Universidade do Algarve

Departamento de Ciências Biomédicas e Medicina

Synthesis and evaluation of polymer nanoparticles for delivery in RPE cells

Diogo Miguel Barros Bitoque

Dissertação de Mestrado para a obtenção do grau de Mestre em Ciências Biomédicas

Trabalho efectuado sob a orientação de: Prof. Dra. Gabriela Silva

Faro, Setembro de 2013

Synthesis and evaluation of polymer nanoparticles for delivery in RPE cells

Declaração de autoria de trabalho:

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Diogo Miguel Barros Bitoque

Copyright

Index

Acknowledgments

Antes de mais, eu quero agradecer à Professora Doutora Gabriela Silva por me ter dado a oportunidade de realizar este trabalho no seu laboratório, e com o seu grupo. Tenho que agradeçer a sua disponibilidade, atenção e boa disposição.

Quero agradecer a todos os elementos do meu grupo laboratorial: Vanessa Oliveira, Sofia Calado, Susana Machado e Sónia Simão. Obrigado pela paciência e pelos conhecimentos que me transmitiram. Um especial agradecimento à Mónica Fernandes e Marinella Ghezzo.

Um especial agradecimento para a minha melhor amiga, obrigado pela tua ajuda e sem a tua amizade seria tudo muito mais difícil, estarás sempre no meu coração.

Não podia deixar de agradecer à minha família, em especial aos meus pais, que têm sido muito pacientes comigo, agradeço toda a ajuda e formação que me deram para a vida. Adoro-vos.

Obrigado a todos vós!!!

Abbreviations

103.3kDa PDMAEMA – PDMAEMA with 103.3kDa

354kDa PDMAEMA – PDMAEMA with 354kDa

AAV - Adeno-associated virus

AIBN - 2,2'-azobisisobutyronitrile

ATRP - Atom transfer radical polymerization

BRB – Blood retinal barrier

B16F10 - Mouse melanoma cell line

CHEMS - Cholesteryl hemisuccinate

CHO - Chinese hamster ovary cell line

COS-7 - African green monkey kidney cell line

CpG – Cytosine-Guanine dinucleotide

CTA - chain transfer agent

D407- Human Retinal Pigment Epithelial cell line

DEAE-dextran – Diethylaminoethyl-dextran

DLS – Dynamic Light Scattering

DNA - Deoxyribonucleic acid

DMAEMA - (2-(N,N-dimethylamino)ethylmethacrylate)

DMEM - Dulbecco's Modified Eagle Medium

DOPE - Dioleylphosphatidyl ethanolamine

DOPC - Dioleylphosphatidyl choline

DOTMA - N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride

EMA - European Medicines Agency

EMSA - Electrophoretic mobility shift assay

FBS- Fetal Bovine Serum

GFP – Green Fluorescent Protein

GGT- Gamma-glutamyltransferase

GPC - Gel Permeation Chromatography

HEK293 - Human Embryonic Kidney cells

HCl - Hydrochloric acid

MCF-7 - Human breast adenocarcinoma cell line

MIDGE – Minimal Immunogenically Defined Gene Expression

MTT- (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide

M_n - Number-average molecular weight

M_w - Weight-average molecular weight

NIPAM - Poly(*N*-isopropylacrylamide)

NLS – Nuclear Localization Signal

N/P - nitrogen/phosphorus

NPC – Nuclear Pore Complex

^{1H}NMR- Nuclear Magnetic Resonance

OCT - Optimal Cutting Temperature compound

OVCAR-3 - Human ovarian carcinoma cell line

PAMAM – Polyamidoamine

PdI- polydispersity index

PDMAEMA - Poly(2-(N,N-dimethylamino)ethylmethacrylate)

pDNA - plasmid DNA

PEG - Polyethylene glycol

PFA - Paraformaldehyde

pFAR – plasmid Free of Antibiotic Resistance gene

PPI - Polypropylenimine

RPE - Retinal Pigmented Epithelium

PEI – Polyethylenimine

RAFT - Reversible Addition-Fragmentation chain Transfer

rPDMAEMA – reducible PDMAEMA

rpm - Revolutions per minute

S/MAR – Scaffold/Matrix Attachment Region

TAE - Tris-Acetate-EDTA buffer

TEM – Transmission Electron Microscopy

THF - tetrahydrofuran

Abstract

Ocular pathologies are among the most debilitating medical conditions affecting all segments of the population. Traditional treatment options are often ineffective, and gene therapy has the potential to become an alternative approach for the treatment of several pathologies.

