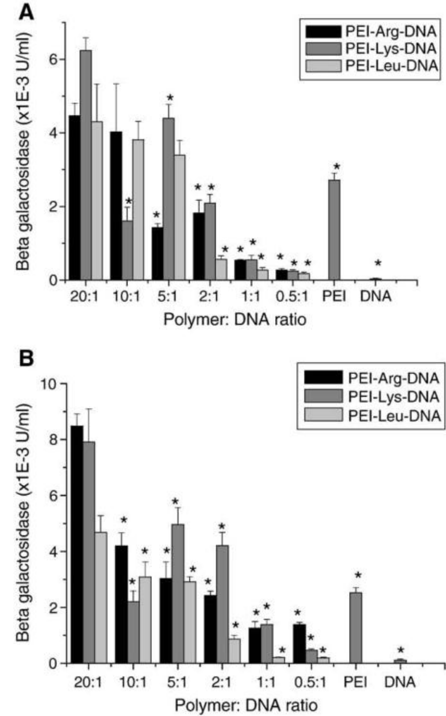

Figure 2. Transfection efficacy of PEI-Arg, PEI-Lys, and PEI-Leu polyplexes at various polymer-to-DNA weight ratios relative to native PEI in A431 **(A)** and T98G cells **(B)**. PEI-DNA was dosed at its optimal carrier-to-DNA ratio of 5:1. Results are expressed as the mean ± SEM of three replicates (n = 15). *P < 0.05 vs. the highest transfection ratio.

three amino acid-bearing PEI polyplexes (Supplementary Figures S3-S5 online). These results demonstrated that complexes can be formed through electrostatic interactions between DNA and amino acid-bearing PEI. The conjugation of amino acids to PEI did not destabilize DNA condensation, which is a prerequisite for the transport of this macromolecule.

Polyplex size and zeta potential measurement

Amino acid-bearing polyplexes displayed average nanoparticle diameter sizes less than 305 nm at all weight ratios tested (Supplementary Figure 6 online). Polyplex size decreased with increasing weight ratios, with the exception of PEI-Arg polyplex, smaller at a polymer-to-DNA weight ratio of 0.5 than at a ratio of 1: 297 nm (polydispersity: 0.416) against 303 nm (polydispersity: 0.398), respectively. This decrease could be explained by the negatively charged DNA partially neutralizing the positively charged PEI shell. PEI will become less water-soluble, shrink and thus drive closer together the molecules in

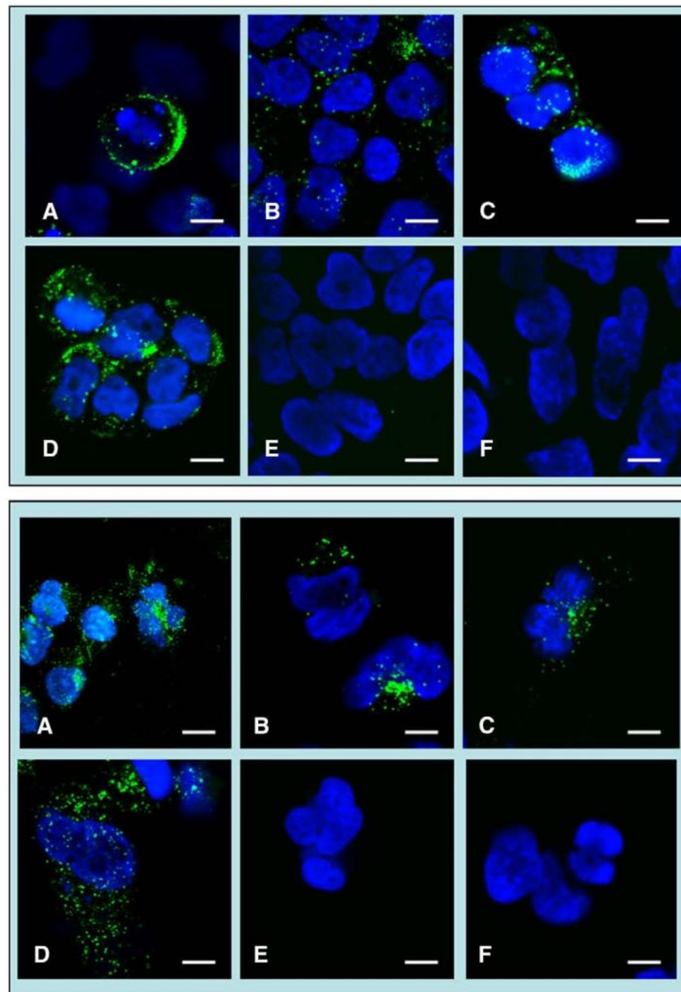

Figure 3. Confocal microscopy imaging of the cellular uptake of Cy3-labeled DNA (6 μg per dish) in complex with PEI-Arg **(A)**, PEI-Lys **(B)**, PEI-Leu **(C)**, PEI **(D)**, or free in solution **(E)** after incubation for 24 hours with A431 (top) and T98G cells (bottom). **(F)** Control, untreated cells. Nuclei stained with DAPI (excitation: 405 nm laser line, bandwidth: 415–491 nm) show as blue;, Cy3-labeled DNA (excitation: 543 nm laser line. bandwidth: 550-620 nm) show as green. Bar, 10 μm).

complex.[17] Among the three amino acid-bearing polyplexes studied, PEI-Arg polyplex was found to be the largest, independently of the weight ratios tested. Its size reached a maximum of 303 nm (polydispersity: 0.398). Below a weight ratio of 2, PEI-Lys polyplex was significantly smaller than PEI-Leu polyplex: 245 nm (polydispersity: 0.311) against 280 nm (polydispersity: 0.407), respectively, for a ratio of 0.5. By contrast, there was no significant difference between PEI-Lys and PEI-Leu sizes over a weight ratio of 2. PEI-Leu polyplex at a weight ratio of 20 was found to be the smallest among those tested, with a size of 199 nm (polydispersity: 0.186). This may be explained by the hydrophobic nature of Leu, compressing the

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complex into smaller and compact particles. The conjugation of amino acids to PEI led to an increase of the polyplexes size compared to that of the unmodified PEI polyplex (147 nm, polydispersity: 0.413). These results showed that amino acid-bearing PEI can condense DNA into nanosized particles appropriate for gene delivery.

Zeta potential experiments demonstrated that the three amino acid-bearing PEI polyplexes were bearing a negative surface charge at a weight ratio of 0.5, suggesting that stable polyplex was not formed yet at that ratio (Supplementary Figure 6 online). This result was consistent with the DNA condensation and the agarose gel electrophoresis results. The zeta potential values of the polyplexes gradually increased with increasing weight ratios, finally reaching their maximum (21 mV, 20 mV and 18 mV for PEI-Arg, PEI-Lys and PEI-Leu polyplexes, respectively) at a weight ratio of 20. The conjugation of Arg, Lys and Leu to PEI increased the overall positive charge of the complexes compared to PEI-DNA (4 mV) for weight ratios over 5. With increasing polymer-to-DNA weight ratios, the nanoparticle size decreased and the zeta potential increased, showing that more charged polymers could more efficiently condense DNA. A net positive charge of polyplex surface would increase cellular uptake due to its electrostatic interactions with the negatively charged cellular membranes.[18] Our results demonstrated that PEI-Arg, PEI-Lys, and PEI-Leu have suitable physicochemical properties for being efficient gene delivery systems.

In vitro biological characterization

In vitro transfection

Transfection efficacy was determined by a β-galactosidase transfection assay. Transfection mainly increased with increasing polymer-to-DNA ratios (Figure 2). The highest transfection levels for the three amino acid-bearing PEI polyplexes were observed at a polymer-to-DNA weight ratio of 20:1 on A431 and T98G cell lines. Based on these findings, along with the DNA condensation results, a 20:1 ratio was selected for the therapeutic efficacy experiments.

In A431 cells, treatment with PEI-Lys polyplex led to the highest transfection ($6.24 \times 10^{-3} \pm 0.34 \times 10^{-3}$ U/mL). Its transfection efficacy was about 1.3 times higher than that of PEI-Arg polyplex ($4.46 \times 10^{-3} \pm 0.34 \times 10^{-3}$ U/mL) and PEI-Leu polyplex ($4.30 \times 10^{-3} \pm 1.02 \times 10^{-3}$ U/mL). In T98G cells, transfection levels were similar for PEI-Lys ($7.91 \times 10^{-3} \pm 1.18 \times 10^{-3}$ U/mL) and PEI-Arg polyplexes ($8.49 \times 10^{-3} \pm 0.43 \times 10^{-3}$ U/mL). They were 1.7 times higher than that obtained with PEI-Leu polyplex ($4.68 \times 10^{-3} \pm 0.59 \times 10^{-3}$ U/mL). The conjugation of Arg, Lys, and Leu to PEI at their optimal polymer-to-DNA ratio led to an improved transfection compared to PEI on both the tested cell lines, with the exception of PEI-Leu, which led to a similar transfection to that observed after treatment with PEI-DNA on A431 cells. On A431 cells, gene expression following treatment with PEI-Lys and PEI-Arg polyplexes was 2.2 times and 1.6 times higher, respectively, than after treatment with PEI polyplex ($2.72 \times 10^{-3} \pm 0.18 \times 10^{-3}$ U/mL). The grafting of Leu to PEI did not bring any significant improvement in the level of transfection compared to unmodified PEI. On T98G cells, gene expression following treatment with PEI-Lys and PEI-Arg polyplexes was 3 times higher than that of PEI polyplex ($2.52 \times 10^{-3} \pm 0.18 \times 10^{-3}$ U/mL). After treatment with PEI-Leu polyplex, it was 1.8 times higher than after treatment with PEI polyplex. No gene expression was observed after treatment with control DNA, as expected.

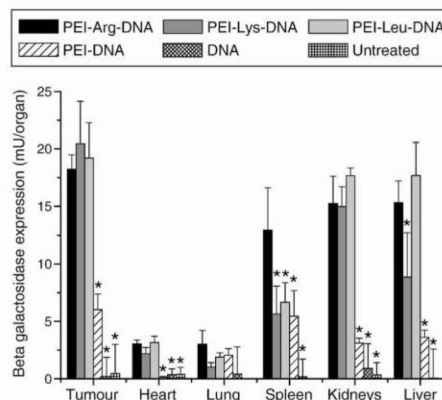

Figure 4. Biodistribution of gene expression after a single intravenous administration of Arg-, Lys-, and Leu-bearing PEI polyplexes (50 μg DNA administered) and unmodified PEI polyplexes (50 μg DNA administered). Results were expressed as milliunits β-galactosidase per organ (n = 5). *P < 0.05: highest gene expression treatment vs. other treatments for each organ.

Cellular uptake

The uptake of Cy3-labeled DNA by A431 and T98G cells was qualitatively confirmed using confocal microscopy (Figure 3). The complexation of plasmid DNA to PEI improved DNA uptake by both cell lines compared to DNA solution, as expected. Co-localization of DNA in the nuclei was clearly visible in both cell lines treated with PEI-Leu polyplex in A431 cells and PEI-Arg polyplex in T98G cells. It was less pronounced in the case of the other treatments. In both cell lines, Cy3-labeled DNA was also disseminated in the cytoplasm after treatment with all PEI formulations. By contrast, cells treated with naked DNA did not show any Cy3-derived fluorescence. The conjugation of Arg and Leu to PEI therefore improved DNA uptake by the nuclei of the tested cell lines, but not the cellular uptake within the cytoplasm. Although PEI-Lys was efficacious for transfection, it did not improve DNA uptake by the cells compared to PEI after 24 hours of incubation, leading to the proposal that the DNA uptake following this treatment occurred at a later time.

In vitro anti-proliferative activity

The conjugation of Arg, Lys, and Leu to PEI led to an increase of the in vitro anti-proliferative activity in A431 cells, respectively, by 34-fold, 51-fold, and 40-fold compared to the unmodified polyplex (IC_{50} of 0.52 ± 0.13 μg/mL, 0.35 ± 0.07 μg/mL, and 0.44 ± 0.16 μg/mL, respectively, for PEI-Arg, PEI-Lys, and PEI-Leu polyplexes; 17.86 ± 5.34 μg/mL for

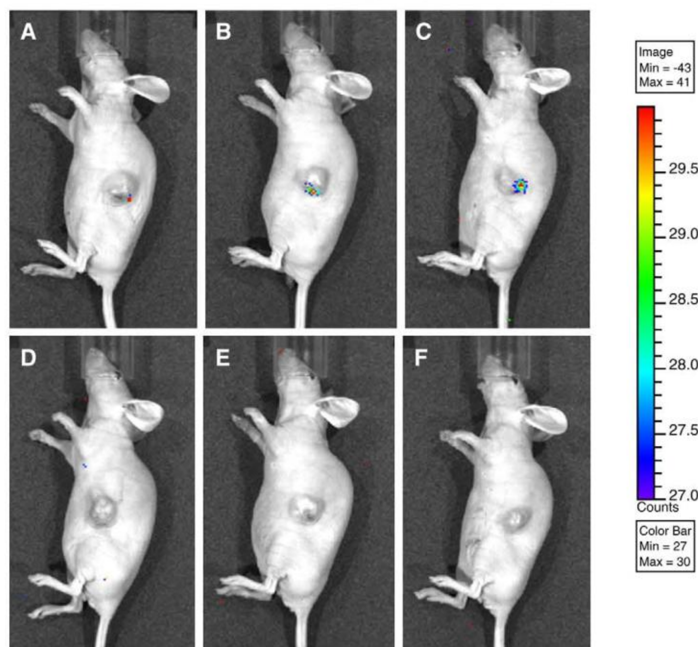

Figure 5. Bioluminescence imaging of gene expression after intravenous administration of luciferase-encoding plasmid DNA in complex with PEI-Arg **(A)**, PEI-Lys **(B)**, PEI-Leu **(C)**, PEI **(D)**, or free in solution **(E)** in living mice (50 μg DNA administered). Control, untreated animal **(F)**. The mice were imaged using the IVIS Spectrum 24 hours after injection of the treatments. The scale indicates surface radiance (photons/s/cm^2/steradian).

unmodified PEI polyplex) (Supplementary Table S1 online). In T98G cells, the conjugation of the amino acids to PEI improved the anti-proliferative activity of the polyplex, by 2-fold for PEI-Arg (IC_{50} 5.08 ± 0.51 μg/mL), by 89-fold for PEI-Lys (IC_{50} 0.12 ± 0.01 μg/mL), and by 269-fold for PEI-Leu (IC_{50} 0.04 ± 0.04 μg/mL). Uncomplexed amino acid-bearing PEI and naked DNA did not exert any cytotoxicity on the cells.

In vivo study

The intravenous administration of PEI-Arg, PEI-Lys, and PEI-Leu polyplexes led to a significant increase of gene expression in the tumor, with a β-galactosidase amount at least 3-fold higher than that obtained after treatment with unmodified PEI polyplex (18.2 ± 1.2, 20.4 ± 3.7, 19.2 ± 3.1 mU β-galactosidase per tumor, respectively, for PEI-Arg, PEI-Lys and PEI-Leu polyplexes, compared to 6 ± 1.8 mU β-galactosidase per tumor for PEI polyplex) (Figure 4). The three amino acid-bearing PEI polyplexes led to similar levels of gene expression in the tumor. The treatments were well tolerated by the mice at the administered doses. Gene expression was also increased in the kidneys (by at least 4-fold) and in the liver (by about 5-fold for PEI-Arg and PEI-Leu, 2-fold for PEI-Lys) after administration of amino acid-bearing PEI polyplexes. These results highlight the need for tumor-targeting moieties conjugated to the polyplexes in future experiments for increasing tumor gene expression specificity. PEI-Arg polyplex also increased gene expression level in the spleen by about 2-fold compared to the other amino acid-bearing and unmodified PEI polyplexes. Gene expression within the heart and lung was weak following all treatments, reaching a maximum of 3.1 ± 0.5 mU β-galactosidase per heart after treatment with PEI-Leu polyplex and 3 ± 1.1 mU β-galactosidase per lung after treatment with PEI-Arg polyplex.

The biodistribution of gene expression was also confirmed by bioluminescence imaging (Figure 5). Co-localization of gene expression in the tumors was clearly visible following treatment with the amino acid-bearing PEI polyplexes. Luciferase expression appeared to be heterogeneously distributed within the tumors, probably concentrated in highly vascularized areas within the tumors. However, it was not visible in the tumors after treatment with PEI polyplex and naked DNA, and appeared to be very limited in the other organs. This might be explained by the chosen parameters for image acquisition and the short imaging times, which probably allowed only the most intensely

luminescent tissues to be analyzed. These data demonstrated that the conjugation of Arg, Lys, and Leu to PEI increased gene expression within the tumors.

Discussion

The possibility of using non-viral gene delivery nanosystems for the treatment of cancer is limited by their lower transfection efficacy compared to viral systems. On the basis that amino acids such as Arg, Lys and Leu were involved in enhancing DNA transport into cells, we hypothesized that the grafting of these amino acids to a model polymer PEI would improve transfection efficacy in cancer cells.

The conjugation of amino acid residues on the primary amines of PEI did not affect the polymer's ability to form a complex to DNA. DNA condensation occurring with PEI-Arg seemed to follow the same pattern as the one described by Kim et al[6] in his study of Arg-bearing PEI generation 2 (PPI). Grafting Arg residues to PEI facilitated the formation of stable polyplexes with DNA due to their strong positive charges at high polymer-to-DNA weight ratios, but reduced the DNA-condensing ability of the polymer at low polymer-to-DNA ratios. In the case of Arg-bearing PPI, this could be explained by the fact that the pKa value of α-amine of Arg in Arg-bearing PPI was lower than that of primary amine in PPI. At the same charge ratio, the number of moles of Arg-PPI was less than that of PPI in polyplex solution, so the self-assembly of Arg-PPI with DNA could proceed less vigorously at the charge ratio. However, this still needs to be elucidated in the case of PEI-Arg. The stronger DNA condensation ability observed with PEI-Lys and PEI-Arg compared to PEI-Leu could be attributed to the enhanced cationic charge density of the PEI-Lys and PEI-Arg conjugates due to the cationic amino acids Lys and Arg, which exceed the loss of positive charges due to the coupling. A lower DNA condensation ability was also similarly described with the conjugation of the hydrophobic Phe to a dendrimer.[9] When combined to the dendrimer chain terminal, Phe residues were able to form a hydrophobic environment in the periphery of the dendrimer, reducing protonation of their amino groups.

Transfection efficacy studies demonstrated that the conjugation of Arg, Lys and Leu to PEI led to an improved transfection compared to the unmodified PEI on both the tested cell lines, with the exception of PEI-Leu in A431 cells. The improved β-gal expression induced by amino acid-bearing PEI compared to PEI most likely resulted from the higher zeta potential of their DNA complexes, as a result of the strong correlation between cellular uptake and positive charge density of polyplexes.[19,20] These results suggest that the grafting of basic amino acids onto a polymer enhances the transfection activity compared to unmodified polymer. To our knowledge, it is the first time that the grafting of Lys residues to gene delivery systems gave such improvement in gene expression. The grafting of Arg residues, which gave rise to improved transfection efficacy in our experiments, has previously been shown to improve gene delivery with mixed success depending on the delivery systems. For example, Gao et al[7] demonstrated that the grafting of Arg to chitosan improved gene expression compared to unmodified chitosan on HEK293 and COS-7 cells. Choi et al[5] also reported that Arg-PAMAM led to much enhanced transfection efficiency. However, Arg oligopeptides did not show a high level of transfection efficacy.[20] The increase of gene expression with Arg-PEI could be attributed to either the cell-penetrating activity during uptake or the nuclear localizing efficacy after entry into the cytosol of the Arg residues, or both effects. The conjugation of the hydrophobic amino acid Leu gave cell line-dependent results, indicative of the influence of cell line-specific factors on the transfection process. In our experiments the grafting of Leu to PEI led to an improved gene expression in glioblastoma T98G cells but not in carcinoma A431 cells. A previous experiment by Kono et al[9] also demonstrated that the grafting of Leu residues on a generation 4 PAMAM dendrimer did not increase its transfection activity compared with the unmodified dendrimer on CV1 kidney fibroblasts.

The grafting of these amino acids to PEI significantly improved the therapeutic efficacy of the system on the two cancer cell lines studied. PEI-Lys polyplex was the most efficacious treatment on the A431 cell line, probably as a result of its highest transfection efficacy on this cell line. Although PEI-Leu was the least efficacious of the three amino acid-bearing polymers for transfection, it led to the highest anti-proliferative activity on T98G cell line. Because this result can be attributed neither to its intrinsic toxicity nor to the enhanced nuclear uptake of DNA following this treatment, it is hypothesized that PEI-Leu polyplex may damage the cells due to the hydrophobic nature of Leu, in conjunction with the efficacy of the therapeutic plasmid.

This work, to the best of our knowledge, corresponds to the first evaluation of gene expression of amino acid-bearing non-viral delivery nanosystems in tumors after intravenous administration. Previously, Fujita et al[21] showed an improved gene expression in tumors following intratumoral injection of tetraarginine-PEG lipid-coated protamine-DNA compared with the control delivery system. Similarly, Kim et al[22] demonstrated that the intratumoral administration of novel glutamate-based cationic lipoplexes led to improved gene expression in the tumors compared to their aspartate-based counterparts. In our study, Arg-, Lys- and Leu-bearing PEI polyplexes administered intravenously led to an improved gene expression in subcutaneous tumors and should therefore have the potential to deliver and express their carried DNA to remote tumors or metastases unsuitable for intratumoral treatments. The administration of Lys-bearing PEI polyplex also led to significantly less gene expression in the spleen and liver than for the two other amino acid-bearing PEI polyplexes, thus making the grafting of Lys particularly promising for the future application of these systems. Most importantly, we did not observe any of the secondary effects generally resulting from treatment with PEI, suggesting that the grafting of Arg, Lys and Leu to PEI could be an alternative to PEG shielding with improved gene expression in the tumors.

Our objective will now be to improve the tumor specificity of gene expression, hopefully leading to an optimized therapeutic effect.

In conclusion, new Arg, Lys and Leu-bearing PEI polymers have been prepared with the aim of improving gene expression

in cancer cells. In vitro, the administration of therapeutic DNA in complex with Arg-, Lys- and Leu-bearing PEI resulted in an enhanced anti-proliferative efficacy compared to native polyplex on both cancer cell lines, by up to 269-fold after treatment by PEI-Leu polyplex on T98G cells. In vivo, the intravenous administration of PEI-Arg, PEI-Lys, and PEI-Leu polyplexes led to an improved tumor gene expression, with a β-galactosidase amount at least 3-fold higher than that obtained after treatment with unmodified PEI polyplex. The three amino acid-bearing PEI polyplexes led to similar levels of gene expression in the tumor. The treatments were well tolerated by the mice. Arg, Lys and Leu-bearing PEI polymers are therefore highly promising gene delivery systems for cancer therapy.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nano.2011.01.016.

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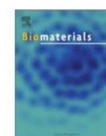

Enhanced gene expression in tumors after intravenous administration of arginine-, lysine- and leucine-bearing polypropylenimine polyplex

Hibah Aldawsari [a], RuAngelie Edrada-Ebel [a], David R. Blatchford [a], Rothwelle J. Tate [a], Laurence Tetley [b], Christine Dufès [a,*]

[a] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, United Kingdom
[b] College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

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ABSTRACT

The possibility of using non-viral gene delivery systems for the treatment of cancer is currently limited by their lower transfection efficacy compared to viral systems. On the basis that amino acids such as arginine, lysine and leucine were involved in enhancing DNA transportation into cells, we hypothesized that the grafting of these amino acids to the highly promising generation 3 diaminobutyric polypropylenimine (DAB) dendrimer would improve its transfection efficacy in cancer cells. In this work we demonstrated that the conjugation of arginine, lysine and leucine to the dendrimer led to an enhanced anti-proliferative activity of the polyplexes, by up to 47-fold for DAB-Lys in T98G cancer cells compared to the unmodified polyplex *in vitro*. *In vivo*, the intravenous administration of amino acid-bearing DAB polyplexes resulted in a significantly improved tumor gene expression, with the highest gene expression level observed after treatment with DAB-Lys polyplex. Arginine, lysine and leucine-bearing generation 3 polypropylenimine polymers are therefore highly promising gene delivery systems for gene transfection in tumors.

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1. Introduction

Gene therapy has become a promising strategy that offers the potential of treating cancer via the production of therapeutic proteins within cells. However, it is currently limited by the lack of safe and efficacious gene delivery carriers able to deliver therapeutic genes selectively to tumors by intravenous administration [1]. In order to remediate to this problem, numerous non-viral gene delivery systems, including cationic polymers and lipids, have been developed, due to advantages such as their low toxicity, ease of manufacturing, stability and high flexibility regarding the size of the transgene delivered [2,3]. Among these non-viral gene delivery systems, generation 3 diaminobutyric polypropylenimine dendrimer (DAB) appears to be particularly promising. We have recently demonstrated that the intravenous administration of this dendrimer combined with Tumor Necrosis Factor (TNFα) expression plasmid driven by a tumor-specific promoter, led to tumor regression in a number of murine models, with excellent long-term response [4]. In order to optimize the efficacy of this system, we proposed 1) to improve its tumor-targeted gene delivery capability and 2) to improve its gene expression efficacy. The conjugation of DAB dendrimer to transferrin, whose receptors are overexpressed on numerous cancer cell lines, resulted in a selective receptor-mediated gene delivery to tumors after intravenous administration and led to an increased therapeutic efficacy [5], therefore fulfilling our first objective. In order to address our second objective, that is the improvement of gene expression following intravenous administration, we hypothesized that the grafting of the amino acids arginine, lysine and leucine to DAB dendrimer would improve its transfection efficacy in cancer cells. Various research groups have previously demonstrated that these amino acids were involved in enhancing DNA transportation into cells [6–13]. To begin to address transfection efficacy concerns, we previously conjugated Arg, Lys and Leu to polyethylenimine (PEI) used as a model non-viral gene delivery system and demonstrated their enhanced gene expression efficacy to tumors after intravenous administration [14].

The objectives of this study were therefore 1) to prepare and characterize arginine, lysine and leucine-bearing DAB dendrimer, 2) to evaluate their transfection and therapeutic efficacies *in vitro* on A431 human epidermoid carcinoma and T98G human glioblastoma cell lines and 3) to evaluate their transfection efficacy *in vivo* after intravenous administration in mice bearing A431 tumors.

* Corresponding author. Tel.: +44 141 548 3796; fax: +44 141 552 2562.
E-mail address: C.Dufes@strath.ac.uk (C. Dufès).