Methacrylate polymers have been described as highly biocompatible and are successfully used in medical applications. Due to their cationic nature, these polymers can be used to form polyplexes with DNA for its delivery. This work aims to study the potential of PDMAEMA (poly(2-(N,N'-dimethylamino)ethyl methacrylate)) as a non viral gene delivery system to the retina.

The first part of this work aimed to study the potential for gene delivery of a previously synthesized PDMAEMA polymer of high molecular weight (354kDa). In the second part, we synthesized by RAFT a PDMAEMA with a lower molecular weight (103.3kDa) and similarly, evaluated its ability to act as a gene delivery vehicle.

PDMAEMA/DNA polyplexes were prepared at 5, 7.5, 10, 12.5 and 20 nitrogen/phosphorous (N/P) ratio for the 354kDa PDMAEMA and at 5 and 7.5 for the 103.3kDa PDMAEMA. Dynamic light scattering and zeta potential measurements confirmed the nanosize and positive charge of polyplexes for all ratios and for both polymers. Both high and low Mw PDMAEMA were able to efficiently complex and protect DNA from DNase I degradation. Their cytotoxicity was evaluated using a non-retinal cell line (HEK293) and a retinal pigment epithelium (RPE) cell line (D407). We have found that cytotoxicity of the free polymer is concentration and time dependent, as expected, and negligible for all the concentrations of the PDMAEMA-DNA polyplexes. Furthermore, for the concentrations to be used *in vivo*, the 354kDa PDMAEMA showed no signs of inflammation upon injection in the intravitreal space of C57BL/6 mice.

The transfection efficiency, as evaluated by fluorescence microscopy and flow cytometry, showed that the D407 retinal cells were transfected by polyplexes of both high and low Mw PDMAEMA, but with varied efficiency, which was dependent on the N/P ratio.

Althogether, these results suggest that PDMAEMA is a feasible candidate for non-viral gene delivery to the retina, and this work constitutes the basis of further studies to elucidate the bottleneck in transfection and further optimization of the material.

Resumo

Desde o século passado que as doenças relacionadas com o genoma têm vindo a ganhar importância. O projecto de mapeamento do genoma humano permitiu descobrir a origem de muitas doenças. Várias abordagens terapêuticas têm sido objecto de estudo, sendo a terapia génica uma das mais promissoras. A terapia génica tem como objectivo usar material genético, em regra DNA, para manipular as células dos pacientes. Antes do aparecimento da terapia génica existia apenas tratamento dos sintomas ou terapia de substituição para várias patologias, e não uma verdadeira cura. Para as doenças oculares, que estão entre as doenças mais debilitantes que afectam todos os segmentos da população, esta realidade era sobretudo relevante.

Em terapia génica existe a necessidade de desenvolver veículos para transporte de material genético, veículo este que tem que proteger o ácido nucleico de degradação, ter especificidade para células/órgão, permitir a expressão do gene de interesse ao longo do tempo e com níveis adequados, não desencadear resposta imunitária (excepto em caso de interesse como no cancro e vacinas) e que seja administrado por injecção sistémica. As duas principais estratégias para entrega de material genético usa vectores virais e não-virais. Os sistemas virais de entrega apresentam diversas vantagens, mas também várias limitações: imunogenicidade, baixa capacidade de empacotamento do material genético, potencial inserção aleatória no genoma hospedeiro e toxicidade. Os vectores não virais, de entre os quais se destacam os polímeros, surgiram como uma alternativa para contornar esses problemas, pois estes apresentam maiores benefícios em termos de segurança, versatibilidade química e estrutural para a manipulação das propriedades fisico-químicas, maior capacidade de empacotamento de genes e estabilidade durante o armazenamento.