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2. Materials and methods

2.1. Cell lines and reagents

Generation 3- diaminobutyric polypropylenimine dendrimer (PPI G3; DAB), arginine (Arg), lysine (Lys), leucine (Leu), dimethylsuberimidate and the other reagents were purchased from Sigma Aldrich (Poole, UK). Quanti-iT™ PicoGreen® dsDNA reagent and tissue culture media were obtained from Invitrogen (Paisley, UK). Vectashield® mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Peterborough, UK). Passive lysis buffer was obtained from Promega (Southampton, UK). Label IT® Cy3 Nucleic Acid Labeling kit was from Cambridge Biosciences (Cambridge, UK). AgarGel H/M agarose was from Continental Laboratory Products (Northampton, UK). HyperLadder I was from Bioline (London, UK). NanoVan was purchased from Nanoprobes (Stony Brook, NY). Firefly D-luciferin was purchased from Caliper Life Sciences (Hopkinton, MA). A431 human epidermoid carcinoma and T98G human glioblastoma were obtained from the European Collection of Cell Cultures (Salisbury, UK). The expression plasmids encoding Tumor necrosis factor (TNF)α (pORF9-mTNFα) and β-galactosidase (pCMVsport β-galactosidase) were obtained respectively from InvivoGen (San Diego, CA) and Invitrogen (Paisley, UK). The expression plasmid encoding luciferase (pEF1α-Luc) was constructed by subcloning the elongation factor 1 alpha (EF1α) promoter from pEF1/*Myc*-His vector (Invitrogen, Paisley, UK) into the promoterless pGL3-Basic vector (Promega, Southampton, UK). They were purified using an Endotoxin-free Giga Plasmid Kit (Qiagen, Hilden, Germany).

2.2. Synthesis and characterization of arginine-, lysine- and leucine-bearing DAB

2.2.1. Conjugation of the amino acids to DAB

Generation 3- diaminobutyric polypropylenimine dendrimer (DAB) was conjugated to arginine, lysine and leucine by using dimethylsuberimidate as a cross-linking agent as previously described [5,14,15]. DAB (5.7 mg, 6.9 mg, 7.7 mg respectively for DAB-Arg, DAB-Lys, DAB-Leu) was added to arginine, leucine or lysine (6 mg) and dimethylsuberimidate (12 mg) in triethanolamine HCl buffer (pH 7.4, 2 mL). The coupling reaction was allowed to take place at 25 °C for 2 h whilst stirring. The final product was purified by size exclusion chromatography using a Sephadex G75 column and freeze-dried. The grafting of the amino acids to DAB was assessed by ^{1}H NMR spectroscopy using a Jeol Oxford NMR AS 400 spectrometer.

2.2.2. Characterization of polyplex formation

The degree of DNA accessibility following complexation with the amino acid-bearing DAB polymers was assessed by PicoGreen® assay, performed according to the protocol from the supplier. PicoGreen® reagent was diluted 200-fold in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 7.5) before the experiment. One mL of complex polymer-DNA at various polymer : DNA weight ratios (20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1, 0:1) was added to 1 mL diluted PicoGreen® solution. The DNA concentration in the cuvette (10 μg/mL) was kept constant throughout the experiment. The fluorescence intensity of the complexes was analyzed at various time points with a Varian Cary Eclipse Fluorescence spectrophotometer (Palo Alto, CA) (λ_{exc} : 480 nm, λ_{em} : 520 nm). Results were represented as percentage of DNA condensation (= 100 - % relative fluorescence to DNA control) and compared with those obtained for DAB-DNA (polymer: DNA weight ratio 5:1) ($n = 4$).

DNA condensation ability of the amino acid-bearing DAB was also examined by agarose gel retardation assay. Polyplexes were prepared at a final DNA concentration of 20 μg/mL. After mixing with loading buffer, the samples (10 μl) were loaded on a 1× Tris-Borate-EDTA (TBE) (89 mM Tris base, 89 mM boric acid, 2 mM Na_2-EDTA, pH 8.3) buffered 0.8% (w/v) agarose gel containing ethidium bromide (0.4 μg/mL), with 1× TBE as a running buffer. The DNA size marker was HyperLadder I. The gel was run at 50 V for 1h and then photographed under UV light.

Nanoparticles of Arg-, Lys- and Leu- bearing DAB complexed with DNA were also visualized by transmission electron microscopy as previously described [5]. Formvar/Carbon-coated 200 mesh copper grids were glow discharged and specimens in distilled water were dried down with filter paper to a thin layer onto the hydrophilic support film. Twenty μL of 1% aqueous methylamine vanadate stain (NanoVan) was applied and the mixture dried down immediately using filter paper. Dried specimens were imaged with a LEO 912 energy filtering transmission electron microscope operating at 120 kV. Contrast enhanced, zero-loss energy filtered digital images were recorded with a 14 bit/2K CCD camera.

2.2.3. Polyplex size and zeta potential measurement

Size and zeta potential of the amino acids-bearing DAB complexed to DNA were measured by photon correlation spectroscopy and laser Doppler electrophoresis using a Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK).

2.3. In vitro biological characterization

2.3.1. Cell culture

A431 and T98G cell lines were grown as monolayers in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine and 0.5% (v/v) penicillin-streptomycin. Cells were cultured at 37 °C in a 5% carbon dioxide atmosphere.

2.3.2. In vitro transfection

Transfection efficacy of the DNA carried by the amino acid-bearing polymers was assessed with a plasmid coding for β-galactosidase (pCMV β-gal), using a β-galactosidase transfection assay. A431 and T98G cells were seeded in quintuplicate at a density of 2000 cells/well in 96-well plates. After 72 h incubation, the cells were treated with DAB-Arg, DAB-Leu and DAB-Lys complexed to plasmid DNA encoding β-galactosidase, at various polymer: DNA weight ratios (20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1, 0:1). Naked DNA served as a negative control, DAB-DNA (polymer: DNA weight ratio 5:1) served as a positive control. DNA concentration (10 μg/mL) was kept constant for all the formulations tested. After 72 h incubation, cells were lysed with 1× passive lysis buffer (PLB) (50 μL/well) for 20 min. The cell lysates were subsequently analyzed for β-galactosidase expression [16]. Briefly, 50 μL of the assay buffer (2 mM magnesium chloride, 100 mM mercaptoethanol, 1.33 mg/mL o-nitrophenol-β-galactopyranoside, 200 mM sodium phosphate buffer, pH 7.3) was added in each well containing the lysates. After 2 h incubation at 37 °C, the absorbance of the samples was read at 405 nm with a plate reader (Thermo Lab Systems, Multiscan Ascent, UK).

2.3.3. Cellular uptake

Imaging of the cellular uptake of the DNA carried by amino acid-bearing DAB was carried out by confocal microscopy. Labeling of plasmid DNA with the fluorescent probe Cy3 was performed using a Label IT® Cy3 Nucleic Acid Labeling kit, as described by the manufacturer. A431 and T98G cells were seeded on microscope slides (0.6×10^6 cells/90-mm Petri dish) at 37 °C for a day before transfection. The cells were then incubated for 24 h with Cy3-labeled DNA (6 μg/dish) complexed to DAB-Arg, DAB-Lys, DAB-Leu and DAB (polymer: DNA weight ratios of 10:1 for amino acid-bearing DAB, 5:1 for DAB). Control slides were treated with naked DNA or remained untreated. The slides were then washed with PBS and fixed in methanol for 30 min. Upon staining of the nuclei with DAPI, the cells were examined using a Leica TCS SP5 confocal microscope. DAPI was excited with the 405 nm laser line (bandwidth: 415–491 nm), whereas Cy3 was excited with the 543 nm laser line (bandwidth: 550–620 nm).

2.3.4. In vitro anti-proliferative activity

Anti-proliferative activity of amino acid-bearing DAB complexed with plasmid DNA encoding TNFα was assessed using a standard MTT assay. A431 and T98G cells were seeded in quintuplicate at a density of 2000 cells/well in 96-well plates 72 h before treatment. They were then incubated for 72 h with the DNA formulations at final concentrations of $6.4.10^{-4}$ to 50 μg/mL. Anti-proliferative activity was evaluated by measurement of the growth inhibitory concentration for 50% of the cell population (IC_{50}). Absorbance was measured at 570 nm using a plate reader. Dose-response curves were fitted to percentage absorbance values to obtain IC_{50} values (three independent experiments, each performed in quintuplicate).

2.4. In vivo study

2.4.1. Animals

Female immunodeficient BALB/c mice were housed in groups of five at 19 °C–23 °C with a 12 h light–dark cycle. They were fed a conventional diet (Rat and Mouse Standard Expanded, B&K Universal, Grimston, United Kingdom) with mains water *ad libitum*. Experimental work was carried out in accordance with UK Home Office regulations and approved by the local ethics committee.

2.4.2. Biodistribution of gene expression

Groups of mice ($n = 4$, initial mean weight 20 g) bearing subcutaneously implanted A431 tumors, were treated intravenously with a single injection of amino acid-bearing and control DAB carrying β-galactosidase expression plasmid (50 μg of DNA). Mice were sacrificed 24 h after injection. Their organs were removed, immediately frozen in liquid nitrogen and analyzed for their β-galactosidase levels as previously described [17].

The biodistribution of gene expression was also visualized by bioluminescence imaging, using an IVIS Spectrum (Caliper Life Sciences, USA). Mice bearing subcutaneous A431 tumors were treated intravenously with a single injection of amino acid-bearing and control DAB carrying luciferase expression plasmid (50 μg of DNA). Twenty-four hours after treatment, they were intraperitoneally injected with the luciferase substrate D-luciferin (150 mg/kg body weight) and anaesthetized by isoflurane inhalation. Light emission was measured 10 min after injection of the D-luciferin solution, for 2 min, using Living Image® software. The resulting pseudo-color images represent the spatial distribution of photon counts within the animal. Identical illumination settings were used for acquiring all images.

2.5. Statistical analysis

Results were expressed as means ± standard error of the mean (S.E.M). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison post-test (GraphPad Prism software). Differences were considered as significant when $P < 0.05$.

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3. Results and discussion

3.1. Synthesis and characterization of arginine-, lysine- and leucine-bearing DAB

3.1.1. Conjugation of the amino acids to DAB

The synthesis of DAB-Arg, DAB-Lys and DAB-Leu was confirmed by ^{1}H NMR (Fig. 1) and spin systems for each moiety were confirmed by ^{1}H-^{1}H COSY : ^{1}H NMR (D_2O) δ: DAB–(H_2N-***CH*₂**-CH_2-CH_2-N-) = 3.20–3.45; DAB (-N-***CH*₂**-CH_2-***CH*₂**-N-) = 2.45–3.00; DAB (O=CHN-***CH*₂**-CH_2) = 3.85–4.00; Arg (1Hα) = 2.54; Arg (2Hβ) = 1.66 and 1.41; Arg (2Hγ) = 1.74; Arg (2Hω) = 3.23, triplet; Lys (1Hα) = 2.54; Lys (2Hβ) = 1.71 and 1.54; Lys (2Hγ2Hω) = 1.74; Lys (H_2N-***CH*₂**) = 3.06, triplet; Leu (1Hα) = 2.54; Leu (2Hβ) = 1.65 and 1.41; Leu (2Hγ) = 1.75; Leu (2CH_3) = 0.99 (overlapping doublets).

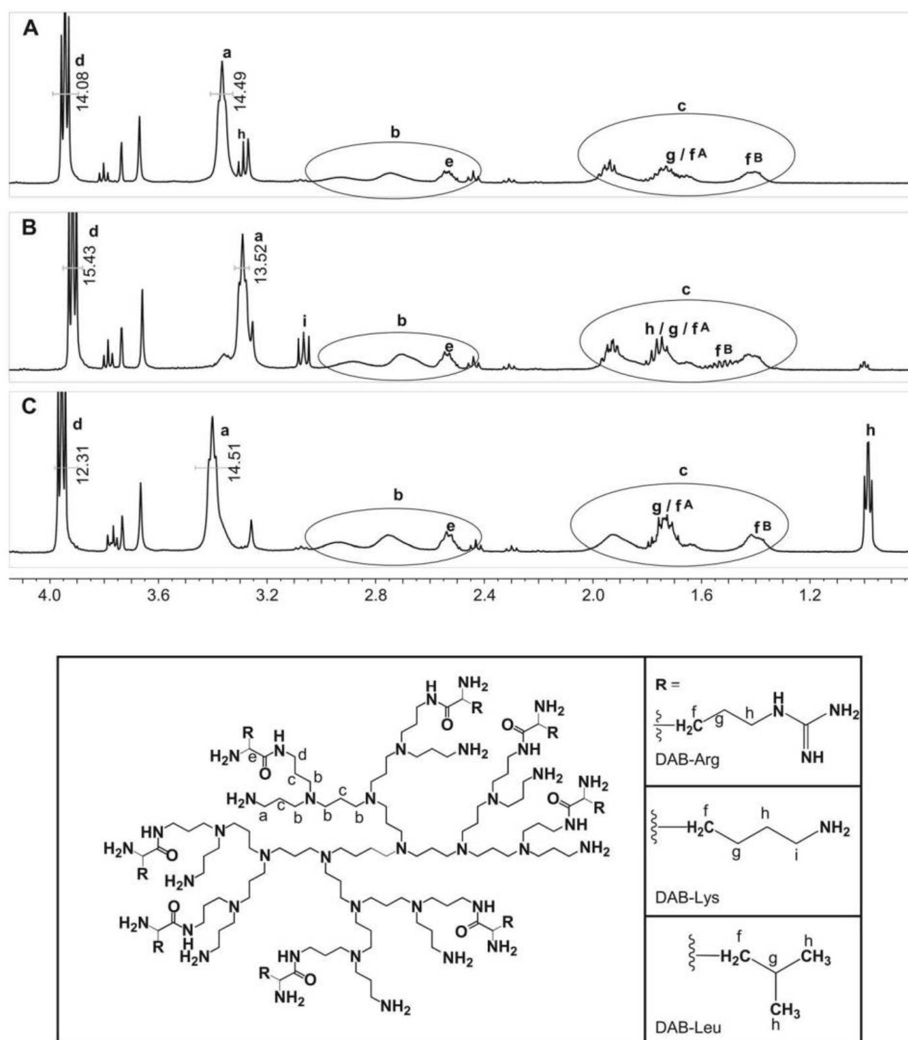

Fig. 1. ^{1}H NMR spectra of DAB-Arg (A), DAB-Lys (B) and DAB-Leu (C).