Dentro da classe dos polímeros, os polímeros naturais como o quitosano e alginato têm ganho importância devido à sua biocompatibilidade, mas são os polímeros sintéticos, como a poli(etilenimina) (PEI), a poli(L-lisina) (PLL) e o poli(2-dimetilamino)etil metacrilato (PDMAEMA), que tem maior relevância

devido ao controlo preciso da sua síntese e consequentemente das suas propriedades. A natureza catiónica de alguns polímeros, como os indicados acima, permite que estes sejam usados para formar poliplexos com material genético.

O objectivo do nosso grupo de investigação é o desenvolvimento de novas estratégias baseadas em terapia génica para tratamento de patologias da retina. Dentro deste enquadramento, o presente trabalho tem como objectivo compreender o potencial do poli(2-dimetilamino)etil metacrilato (PDMAEMA) como sistema de entrega de material genético na retina. Na primeira parte deste trabalho um polímero PDMAEMA com 354kDa, que tinha sido previamente sintetizado, foi avaliado para o seu potencial como veículo para terapia génica. Na segunda parte, sintetizamos um PDMAEMA com um menor peso molecular (103.3kDa) via RAFT (Reversible addition-fragmentation chain-transfer) para efectuar uma comparação entre a eficiência de transfecção de PDMAEMAs com diferentes pesos moleculares.

Poliplexos de PDMAEMA/DNA foram preparados nos rácios amina(N)/fosfato(P) de 5, 7.5, 10, 12.5 e 20 com o PDMAEMA 354kDa e nos rácios 5 e 7.5 com o PDMAEMA 103.3kDa. Medições de DLS (Dynamic light scattering) e potencial zeta confirmam que foram preparados poliplexos à escala nanométrica com carga positiva, em todos os rácios. Ambos os polímeros PDMAEMA são capazes de complexar eficientemente e proteger o DNA da degradação por parte da DNAse I.

A citoxicidade de ambos os polímeros foi avaliada usando duas linhas celulares: a HEK293 (linha celular de rim embriónico humano) e a D407 (linha celular de epitélio pigmentar da retina). Os resultados revelam que a citoxicidade do polimero livre (não-complexado) é dependente da concentração e do tempo de exposição, como esperado, e os poliplexos PDMAEMA/DNA não apresentam citoxicidade. Foi também observado que nas concentrações usadas *in vivo* a citoxicidade do PDMAEMA 354kDa é negligível, como demonstrado pela ausência de inflamação após a injecção no espaço intravítreo de ratinhos C57BL6.

A eficiência de transfecção foi avaliada qualitativamente por microscopia de fluorescência e quantitativamente por citometria de fluxo e mostrou que os poliplexos PDMAEMA/DNA são capazes de transfectar, e que a eficiência varia de acordo com o rácio N/P utilizado, para os polimeros de alto e baixo peso molecular.

Em suma, estes resultados sugerem que o PDMAEMA é um candidato viável para a entrega de genes na retina, e este trabalho constitui a base de estudos futuros que visam elucidar o passo limitante na transfecção e desse modo permitir a optimização deste vector não-viral.

Palavras-Chave: Terapia Génica, Polímero, Poliplexos, Retina, RPE, D407

Part of this work was submitted to the Journal of Tissue Engineering and Regenerative Medicine, "Efficiency of RAFT-synthesized PDMAEMA in gene transfer to the retina", 2013 and was also presented as a poster in the American Society of Gene and Cell Therapy Annual Meeting 2012, "PDMAEMA as a delivery vector for gene therapy of the retina"; in the 8th International Meeting of the Portuguese Society for Stem Cells and Cell Therapies, "Hybrid Strategies For Retinal Gene Therapy"; and in the European Society of Gene and Cell Therapy British Society for Gene Therapy Collaborative Congress 2013, "PDMAEMA is an efficient retinal gene therapy vector".

Introduction

The aim of gene therapy is treat diseases by delivering therapeutic genes to diseased cells or blocks the expression of a dysfunctional gene. This therapeutic strategy has been heralded as the next development in modern medicine [1, 2], since it has the potential to cure genetic diseases and other diseases such as cancer that are responsible for a large number of deaths and decreased quality of life. There are two very important components of gene therapy that are absolutely necessary for a successful gene therapy approach: an efficient and safe delivery system, coupled to an effective gene expression system that can be expressed over time at the target site [1].

In gene therapy, there are two delivery approaches: viral and non-viral. The first one relies on modified virus to deliver the genetic material, since viruses have a natural capability to infect cells and to express their genes in the host. Viral gene delivery can be achieved by modifications in adenoviruses, retroviruses, lentiviruses and adeno-associated viruses (AAVs), among others. These viral vectors can be classified as nonlytic (i.e. virus that produce virions and leave the host cell intact) and lytic (i.e. virus that produce virions and cause death to the host cell) [3]. The modifications made on viruses aim to render them replication-deficient, which do not allow them to replicate and exit the cell as mature viral particles that can further infect other cells. Viral vectors have several advantages, such as sustained expression due to DNA integration into the host genome, some viruses have tropism for specific cell types, and high levels of gene expression [4]. However, there are some drawbacks, including immunogenicity, low capacity of gene packaging, random transgen insertion into the host genome, toxicity and limitations to large-scale production [1, 5]. Despite of these disadvantages, several viral vectors have reached the clinical trial stage (Clinicaltrials.gov NCT01024998, NCT00999609, NCT01461213) and a adenoviral based product – Gendicine® – has been approved in Chine and U.S. In Europe, glybera®, a product based on AAVs, has recently been

approved by EMA (European Medicines Agency) for the treatment of adults with lipoprotein lipase deficiency [6].

Despite the success of viral vectors, the safety issues have motivated the search for safer, less immunogenic and pathogenic gene delivery alternatives, which include polymer and lipid-based vectors, and inorganic materials [1, 2]. Other approaches that are also being studied is the use of a physical mean to deliver the DNA, like electroporation or gene gun [7], which shows several limitations in clinical practice.

The new potential delivery systems bring the promise of safety benefits, structural and chemical versatility for manipulating physicochemical properties, bigger gene capacity and vector stability upon storage.

1. Non-viral vectors for gene therapy

The non-viral approach to the development of a gene delivery system must surpass two main barriers before achieving therapeutic sucess: limited internalization into the cell and the nucleus and short term gene expression.

2. Cellular barriers to non-viral gene delivery

An efficient delivery system must accomplish several steps to be able to express the gene of interest (figure 1): (I) enter the cell by crossing the cell membrane; (II) escape the endo-lysosomal degradation pathway; (III) release the genetic material; (IV) traffic through the cytoplasm and enter the nucleus; and last (V), the gene expression has to occur for synthesis of the protein of interest [1].
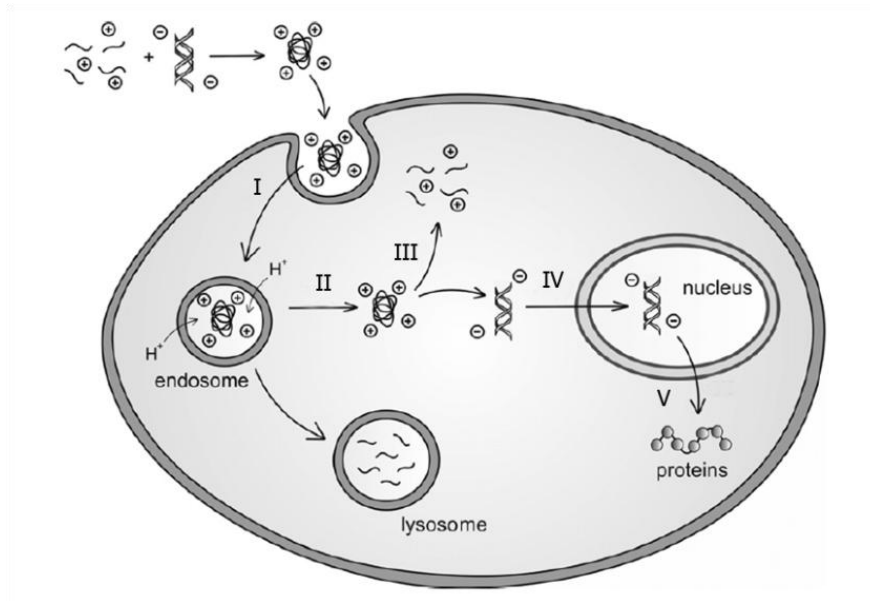
Figure 1 – Representation of the main barriers to gene delivery: (I) enter the cell membrane; (II) escape the endo-lysosomal pathway; (III) the genetic material must be released from the carrier; (IV) traffic through the cytoplasm and into the nucleus; and last (V), the gene expression has to occurred and expression of the protein of interest (Adapted from [1]).