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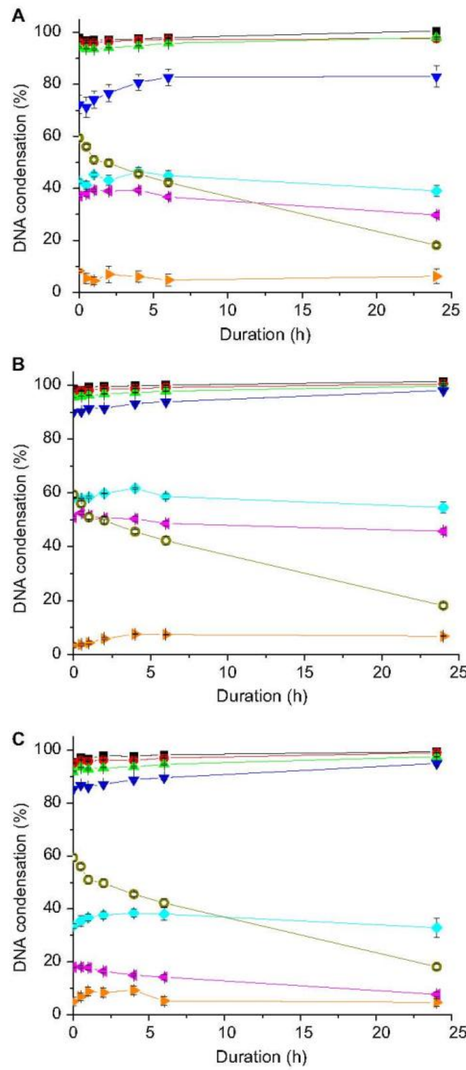

Fig. 2. DNA condensation of DAB-Arg (A), DAB-Lys (B) and DAB-Leu (C) polyplexes using PicoGreen® reagent at various durations and polymer: DNA weight ratios : 20:1 (■, black), 10:1 (●, red), 5:1 (▲, green), 2:1 (▼, blue), 1:1 (◆, cyan), 0.5:1 (◀, pink), DNA only (▶, orange) (empty symbol, dark yellow : DAB-DNA, dendrimer: DNA weight ratio: 5:1). Results are expressed as mean ± SEM ($n = 4$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The characteristic broad triplet peak for the CH_2 adjacent to peripheral primary amino group of DAB at δ 2.65 was shifted to 3.20–3.45 ppm in the NMR spectrum of a conjugated DAB-amino acid analogue. An additional triplet at the region of δ 3.85–4.00 was observed which is compatible to methylene protons adjacent to an amide unit indicating that some of the peripheral amino groups had formed an amide linkage with DAB. These results demonstrated that DAB has been successfully conjugated with the respective amino acids. ^{1}H NMR spectra showed that 50% of the surface group of dendrimer DAB-16 is bound to 8 units of amino acids as signified by the ratio of the integrals of resonances at ca. δ 3.90 and 3.30 for methylene units (d and a) attached to the amide-linked bound amino acid and unbound free amine, respectively. Percentage conjugation of the amino acids with the polymer was found to be 49.28, 53.49 and 45.90 for arginine, lysine and leucine, respectively. The molecular weights of amino acids-bearing DAB were then calculated to be 2.9 KDa for arginine, 2.7 KDa for lysine and 2.6 KDa for leucine. All formulations produced high yields: 99.1% for DAB-

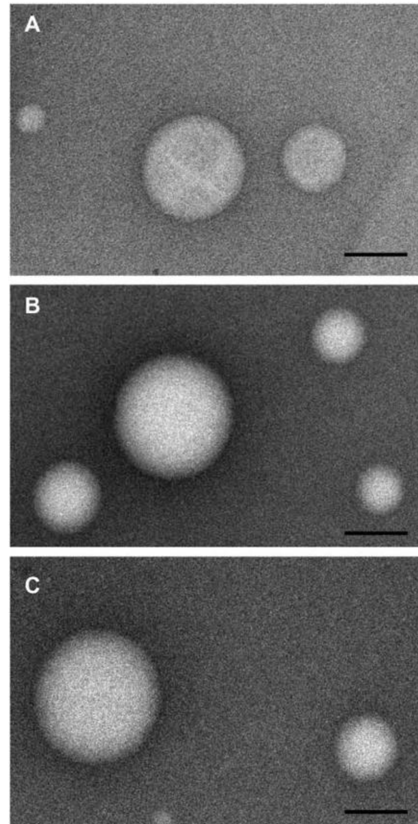

Fig. 3. Transmission electron micrographs of arginine- (A), lysine- (B) and leucine- (C) bearing DAB polyplexes (Bar: 100 nm).

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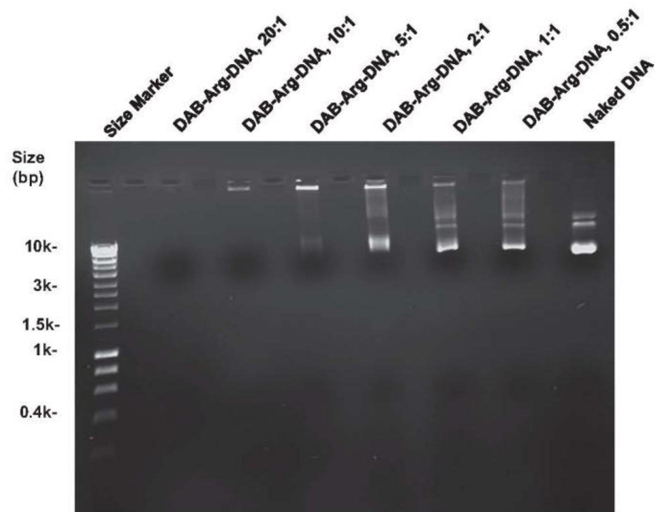

Fig. 4. Gel retardation assay of DAB-Arg polyplexes at various polymer: DNA weight ratios: 20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1 and DNA only.

Arg, 99.9% for DAB-Lys and 98.9% for DAB-Leu, as previously observed when preparing amino acid-bearing PEI and transferrin-bearing DAB using the same simple one-step synthesis [5,14].

3.1.2. Characterization of polyplex formation

The three amino acid-bearing DAB were able to condense more than 70% of the DNA at dendrimer: weight ratios of 2:1 or higher (Fig. 2). DNA condensation occurred almost instantaneously and was found to be stable over at least 24 h. It increased with increasing weight ratios and was almost complete at a dendrimer: DNA weight ratio of 20:1 for the 3 dendrimers. The DNA condensation observed for dendrimer: DNA weight ratios of 2:1 or higher was much higher than that observed for the unmodified dendrimer, which was of 60% at its best and decreasing with time.

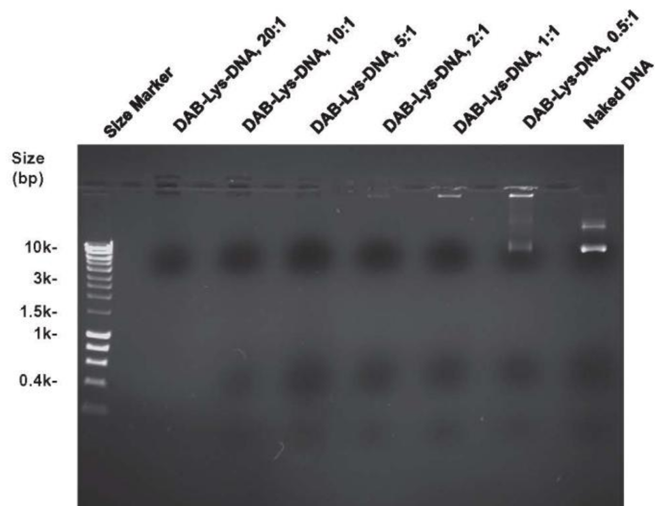

Fig. 5. Gel retardation assay of DAB-Lys polyplexes at various polymer: DNA weight ratios: 20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1 and DNA only.

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The formation of nanoparticles of amino acid-bearing DAB complexed to DNA was also demonstrated by transmission electron microscopy (Fig. 3). A gel retardation assay confirmed the complete and partial DNA condensation by the 3 amino acid-bearing dendrimers (Figs. 4–6). At dendrimer: DNA weight ratios of 20:1 and 10:1, DNA was fully condensed by DAB-Arg, thus preventing ethidium bromide to intercalate with DNA. No free DNA was therefore visible at this ratio. DNA was however partially condensed by DAB-Arg dendrimer for the other dendrimer: DNA ratios. Ethidium bromide could therefore intercalate with DNA and a band corresponding to free DNA was visible. DAB-Lys and DAB-Leu seemed to be able to condense DNA at dendrimer: DNA ratios higher than 1:1, as free DNA bands were only visible at a dendrimer: DNA ratio of 0.5:1. These results demonstrate that amino acid-bearing DAB can condense DNA via electrostatic interactions between the positive charges of amino acid-bearing DAB and the negative charges of DNA phosphates.

DNA condensation occurring with generation 3 DAB-Arg seemed to follow the same pattern as the one described by Kim et al. [12] when using generation 2 polypropylenimine dendrimer after 2 min incubation. Although DNA condensation was similar for generation 2 DAB-Arg and DAB polyplexes at higher weight ratios, generation 3 DAB did not behave the same way: the grafting of Arg, as well as Lys and Leu, led to a much improved DNA condensation compared with native generation 3 DAB polyplexes for weight ratios higher than 2:1. The formation of stable polyplexes at high polymer: DNA weight ratios could be explained by the fact that the pKa value of α-amine of arginine in DAB-Arg was lower than that of primary amines in DAB. Consequently, the number of moles of DAB-Arg was smaller than that of DAB at the same charge ratio, thus resulting in the formation of less stable DAB-Arg polyplexes for smaller weight ratios.

DAB-Leu presented the weakest DNA condensation ability at low polymer: DNA weight ratios, as already observed previously with PEI-Leu [14]. This could be explained by the fact that the conjugation of the dendrimer with cationic Arg and Lys exceeded the loss of positive charges due to the coupling, unlike what happened with hydrophobic Leu.

3.1.3. Polyplex size and zeta potential measurement

Amino acid-bearing polyplexes displayed average sizes less than 300 nm, at all weight ratios tested (Fig. 7). Polyplex size decreased with increasing weight ratios, independently of the amino acid conjugated to the dendrimer. Among the 3 amino acid-bearing polyplexes tested, DAB-Lys polyplex at a polymer: weight ratio of 0.5:1 was found to be the largest, with an average size of 267 nm (polydispersity: 0.659), while DAB-Leu polyplex at a polymer: weight ratio of 20:1 was the smallest, with an average size of 214 nm (polydispersity: 0.363). The conjugation of amino acids to the periphery of DAB led to a slight increase of the polyplex size compared to the unmodified DAB polyplex, which had an average size of 196 nm (polydispersity index: 0.683). As the cut-off size for extravasation has been found to be 400 nm for most tumors [18], amino acid-bearing DAB polyplexes have therefore the required size to access the tumor cells.

Zeta potential experiments demonstrated that the three amino acid-bearing DAB were bearing a negative surface charge (−3 for DAB-Arg and DAB-Lys polyplexes, −5 for DAB-Leu polyplex) at a weight ratio of 0.5, indicating that negatively charged DNA was not condensed yet with amino acid-bearing DAB at this ratio (Fig. 7). This result was consistent with the DNA condensation and the agarose gel electrophoresis results. The zeta potential values of the polyplexes increased with increasing weight ratios, finally reaching their maximum (24 mV, 19 mV and 16 mV respectively for DAB-Arg, DAB-Lys and DAB-Leu polyplexes) at a weight ratio of 20. These results were in accordance with the zeta potentials previously obtained when grafting Arg, Lys and Leu to PEI [14]. The conjugation of amino acids to DAB increased the overall positive charge of the complexes compared to unmodified DAB-DNA (6 mV) for weight ratios over 2:1. The particle size decreased and the zeta potential increased with increasing polymer: DNA weight ratios, thus indicating that more positively charged polymers could more efficiently condense DNA. Positively charged surfaces of polyplexes are considered to be important for their electrostatic interactions with negatively charged cellular membranes, resulting in an improved cellular uptake through internationalization mechanisms

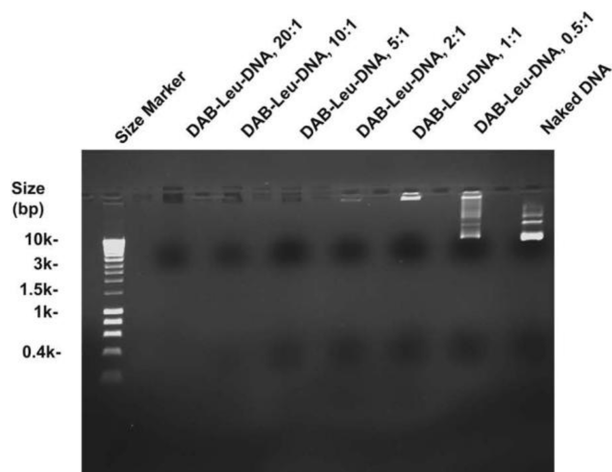

Fig. 6. Gel retardation assay of DAB-Leu polyplexes at various polymer: DNA weight ratios: 20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1 and DNA only.