2.1. Crossing of the cell membrane

The cell membrane has a negatively net charge, due to the presence of certain lipids and proteoglycans. Since DNA is a molecule with a negative charge, "naked" DNA cannot entry the cell by electrostatically interaction with the membrane [8]. Therefore, a positively charged system might electrostatically interact with the cellular membrane, and this will permit the entry in the cell by a well described process of endocytosis.

Endocytosis is a process by which cells absorb extracellular molecules by forming invaginations in the cell membrane and the vesicles enter the cytoplasm [9]. This process exists in several forms: macropinocytosis, phagocytosis and receptor- mediated endocytosis.

- Macropinocytodis is the formation of large uncoated vesicles with, approximately 200 nm–5 mm;
- Phagocytosis is normally carried out by specialized cells, such as macrophages and retinal pigment epithelium cells;
- Receptor-mediated endocytosis (also called clathrin-dependent endocytosis) is believed to be the predominant process of polyplex uptake [8].

2.2. Endo- lysosomal escape

The early endosomal escape of non-viral vectors is a critical step towards the delivery of a therapeutic gene. In order to explain this process, the proton sponge hypothesis (figure 2) was proposed for cationic polymers with buffering capacity, like PEI [10], and polyamidoamine (PAMAM) dendrimers [11]. The proton sponge effect states that after endocytosis, the membrane-bound ATPase proton (H^+) pump starts to pump protons into the endosomes which leads to an acidification of these compartments. At this time, polymers with protonable amines will become protonated and resist the acidification. As a consequence, more protons (H^+) will enter the endosome in an attempt to lower the pH. This leads to a passive entry of chloride ions (Cl^-), which increases the ionic concentration and leading to a water influx. The osmotic pressure eventually causes swelling and endosomal rupture, delivering its content to the cytosol [10].