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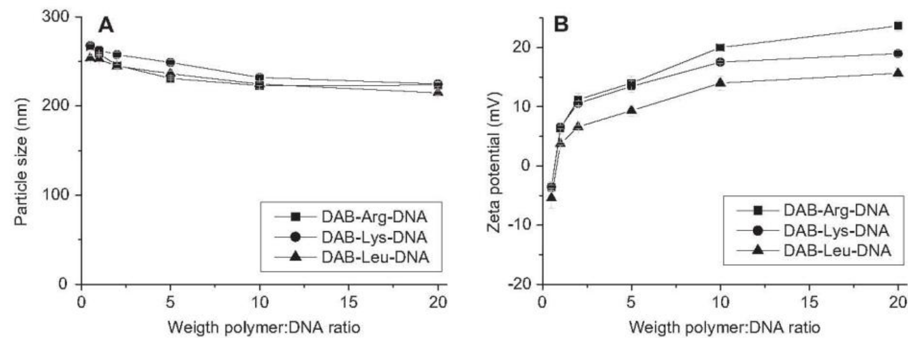

Fig. 7. Size (A) and zeta potential (B) of DAB-Arg, DAB-Lys, and DAB-Leu polyplexes at various polymer: DNA weight ratios: 20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1. Results are expressed as mean ± SEM ($n = 4$).

[19]. These results therefore demonstrated that DAB-Arg, DAB-Lys and DAB-Leu have suitable physicochemical properties for being efficient gene delivery systems.

3.2. In vitro biological characterization

3.2.1. In vitro transfection

The highest transfection level after treatment with the 3 amino acid-bearing DAB polyplexes was obtained at a polymer: DNA weight ratio of 10:1 on A431 (Fig. 8A) and T98G cells (Fig. 8B). At this ratio, in A431 cells, treatment with DAB-Lys polyplex led to the highest transfection ($10.89 \times 10^{3} \pm 0.27 \times 10^{3}$ U/mL). Its transfection efficacy was slightly higher than that observed with DAB-Arg polyplex ($9.79 \times 10^{3} \pm 0.24 \times 10^{3}$ U/mL) and was about 1.2-fold higher than that of DAB-Leu polyplex ($9.11 \times 10^{3} \pm 0.18 \times 10^{3}$ U/mL). In T98G cells as well, the highest transfection was observed after treatment with DAB-Lys polyplex ($11.96 \times 10^{3} \pm 0.22 \times 10^{3}$ U/mL), closely followed by DAB-Arg polyplex ($11.24 \times 10^{3} \pm 0.23 \times 10^{3}$ U/mL). It was 1.2-fold higher than that of DAB-Leu polyplex ($9.82 \times 10^{3} \pm 0.16 \times 10^{3}$ U/mL).

The conjugation of Arg, Lys and Leu to DAB at their optimal polymer: DNA ratio of 10:1 led to an improved transfection compared to DAB on both the tested cell lines. Gene expression following treatment with DAB-Arg polyplex was respectively 1.7 and 1.8-fold higher than after treatment with DAB polyplex on A431 and T98G cells ($5.49 \times 10^{3} \pm 0.11 \times 10^{3}$ U/mL on A431, $6.09 \times 10^{3} \pm 0.11 \times 10^{3}$ U/mL on T98G). Following treatment with DAB-Lys and DAB-Leu polyplexes, it was respectively 1.9 and 1.6-fold higher than that of DAB-DNA on both cell lines. No gene expression was observed after treatment with control DNA, as expected.

The improved β-gal expression following treatment with amino acid-bearing DAB most likely resulted from the higher zeta potential of these polyplexes, as there is a strong correlation between cellular uptake and positive charge density of polyplexes [20]. The grafting of arginine residues which gave rise to improved transfection efficacy in our experiments has previously been shown to improve gene delivery with mixed success depending of the polymers. For example, Choi *et al.* [8] demonstrated that PAMAM-Arg improved gene expression in comparison with unmodified PAMAM, with results comparable to those obtained with PEI. In the same study, the transfection results obtained with PAMAM-Lys were slightly improved compared with those observed with unmodified PAMAM. Kim and colleagues [12] previously

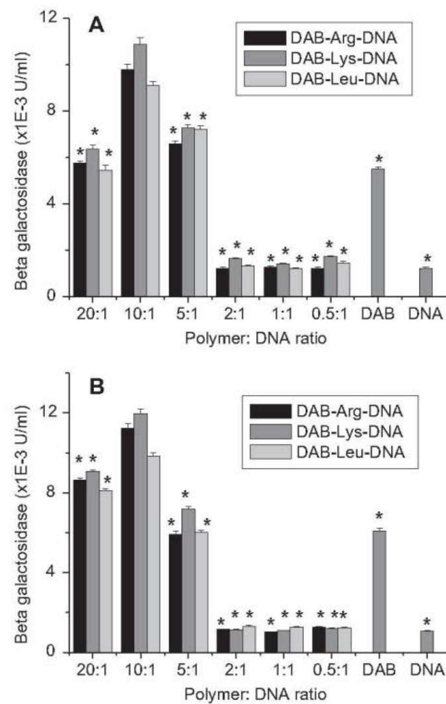

Fig. 8. Transfection efficacy of DAB-Arg, DAB-Lys and DAB-Leu polyplexes at various polymer: DNA weight ratios relative to native DAB in A431 (A) and T98G cells (B). DAB-DNA was dosed at its optimal polymer: DNA ratio of 5:1. Results are expressed as the mean ± SEM of three replicates ($n = 15$). * : $P < 0.05$ *vs* the highest transfection ratio.

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demonstrated that the transfection efficacy of Arg-bearing generation 2 PPI was increased compared to native PPI, with maximum efficacies reaching 27–54% of that of PEI 25 kDa. In our previous experiments, we showed that gene expression following treatment with PEI-Arg and PEI-Lys polyplexes was 3-fold higher than following treatment with PEI polyplex on T98G cells [14]. The use of amino acid-bearing DAB now further increases the gene expression level, to reach a maximum increase of 4.7-fold after treatment with DAB-Lys polyplex compared with native PEI. This favorable transfection profile could also be due to the ability of Arg and Lys to enable endosomal escape of the polyplex to the cytoplasm [21]. The grafting of hydrophobic moieties such as Leu has previously been shown to improve the transfection activity of cationic polymers, probably as a result of destabilization of cell membrane [11,22].

3.2.2. Cellular uptake

The cellular uptake of Cy3-labeled DNA carried by amino acid-bearing DAB was qualitatively confirmed in both cell lines by confocal microscopy (Fig. 9). Cy3-labeled DNA was disseminated in the cytoplasm after treatment with all DAB formulations in both cell lines. The DNA uptake appeared to be more pronounced in A431 cells treated with DAB-Lys polyplex and in T98G cells treated

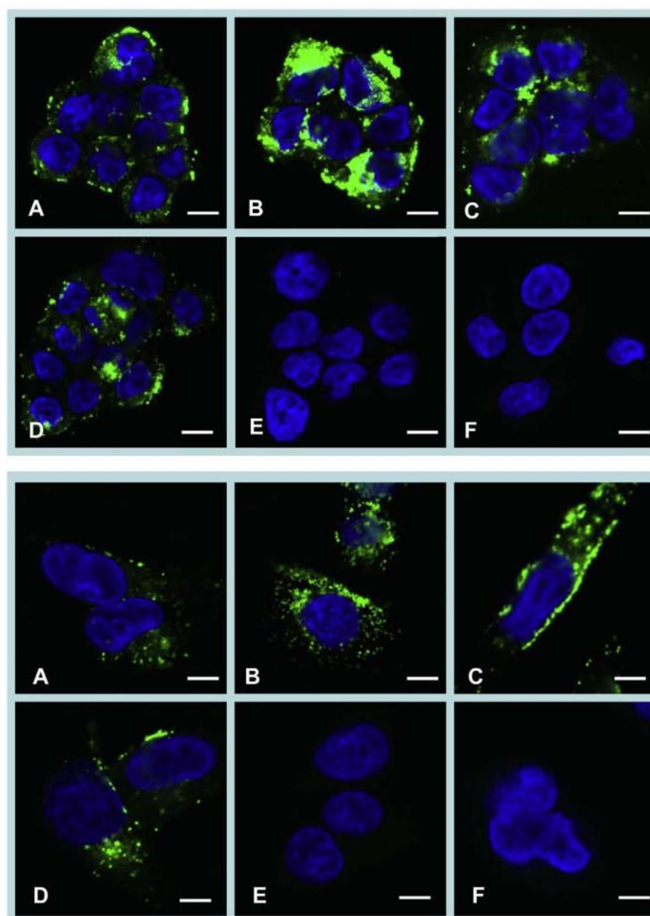

Fig. 9. Confocal microscopy imaging of the cellular uptake of Cy3-labeled DNA (6 μg/dish) either complexed with DAB-Arg (A), DAB-Lys (B), DAB-Leu (C), DAB (D) or in solution (E) after incubation for 24 h with A431 (top) and T98G cells (bottom) (control: untreated cells (F)) (Blue: nuclei stained with DAPI (excitation: 405 nm laser line, bandwidth: 415–491 nm), green: Cy3-labeled DNA (excitation: 543 nm laser line, bandwidth: 550–620 nm) (Bar: 10 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

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with both DAB-Lys and DAB-Leu polyplexes. By contrast, cells treated with naked DNA did not show any Cy3-derived fluorescence. No co-localization of DNA in the nuclei was visible in any cell lines after 24 h incubation, leading to the hypothesis that the nuclear uptake of DNA occurred at a latter time.

3.2.3. *In vitro anti-proliferative activity*

The conjugation of Arg, Lys and Leu to DAB led to a significant increase of *in vitro* anti-proliferative activity in A431 cells, respectively by 30-fold, 22-fold and 20-fold compared to the unmodified polyplex (IC_{50} of 0.32 ± 0.04 μg/mL, 0.43 ± 0.09 μg/mL and 0.48 ± 0.16 μg/mL respectively for DAB-Arg, DAB-Lys and DAB-Leu polyplexes, 9.47 ± 1.15 μg/mL for unmodified DAB polyplex) (Table 1). In T98G cells, the conjugation of the amino acids to DAB improved the anti-proliferative activity of the polyplex, by 43-fold for DAB-Arg (IC_{50}: 0.23 ± 0.01 μg/mL), by 47-fold for DAB-Lys (IC_{50}: 0.21 ± 0.01 μg/mL) and by 35-fold for DAB-Leu (IC_{50}: 0.28 ± 0.02 μg/mL) compared to the unmodified DAB polyplex (IC_{50}: 9.84 ± 2.79 μg/mL). Uncomplexed amino acid-bearing DAB and naked DNA did not exert any cytotoxicity to the cells. These results may be attributed to the improved transfection efficacy when treated with Arg-, Lys- and Leu-bearing DAB polyplex. DAB-Lys polyplex was the most efficacious treatment on T98G cells, probably as a result of its highest transfection efficacy on the same cell line.

3.3. *In vivo study*

The intravenous administration of DAB-Arg, DAB-Lys and DAB-Leu polyplexes led to a significant increase of gene expression in the tumor compared to that obtained after treatment with unmodified DAB polyplex (34.9 ± 0.4, 39.9 ± 0.8, 36.7 ± 0.2 mU β-galactosidase per tumor respectively for DAB-Arg, DAB-Lys and DAB-Leu polyplexes, compared to 25.8 ± 5.6 mU β-galactosidase per tumor for DAB polyplex) (Fig. 10). Among the 3 amino acid-bearing polymers tested, DAB-Lys led to the highest level of gene expression in the tumor. The treatments were well tolerated by the mice at the administered doses. Gene expression was also decreased in the heart (by about 15-fold for DAB-Arg, 7-fold for DAB-Lys and 5-fold for DAB-Leu) and in the spleen (by about 3-fold for DAB-Arg, 2-fold for DAB-Lys and 1.5-fold for DAB-Leu) compared with that of DAB polyplex. The results obtained in the lung and the kidneys were more amino acid dependent: while the grafting of Arg led to a 5-fold decrease of gene expression compared to that of the unmodified polymer, the grafting of Lys did not modify it, and the grafting of Leu even increased it by more than 9-fold. A similar pattern was observed in the kidneys, where only the grafting of Arg led to a 2-fold decrease in the level of gene expression. In the liver, the grafting of Arg seemed to lead to an increase of gene expression compared to the other treatments. This increase was however not significantly different. The predominant gene expression in the liver compared to the other organs has been

Table 1
Therapeutic efficacy of TNFα- expressing DNA complexed with DAB-Arg, DAB-Lys, DAB-Leu and DAB in A431 cells and T98G cells, expressed as IC_{50} values ($n = 15$).

IC_{50} (μg/mL) (mean ± S.E.M.)		
Formulation	A431	T98G
DAB-Arg-DNA	0.32 ± 0.04	0.23 ± 0.01
DAB-Lys-DNA	0.43 ± 0.09	0.21 ± 0.01
DAB-Leu-DNA	0.48 ± 0.16	0.28 ± 0.02
DAB-DNA	9.47 ± 1.15	9.84 ± 2.79
DAB-Arg	>50	>50
DAB-Lys	>50	>50
DAB-Leu	>50	>50
DNA	>50	>50

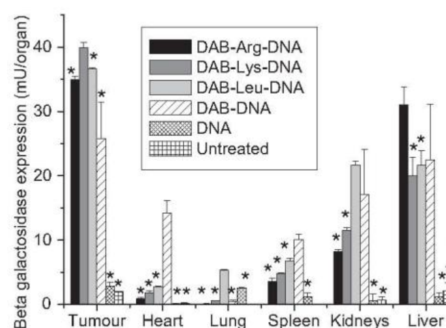

Fig. 10. Biodistribution of gene expression after a single intravenous administration of Arg-, Lys- and Leu-bearing DAB polyplexes and unmodified DAB polyplex (50 μg DNA administered). Results were expressed as milliunits β-galactosidase per organ ($n = 5$). *: $P < 0.05$: highest gene expression treatment vs. other treatments for each organ.

previously reported following intravenous administration of DAB polyplex, due to their removal by macrophages [23]. It is unusual, as particulate cationic gene transfer agents are generally reported to preferentially transfer genes to the lung, probably due to particle aggregation and accumulation in the small lung capillaries [24].

The biodistribution of gene expression was also confirmed by bioluminescence imaging (Fig. 11). Co-localization of gene expression in the tumors was clearly visible following treatment with the amino acid-bearing DAB polyplexes. Luciferase expression appeared to be heterogeneously distributed within the tumors, probably concentrated in highly vascularized areas within the tumors. It was however not visible in the tumors after treatment with DAB polyplex and naked DNA and appeared to be very limited in the other organs, with the exception of the liver after treatment with DAB-Arg polyplex. This might be explained by the chosen image acquisition parameters that probably allowed only the most intensely luminescent tissues to be analyzed. These data demonstrated that the conjugation of Arg, Lys and Leu to DAB increased gene expression within the tumors. However, the significantly elevated gene expression in the liver highlights the need of tumor-targeting moieties conjugated to the polyplexes in future experiments for increasing the specificity of tumor gene expression.

This communication presented evidence that amino acid-bearing DAB polyplexes can lead to improved gene expression in the tumors after intravenous administration. Other groups have already demonstrated gene transfer capabilities of amino acid-bearing gene delivery systems, but following intratumoral rather than intravenous administration. Tetraarginine-PEG lipid coated protamine DNA has been previously reported to improve gene expression in tumors following intratumoral injection, compared to the control delivery system [25]. Similarly, the intratumoral administration of glutamate-based cationic lipoplexes improved the level of gene expression in the tumors compared to their aspartate-based counterparts [26]. Our previous study presented evidence of the improved gene expression in the tumors after intravenous administration of amino acid-bearing PEI polyplexes, used as a model polymer [14]. In this study we wanted to evaluate if this improvement could be generalized to other types of gene delivery systems, such as dendrimers and especially generation 3 DAB dendrimer, which has been shown to be an extremely promising non-viral gene delivery system *in vitro* as well as *in vivo*. We demonstrated that this is indeed the case. Our objective will now be to improve the tumor specificity of gene expression, by conjugating

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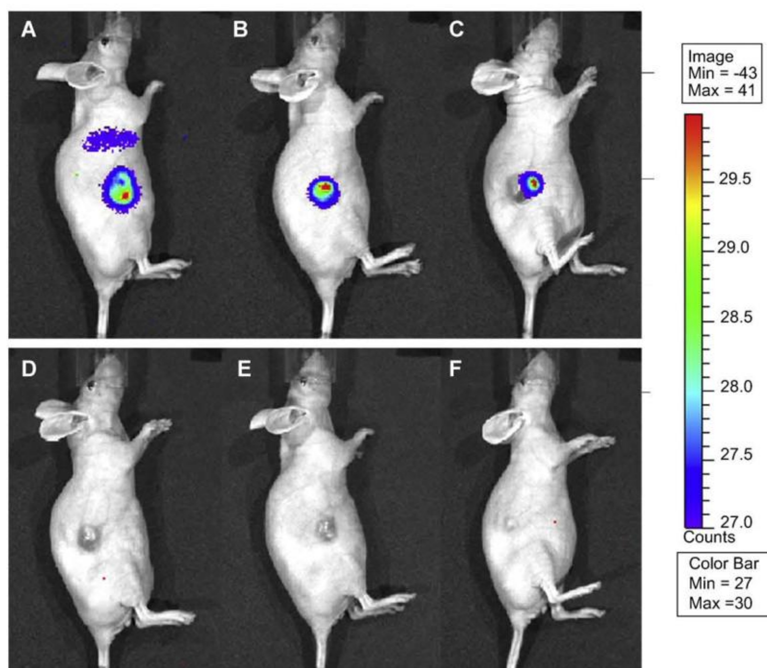

Fig. 11. Bioluminescence imaging of gene expression after intravenous administration of luciferase-encoding plasmid DNA complexed with DAB-Arg (A), DAB-Lys (B), DAB-Leu (C), DAB (D) or free in solution (E) in living mice (50 μg DNA administered) (control: untreated cells (F)). The mice were imaged using the IVIS Spectrum 24 h after injection of the treatments. The scale indicates surface radiance (photons/s/cm^2/steradian).

a tumor-targeting ligand together with amino acids, with the hope to optimize the therapeutic effect.

4. Conclusions

The grafting of arginine, lysine and leucine to DAB dendrimer has been shown to enhance the anti-proliferative activity of the polyplex *in vitro*, by up to 47-fold for DAB-Lys in T98G cells compared to the unmodified polyplex. *In vivo*, the intravenous administration of amino acid-bearing DAB polyplexes resulted in an improved tumor gene expression, with the highest gene expression level observed after treatment with DAB-Lys polyplex. These transfection effects, together with the lack of toxicity, make arginine-, lysine- and leucine-bearing generation 3 polypropylenimine polymers highly promising gene delivery systems for both *in vitro* and *in vivo* tumor gene expression.

Acknowledgments

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IN VITRO EVALUATION OF NOVEL GENE DELIVERY SYSTEMS FOR CANCER THERAPY

Hibah Aldawsari

University of Strathclyde

Glasgow, United Kingdom

Email: hibah.aldawsari@strath.ac.uk

Christine Dufès

University of Strathclyde

Glasgow, United Kingdom

Email: C.Dufes@strath.ac.uk

ABSTRACT

Gene therapy is currently limited by the lack of delivery systems able to efficiently carry therapeutic DNA to their site of action. As amino acids receptors are overexpressed on most cancer cell lines, we hypothesize that amino acids-bearing gene delivery systems might lead to more efficacious gene delivery. To this end, various amino acids- bearing polyethylenimine conjugates have been prepared. The evaluation of their transfection and therapeutic efficacy demonstrated that Lys-bearing polyethylenimine was more efficacious than its non-targeted counterpart on the cancer cell lines studied. Amino acids bearing polymers thus seem to be promising gene delivery systems for cancer therapy.

Keywords: non-viral gene delivery, amino acids, polyethylenimine, cancer therapy.

INTRODUCTION

The possibility of using genes as medicines for the treatment of cancer is currently hampered by the inability to efficiently delivery therapeutic genes to remote tumours and metastases by intravenous administration, without secondary effects to healthy tissues. As amino acids receptors are overexpressed on most cancer cell lines, we hypothesize that grafting amino acids on gene delivery systems might lead to more efficacious systems. We chose to use polyethylenimine (PEI) as a model of non-viral delivery system, as it has been extensively studied and exhibited a consistent, high transfection efficacy on numerous cancer cell lines. The objectives of this study was thus to prepare and evaluate *in vitro* the transfection and therapeutic efficacies of arginine, lysine and leucine bearing polyethylenimine on various cancer cell lines.

METHODS

The synthesis of arginine, lysine and leucine-bearing PEI was confirmed by ^{1}H NMR spectroscopy. DNA condensation by the amino acids-bearing polymer was evaluated by PicoGreen® assay. The size and zeta potential of the targeted polymer complexed with DNA were measured by photon correlation spectroscopy. A qualitative analysis of the uptake of the DNA carried by the delivery systems was performed by confocal microscopy. The transfection efficiency of DNA carried by arginine, lysine and leucine-bearing PEI was assessed at various polymer: DNA ratios and compared to that observed with non-targeted PEI and DOTAP. The therapeutic efficacy of these systems was assessed by MTT assay.

RESULTS AND CONCLUSION

Arginine, lysine and leucine-bearing PEI were successfully prepared, as demonstrated by ^{1}H NMR. The size of all formulations was below 400 nm, which is suitable for gene delivery. All amino acid-bearing PEI complexed with DNA were bearing a positive surface charge, which should facilitate electrostatic interaction with the anionic cell surface. Among the amino acid-bearing polymers tested, lysine-bearing PEI led to a better transfection than arginine and leucine-bearing PEI. Lysine-bearing PEI also led to an enhanced transfection compared to non-targeted PEI and DOTAP. Confocal microscopy experiments showed that DNA uptake was improved following treatment of the cancer cells with targeted PEI compared to co-targeted PEI, whereas no DNA solution was taken up by the cells. The therapeutic efficacy observed after treatment with lysine and leucine-bearing PEI polyplexes was improved compared to non-targeted PEI polyplexes on the cancer cell lines studied. Amino acids bearing polymers thus seem to be highly promising gene delivery systems for cancer therapy and will be further investigated.

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ENHANCED GENE EXPRESSION OF POLYETHYLENIMINE BY SURFACE MODIFICATION WITH ARGININE, LYSINE AND LEUCINE

Hibah Aldawsari*, Behin Sundara Raj, RuAngelie Edrada-Ebel, David R. Blatchford, Christine Dufès

University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR, United Kingdom

Gene therapy has become a promising strategy for the treatment of many inheritable or acquired diseases that are currently considered incurable. However, the potential of gene therapy is currently limited by the lack of delivery systems able to efficiently carry therapeutic DNA to their site of action. In this study, we show that the grafting of arginine, lysine and leucine residues onto the surface of the cationic polymer polyethylenimine, chosen as a model non-viral gene delivery system, improves the gene expression and therapeutic efficacies of this delivery system on A431 epidermoid carcinoma and T98G glioma *in vitro*. Novel arginine, lysine and leucine-bearing polyethylenimine were able to condense DNA into particles with positive surface charges and average sizes inferior to 300 nm, attributes favourable for efficient gene delivery *in vitro*. The conjugation of arginine and lysine residues to polyethylenimine led to an improved transfection compared to the native polyethylenimine and DOTAP on both the tested cell lines, with improvements of up to 11.7 times following treatment with lysine-bearing polyethylenimine polyplex compared to DOTAP-DNA in A431 cells. The grafting of leucine to polyethylenimine improved the level of transfection only on T98G cells, by 4 times compared to DOTAP-DNA. The administration of therapeutic DNA complexed with lysine and leucine-bearing polyethylenimine led to an improved therapeutic efficacy compared to the unmodified polymer on both the cancer cell lines tested, by up to 51 times and 127 times respectively in A431 and T98G cells. Arginine-bearing polyethylenimine was more efficacious than the native polymer only on A431 cell line. These results show that the grafting of arginine, lysine and leucine to a model polyethylenimine delivery system is highly promising for improving transfection and therapeutic efficacies to cancer cells. The potential of these amino acid-bearing delivery systems will be further investigated.

Enhanced transfection efficacy of polyethylenimine by surface modification with arginine, lysine and leucine

Hibah Aldawsari , Behin Sundara Raj, RuAngelie Edrada-Ebel, David R. Blatchford, and Christine Dufès

Strathclyde Institute of Pharmacy and Biomedical Sciences, Glasgow, G4 0NR, United Kingdom
hibah.aldawsari@strath.ac.uk

ABSTRACT SUMMARY

The potential of gene therapy is currently limited by the lack of delivery systems able to efficiently carry therapeutic DNA to their site of action. Non-viral vectors are receiving increasing attention as gene delivery vehicles due to their favourable properties compared to viral systems. Unfortunately, their use is still hampered by their lower transfection efficacy compared to viral systems. The present study investigates the possibility of creating an improved transfection agent by grafting arginine, lysine and leucine residues onto the surface of a non-viral gene delivery system. As a model delivery system, we chose to use the cationic polymer polyethylenimine (PEI), as it has been widely used for non-viral transfection *in vitro* and *in vivo* and combine strong DNA compaction capacity with an intrinsic endosomolytic activity also known as the proton sponge effect [1]. It is hypothesized that arginine, lysine and leucine-bearing polyethylenimine would lead to an improved transfection efficacy by synergistic actions of the proton sponge effect and hydrophobic interactions with the cellular membranes. The objectives of this study were 1) to prepare and characterize arginine, lysine and leucine-bearing polyethylenimine, 2) to evaluate their transfection and therapeutic efficacies *in vitro* on an epidermoid carcinoma (A431) and a glioma (T98G) cell lines.

INTRODUCTION

Gene therapy, the technology that help delivering the therapeutic genes (DNA or RNA) for the purpose of correcting or replacing the defected genes, has become one of the most attractive concepts for a broad variety of biomedical applications in clinical research. Although the majority of gene delivery approaches so far have involved viral delivery systems, several non-viral vectors have been developed and investigated as an alternatives to the viral vectors due to their several advantages [2]. The major drawback for the use of non-viral vectors is their lower transfection efficacy compared to viral systems. Many techniques have been investigated to overcome this problem. Among them, the conjugation of cell-specific ligands to gene delivery systems seems particularly promising. It has recently been demonstrated that some peptide sequences known as transduction domains or membrane translocation signals contain positively charged amino acid residues such as arginine and lysine, which have been reported to enhance transportation into cells by different groups [3-5]. Another delivery strategy using amino acids involves the use of their transporter systems. As amino acids are essential for the proliferation of cancer cells, the system L-amino acid transporter, which transports large neutral amino acids such as leucine but also amino acid-related drugs such as the anti-cancer phenylalanine mustard melphalan, are overexpressed in brain cancer cells and other malignant tumours [5]. It is proposed that this system transporter may facilitate the uptake of leucine-bearing gene delivery systems to cancer cells.

EXPERIMENTAL METHODS

Amino acid coupling to PEI was performed using a cross-linking agent according to a previously described method [(6)]. The grafting of the amino acids to PEI was assessed by ^{1}H NMR spectroscopy. DNA condensation assay using PicoGreen® reagent was performed to assess the polyplex formation of PEI with plasmid DNA at different polymer: DNA weight ratios. Size and zeta potential of the polyplexes were measured by photon correlation spectroscopy. *In vitro*, the uptake of selected polyplexes were observed under confocal microscopy on T98G and A431 cells. The transfection efficacy of amino acids-grafted PEI complexed to DNA was investigated at different weight ratios and compared to that of unmodified PEI and DOTAP. Cytotoxicity was determined by means of measuring IC_{50} using MTT assay on A431 and T98G cell lines. Results were expressed as mean ± standard error of the mean (S.E.M). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison post-test (GraphPad Prism software). Differences were considered as significant when $P < 0.05$.

RESULTS AND DISCUSSION

The grafting of arginine, lysine and leucine residues onto PEI was confirmed by ^{1}H NMR spectrum. PicoGreen® assay showed that the three amino acid-bearing polymers were able to condense DNA at polymer to DNA ratio higher than 2:1. DNA condensation was higher than 80%, independently of the weight ratio tested. All tested amino acid-bearing polyplexes displayed average sizes inferior to 300 nm, which is suitable for gene delivery. The zeta potential values were favourably positive at higher polymer: DNA weight ratios which are advantageous to facilitate the interaction with the negatively charged cell membrane.

In vitro, the highest transfection levels for the three amino acid-bearing PEI polyplexes were observed at a polymer: DNA ratio of 20:1 on A431 and T98G cell lines. Further transfection experiments were performed to evaluate the efficacy of the three amino acid-bearing PEI polyplexes

compared to that of DOTAP-DNA and unmodified PEI polyplex, on A431 and T98G cell lines. The conjugation of arginine and lysine residues to polyethylenimine led to an improved transfection compared to the native polyethylenimine and DOTAP on both the tested cell lines, with improvements of up to 11.7 times following treatment with lysine-bearing polyethylenimine polyplex compared to DOTAP-DNA in A431 cells. The grafting of leucine to polyethylenimine improved the level of transfection only on T98G cells, by 4 times compared to DOTAP-DNA. The administration of therapeutic DNA complexed with lysine and leucine-bearing polyethylenimine led to an improved therapeutic efficacy compared to the unmodified polymer on both the cancer cell lines tested, by up to 51 times and 127 times respectively in A431 and T98G cells. Arginine-bearing polyethylenimine was more efficacious than the native polymer only on A431 cell line.

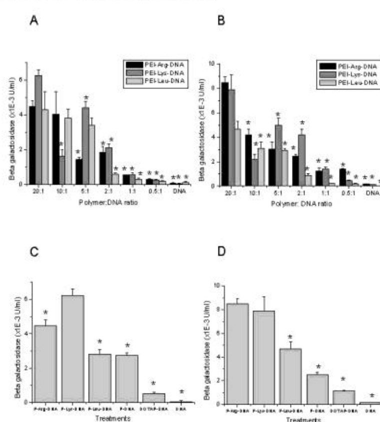

Figure 1: Transfection efficacy of PEI-Arg, PEI-Lys and PEI-Leu polyplexes at various polymer: DNA weight ratios (A, B), relative to DOTAP and native PEI (C, D) in A431 (A, C) and T98G cells (B, D). DOTAP-DNA and PEI-DNA were dosed at their optimal carrier: DNA ratio of 5:1. Results are expressed as the mean ± SEM of three replicates (n=15). *: P <0.05 vs the highest transfection ratio (A, B) or treatment (C, D).

Table 1: Therapeutic efficacy of TNFα- expressing DNA complexed with PEI-Arg, PEI-Lys, PEI-Leu and PEI in A431 cells and T98G cells, expressed as IC_{50} values (n=15)

	IC_{50} ± SEM (µg/ml)	
Formulation	**A431 cells**	**T98G cells**
PEI-Arg-DNA	0.52 ± 0.13	10.78 ± 1.66
PEI-Lys-DNA	0.35 ± 0.07	0.12 ± 0.01
PEI-Leu-DNA	0.44 ± 0.16	0.04 ± 0.04
PEI-DNA	17.86 ± 5.34	5.08 ± 0.51

CONCLUSION

New arginine, lysine and leucine-bearing polyethylenimine polymers have been prepared with the aim of producing effective gene transfection levels *in vitro*. The three amino acid-bearing polymers were able to condense DNA into particles with positive surface charges and average sizes inferior to 300 nm, attributes that have been recognized as being favourable for efficient gene delivery *in vitro*. The therapeutic efficacy of DNA encoding TNF-α was improved when delivered with PEI-Lys and PEI-Leu by at least 34-times compared to unmodified PEI on the two cancer cell lines tested. Arginine, lysine and leucine-bearing polyethylenimine polymers are thus highly promising gene delivery systems for cancer therapy and will be further investigated.

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Enhanced gene expression of polyethylenimine by surface modification with arginine, lysine and leucine

Hibah Aldawsari*, Behin Sundara Raj, RuAngelie Edrada-Ebel, David R. Blatchford, Christine Dufès

Drug Delivery/ Strathclyde Institute of Pharmacy and Biomedical Sciences/ University of Strathclyde

Glasgow/ United Kingdom

hibah.aldawsari@strath.ac.uk

Abstract

The potential of gene therapy to treat cancer is currently limited by the lack of delivery systems able to efficiently carry therapeutic DNA to its site of action, with a resulting low expression of therapeutic genes in tumours. As amino acids are known to have excellent properties in cell penetration and gene expression regulation, we investigated if the grafting of arginine, lysine and leucine residues onto the surface of the cationic polymer polyethylenimine, chosen as a model non-viral gene delivery system, could improve the gene expression in tumours after intravenous administration.

Key words: Gene delivery; Amino acids; Gene expression; cancer therapy; polyethylenimine.

Introduction

Gene therapy has become a promising strategy for the treatment of many inheritable or acquired diseases that are currently considered incurable. However, the lack of effective vectors is a major barrier for the progress of human gene therapy. Non-viral gene delivery systems have been proposed as safer alternatives to viral vectors because they have the potential to be administered repeatedly with minimal host immune response, are stable in storage and can be easily produced in large quantities. Among non-viral vectors, cationic polymers have gained increased attention because of their ability to mediate transfection via condensing DNA into small particles, which protects DNA from enzymatic degradation and facilitate the cell uptake and endolysosomal escape (Dang and Leong, 2006). Polyethylenimine (PEI) is regarded as one of the most effective non-viral vectors. Its high gene transfection efficiency which is believed to come from its intrinsic ''proton sponge'' effect have granted PEI the reputation of being considered as the "golden standard" among other non-viral vectors (Deng *et al.*, 2009). Recently, some peptide sequences known as protein transduction domains or membrane translocation signals were recognised and introduced to enhance the delivery of plasmid DNA (Futaki, 2005). These sequences usually contain positively charged amino acids residues such as arginine, lysine which have been reported to enhance the transportation into cells (Nakanishi *et al.,* 2003). In the present study, we investigated if the conjugation of arginine, lysine and leucine residues onto the surface of polyethylenimine could lead to an improved gene expression in tumours after intravenous administration.

Materials and Methods:

Cell lines and reagents:

Branched polyethylenimine (PEI) (molecular weight 25 kDa), arginine (Arg), lysine (Lys), leucine (Leu), dimethylsuberimidate (DMSI), triethanolamine, phosphate buffered saline (PBS), Sephadex G75, deuterated water (D2O), magnesium chloride, mercaptoethanol, o- nitrophenol-β-galactopyranoside (ONPG) and 3-(4,5 dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (Poole, UK). Quanti-iTTM PicoGreen® dsDNA reagent and tissue culture media were obtained from Invitrogen (Paisley, UK). (*N*-[1-(2,3-Dioleoyloxy)]-*N,N,N*-trimethylammonium methyl-sulphate (DOTAP) liposomal transfection reagent was purchased from Roche (Burgess Hill, UK). Vectashield® mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories (Peterborough, UK). Passive lysis buffer was purchased from

Promega (Southampton, UK). Label IT® Cy3 Nucleic Acid Labelling kit was from Cambridge Biosciences (Cambridge, UK). Firefly D-luciferin was purchased from Caliper Life Sciences (Hopkinton, MA). A431 epidermoid carcinoma and T98G glioblastoma were obtained from the European Collection of Cell Cultures (Salisbury, UK). The expression plasmids encoding Tumour necrosis factor (TNF)α (pORF9-mTNFα) and β-galactosidase (pCMVsport β-galactosidase) were obtained respectively from InvivoGen (San Diego, CA) and Invitrogen (Paisley, UK).

Synthesis of PEI-Arg, PEI-Lys and PEI-Leu

Amino acid coupling to PEI was performed by using DMSI as a cross-linking agent in a similar manner to that reported for transferrin-bearing dendrimers and vesicles (Koppu et al., 2010). PEI was added to the amino acids arginine, leucine or lysine and DMSI in triethanolamine HCl buffer (pH 7.4). The final product was purified by size exclusion chromatography using a Sephadex G75 column and freeze-dried. The grafting of the amino acids to PEI was assessed by ^{1}H NMR spectroscopy using a Jeol Oxford NMR AS 400 spectrometer.

Characterization of polyplex formation by PicoGreen® assay

The degree of DNA accessibility following complexation with the amino acid-bearing PEI polymers was assessed by PicoGreen® assay, performed according to the protocol from the supplier. The electrostatic interaction between the anionic DNA and cationic group of the polymer on formation of the DNA-PEI-amino acids complex condenses the DNA and reduces the number of PicoGreen® binding sites, ultimately reducing the fluorescence intensity for the PicoGreen® solution. PicoGreen® reagent was diluted 200-fold in Tris-EDTA (TE) buffer (10 mM Tris, 1mM EDTA, pH 7.5) on the day of the experiment. Polymer : DNA weight ratios (20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1, 0:1) were used. The fluorescence intensity of the complexes in the presence of PicoGreen® were analyzed at various time points with a Varian Cary Eclipse Fluorescence spectrophotometer (λ_{exc} : 480 nm, λ_{em} : 520 nm). Results were represented as percentage of DNA condensation (= 100 - % relative fluorescence to DNA control) and compared with those obtained for PEI-DNA (polymer: DNA weight ratio 5:1) (n=4).

Polyplex size and zeta potential measurement

Size and zeta potential of the amino acids-bearing PEI complexed to DNA were measured by photon correlation spectroscopy and laser Doppler electrophoresis using a Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK).

In vitro biological characterization

In vitro transfection

Transfection efficacy of the DNA carried by the amino acid-bearing polymers was assessed with a plasmid coding for β-galactosidase (pCMV βgal), using a β-galactosidase transfection assay on A431 and T98G cell lines. PEI-Arg, PEI-Leu and PEI-Lys complexed to plasmid DNA encoding β-galactosidase, at the polymer: DNA weight ratios used for the DNA condensation experiment. Naked DNA served as a negative control, formulations of DOTAP-DNA (DOTAP: DNA ratio 5:1, according to the manufacturer's protocol) and PEI-DNA (polymer: DNA weight ratio 5:1), served as positive controls.

Cellular uptake

Imaging of the cellular uptake of the DNA carried by amino acid-bearing PEI was done by confocal microscopy. Labelling of plasmid DNA with the fluorescent probe Cy3 was performed using a Label IT® Cy3 Nucleic Acid Labelling kit, as described by the manufacturer.

In vitro anti-proliferative activity

Anti-proliferative activity of amino acid-bearing PEI complexed with plasmid DNA encoding TNFα was assessed in A431 and T98G cell lines. Anti-proliferative activity was evaluated by measurement of the growth inhibitory concentration for 50% of the cell population (IC_{50}) in a standard MTT assay.

In vivo study

Biodistribution of gene expression

Groups of mice (n=4, initial mean weight 20 g) bearing subcutaneously implanted A431 tumors, were treated intravenously with a single injection of amino acid-bearing and control PEI carrying β-galactosidase expression plasmid (50 µg of DNA). Mice were sacrificed 24 h after injection. Their organs were removed, immediately frozen in liquid nitrogen and analyzed for their β-galactosidase levels as previously described (Zinselmeyer *et al.,* 2003).

The biodistribution of gene expression was also visualized by bioluminescence imaging, using an IVIS Spectrum (Caliper Life Sciences, USA).

Statistical Analysis

Results were expressed as means ± standard error of the mean (S.E.M). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison post-test (GraphPad Prism software). Differences were considered significant for P value < 0.05.

Results and discussion

New arginine, lysine and leucine-bearing polyethylenimine polymers have been successfully prepared. Percentage conjugation of the amino acids with the polymer was found to be 14.9%, 12.6% and 10.6% for arginine, leucine and lysine, respectively as shown from NMR. The three amino acid-bearing polymers were able to condense DNA into particles with positive surface charges and average sizes inferior to 300 nm, attributes that have been recognized as being favourable for efficient gene delivery.

Characterization of polyplex formation by PicoGreen® assay

The conjugation of amino acid residues on the primary amines of PEI may affect the polymer's ability to complex DNA. For this reason, we examined the influence of the conjugation of amino acids on the polymer ability to form complexes with DNA, by using a PicoGreen® assay.The three amino acid-bearing polymers were able to condense more than 80% of DNA, at polymer: DNA weight ratios of 2:1 for PEI-Arg, 0.5: 1 for PEI-Lys and 1:1 for PEI-Leu (Fig. 1).

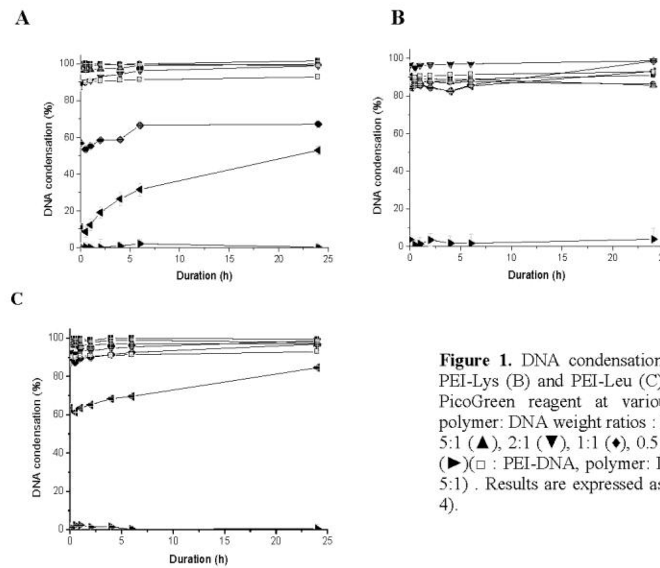

Figure 1. DNA condensation of PEI-Arg (A), PEI-Lys (B) and PEI-Leu (C) polyplexes using PicoGreen reagent at various durations and polymer: DNA weight ratios : 20:1 (■), 10:1 (●), 5:1 (▲), 2:1 (▼), 1:1 (♦), 0.5:1 (◄), DNA only (►)(□ : PEI-DNA, polymer: DNA weight ratio: 5:1) . Results are expressed asmean ± SEM (n= 4).

In vitro transfection

Transfection efficacy was determined by quantifying the expression of β-galactosidase encoded by the plasmid. A first transfection experiment was performed to determine the polymer: DNA weight ratio leading to optimized

transfection on A431 (Fig. 2A) and T98G cells (Fig. 2B), for each of the three amino acid-bearing PEI polyplexes. Transfection mainly increased with increasing polymer: DNA ratios. The highest transfection levels for the three amino acid-bearing PEI polyplexes were observed at a polymer: DNA ratio of 20:1 on A431 and T98G cell lines. Based on these findings, along with the DNA condensation results; 20:1 ratio was selected for transfection and therapeutic efficacy experiments.

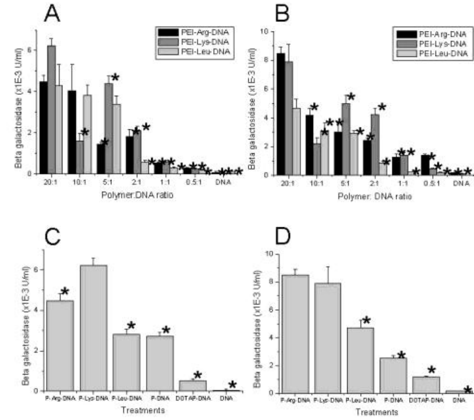

Figure 2. Transfection efficacy of PEI-Arg, PEI-Lys and PEI-Leu polyplexes at various polymer: DNA weight ratios (A, B), relative to DOTAP and native PEI (C, D) in A431 (A, C) and T98G cells (B, D). DOTAP-DNA and PEI-DNA were dosed at their optimal carrier: DNA ratio of 5:1. Results are expressed as the mean ± SEM of three replicates (n=15). * :P <0.05 vs the highest transfection ratio (A, B) or treatment (C, D).

Further transfection experiments were performed to evaluate the efficacy of the three amino acid-bearing PEI polyplexes compared to that of DOTAP-DNA and unmodified PEI polyplex, on A431 and T98G cell lines. In A431 cells, treatment with PEI-Lys polyplex led to the highest transfection (6.24.10-3 ± 0.34.10-3 U/mL). Its transfection efficacy was 1.3 times higher than that of PEI-Arg polyplex (4.46.10-3 ± 0.34.10-3 U/mL) and more than 2 times higher than that of PEI-Leu polyplex (2.81.10-3 ± 0.27.10-3 U/mL) (Fig. 2C). In T98G cells, transfection levels were similar for PEI-Lys (7.91.10-3 ± 1.18 .10-3 U/mL) and PEI-Arg polyplexes (8.49.10-3 ± 0.43.10-3 U/mL). They were 2.8 times higher than that obtained with PEI-Leu polyplex (2.81.10-3 ± 0.27.10-3 U/mL) (Fig. 2D)..The conjugation of Arg, Lys and Leu to PEI led to an improved transfection compared to the unmodified PEI and DOTAP on both the tested cell lines, with the exception of PEI-Leu which led to a similar transfection than that observed after treatment with PEI-DNA on A431 cells. On A431 cells, gene expression following treatment with PEI-Lys polyplex was 2.2 times higher than that of unmodified PEI polyplex (2.72.10-3 ± 0.18.10-3 U/mL) and 11.7 times higher than that of DOTAP-DNA (0.53.10-3 ± 0.08.10-3 U/mL) (Fig. 2C). Following treatment with PEI-Arg polyplex, it was respectively 1.6 and 8.4 times higher than after treatment with unmodified PEI polyplex and DOTAP-DNA. The grafting of Leu to PEI did not bring any significant improvement in the level of transfection compared to unmodified PEI, but was still 5.3 times more efficacious than DOTAP-DNA. On T98G cells, gene expression following treatment with PEI-Lys and PEI-Arg polyplexes were 3 times higher than that of unmodified PEI polyplex (2.52.10-3 ± 0.18.10-3 U/mL) and 6.7 times higher than that of DOTAP-DNA (1.17.10-3 ± 0.05.10-3 U/mL) (Fig. 2D). After treatment with PEI-Leu polyplex, it was respectively 1.8 and 4 times higher than after treatment with unmodified PEI polyplex and DOTAP-DNA. No gene expression was observed after treatment with control DNA, as expected. The improved β-gal expression induced by amino acid-bearing PEI compared to unmodified PEI most likely resulted from the higher zeta-potential of their DNA complexes, due to the strong correlation between cellular uptake and positive charge density of polyplexes (Choi *et al.*, 2006). These results suggest that the grafting of basic amino acids on a polymer enhances the transfection activity compared to unmodified polymer. To our knowledge, it is the first time that the grafting of Lys residues to gene delivery systems gave such improvement in gene expression. The conjugation of the hydrophobic amino acid Leu gave cell line-dependent results, indicative of the influence of cell line specific factors on the transfection process. In our experiments, the grafting of Leu to PEI led to an improved gene expression in glioma T98G cells, but not in carcinoma A431 cells.

Cellular uptake:

The uptake of Cy3-labelled DNA by A431 and T98G cells was qualitatively confirmed using confocal microscopy (Fig. 3). The complexation of plasmid DNA to PEI improved DNA uptake by both cell lines compared to DNA solution, as expected. Co-localization of DNA in the nuclei was clearly visible in both cell lines treated with PEI-Leu polyplex in A431 cells and PEI-Arg polyplex in T98G cells. It was less pronounced in the case of the other treatments.

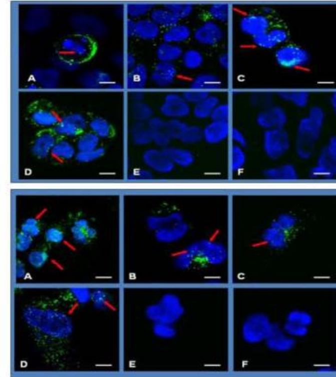

Figure 3. Confocal microscopy imaging of the cellular uptake of Cy3- labeled DNA either complexed with PEI-Arg (A), PEI-Lys (B), PEI-Leu (C), PEI (D) or free in solution (E) after incubation for 24 hours with A431 (top) and T98G cells (bottom) (control: untreated cells (F)) (Blue: nuclei stained with DAPI (excitation: 405 nm laser line, bandwidth: 415-491nm), green: Cy3-labelled DNA (excitation: 453 nm laser line. bandwidth: 550-620 nm) (Magnification: x40).

In vitro anti-proliferative activity

The conjugation of Arg, Lys and Leu to PEI led to an increase of the *in vitro* antiproliferative activity in A431 cells, respectively by 34-fold, 51-fold and 40-fold compared to the unmodified polyplex. In T98G cells, the conjugation of the amino acids to PEI improved the anti-proliferative activity of the polyplex, by 2-fold for PEI-Arg (IC50: 5.08 ± 0.51 µg/mL), by 89-fold for PEI-Lys (IC50: 0.12 ± 0.01 µg/mL) and by 269-fold for PEI-Leu (IC50: 0.04 ± 0.04 µg/mL). Uncomplexed amino acid-bearing PEI and naked DNA did not exert any cytotoxicity to the cells. The grafting of these amino acids to PEI significantly improved the therapeutic efficacy of the system on the two cancer cell lines studied.

In vivo study

The intravenous administration of PEI-Arg, PEI-Lys and PEI-Leu polyplexes led to a significant increase of gene expression in the tumour, with a β-galactosidase amount at least 3-fold higher than that obtained after treatment with unmodified PEI polyplex (18.2 ± 1.2, 20.4 ± 3.7, 19.2 ± 3.1mU β-galactosidase per tumour respectively for PEI-Arg, PEI-Lys and PEI-Leu polyplexes, compared to 6 ± 1.8 mU β-galactosidase per tumour for PEI polyplex) (Fig. 4).

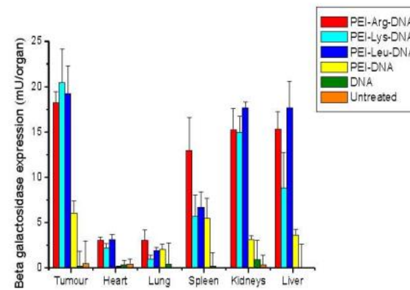

Figure 4. Biodistribution of gene expression after a single intravenous administration of Arg-, Lys- and Leu-bearing PEI polyplexes and unmodified PEI polyplexes (50 µg DNA administered). Results were expressed as milliunits β-galactosidase per organ (n=5). * : P <0.05 : highest gene expression treatment vs. other treatments for each organ.

The 3 amino acid-bearing PEI led to similar levels of gene expression in the tumour. The treatments were well tolerated by the mice at the administered doses. Gene expression was also increased in the kidneys (by at least 4-fold) and in the liver (by about 5-fold for PEI-Arg and PEI-Leu, 2-fold for PEI-Lys) after administration of amino acid-bearing PEI polyplexes. These results highlight the need of tumour-targeting moieties conjugated to the polyplexes in future experiments for increasing tumour gene expression specificity. PEI-Arg polyplex also increased gene expression level in the spleen by about 2-fold compared to the other amino acid-bearing and unmodified PEI polyplexes. Gene expression within the heart and lung was weak following all treatments, reaching a maximum of 3.1 ± 0.5 mU β-galactosidase per heart after treatment with PEI-Leu polyplex and 3 ± 1.1 mU β-galactosidase per lung after treatment with PEI-Arg polyplex.

The biodistribution of gene expression was also qualitatively confirmed by bioluminescence imaging. Co-localization of gene expression in the tumours was clearly visible following treatment with the amino acid-bearing PEI polyplexes. Luciferase expression appeared to be heterogeneously distributed within the tumours, probably concentrated in highly vascularised areas within the tumours. It was however not visible in the tumours after treatment with PEI polyplex and naked DNA and appeared to be very limited in the other organs. This might be explained by the chosen parameters for image acquisition and the short imaging times, which probably allowed only the most intensely luminescent tissues to be analysed. These data demonstrated that the conjugation of Arg, Lys and Leu to PEI increased gene expression within the tumours. This work corresponds to the first evaluation of gene expression of amino acid-bearing non-viral delivery systems in tumours after intravenous administration. Previously, Fujita *et al.* 2008, showed an improved gene expression in tumours following intratumoral injection of tetraarginine-PEG lipid coated protamine / DNA compared with the control delivery system. In our study, Arg-, Lys- and Leu-bearing PEI polyplexes administered intravenously led to an improved gene expression in subcutaneous tumours and should therefore have the potential to deliver and express their carried DNA to remote tumours or metastases unsuitable for intratumoral treatments. Our objective will now be to improve the tumour specificity of gene expression, hopefully leading to an optimized therapeutic effect.

In conclusion, new arginine, lysine and leucine-bearing polyethylenimine polymers have been prepared with the aim of producing effective gene transfection levels *in vitro*. The three amino acid-bearing polymers were able to condense DNA into particles with positive surface charges and average sizes inferior to 400 nm, attributes that have been recognized as being favourable for efficient gene delivery *in vitro*. The conjugation of arginine and lysine to PEI led to an improved transfection compared to the unmodified PEI and DOTAP on both the tested cell lines. The therapeutic efficacy of DNA encoding TNF-α was improved when delivered with PEI-Lys and PEI-Leu by at least 34-times compared to unmodified PEI on the two cancer cell lines tested. Arginine, lysine and leucine-bearing polyethylenimine polymers are thus highly promising gene delivery systems for cancer therapy and will be further investigated.

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