This effect has two main functions: inhibit the activity of lysosomal nucleases and change the osmolarity of acidic vesicles allowing endosomal swelling and burst.
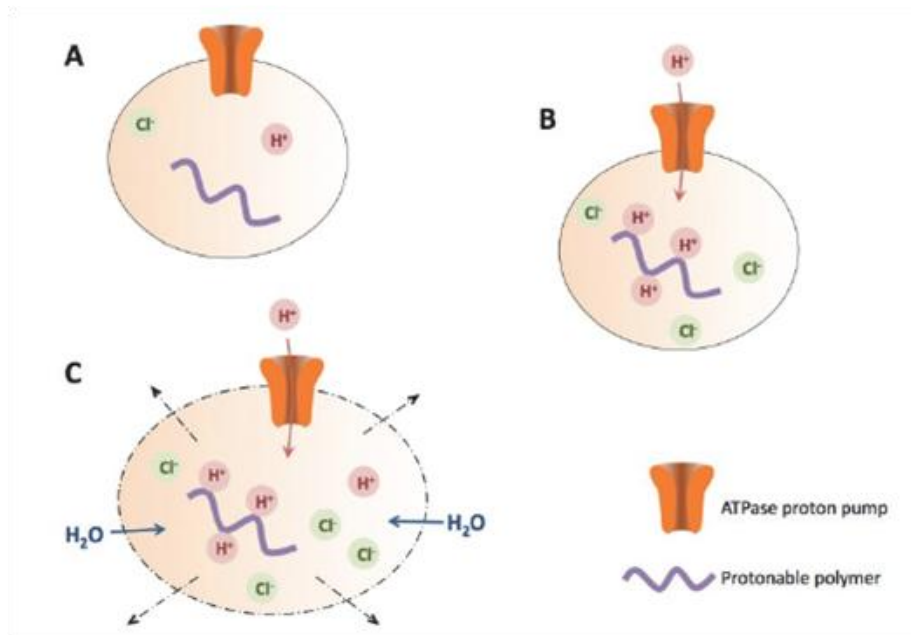
Figure 2 - Proton Sponge Effect: A - Polymer is entrapped inside the endosome; B - ATPase pumps H$^+$ from the cytosol and passive entry of Cl$^-$ ions; C - The increased ionic concentration leads to a influx of water which will lead to swelling and burst of endosomes [12].

2.3. Vector/DNA dissociation

Before gene expression can happen, the genetic content present in the delivery system must be released. Gene expression can be enhanced if the DNA dissociates from the vector insisde the nucleus, preventing the action of nucleolytic enzymes. Several strategies were devised toward this objective. 1) Researchers have demonstrated that thermoresponsive polymers like poly(*N*-isopropylacrylamide) (NIPAM) can enhance transfection efficiency in a temperature-dependent manner [13, 14]. 2) The incorporation of disulfide bonds in polymers can improve the release of genetic material. These bonds can be reduced and cleaved in two thiol groups (-SH) through the action of a very common cytosolic peptide, glutathione [15]. 3) The reduction of the polymer backbone into smaller molecules promotes the release of the genetic content and the cytotoxicity is also reduced [16, 17]. 4) Conjugation of a hydrolytically-sensitive ester bond as cross-linker inside the polymer structure. Since water molecules are the cleaving agents of these bonds, hydrolysis can happen on the first exposure to physiological conditions, thus releasing the DNA. In order

to promote a controlled and sustained release, efforts have been made to accomplish that (e.g. molecular weight and crosslinking density) [18, 19]. However, the requirements for an optimal release have yet to be fully determined [20, 21].

2.4. Cytosolic trafficking and nuclear import

The cytosol is a metabolic and physical hostile environment for nucleic acids, since it is full of nucleolytic enzymes that degradate unprotected DNA. Microtubules, intermediate filaments and microfilaments are part of a dense network that forms the cytoskeleton [22]. Dauty *et al* showed that naked DNA with an extended linear length of approximately 85nm hardly diffuses to the nucleus [23]. This internal degradation barrier is a major obstacle to a free diffusion of polyplexes, with sizes up to 200nm. Another obstruction is the nuclear pore complexes (NPC) which enable the transport of macromolecules greater than 39 nm in diameter into the nuclear space [24].

In order to overcome this problem, researchers tried to use natural endogenous cytosolic factors to promote the nuclear import. One of the most studied factors is nuclear localization signals (NLS). These signals are a distinct short amino sequence present in cytosolic proteins destined to the nucleus, which are identified by import proteins promoting nuclear transport [25].
In addition, NLSs are cationic peptide sequences that can electrostatically interact with DNA or can be attached to a polymer that complex with DNA [26, 27], which constitutes an interesting alternative to increase nuclear import of gene expression systems.

2.5. Gene expression

As stated before, a fundamental characteristic for gene therapy to work is the existence of prolonged gene expression. The two major disadvantages of non-viral systems are the loss of the plasmid during mitosis and gene silencing. Several advances have been made in this field to overcome these disadvantages, mostly in terms of expression systems. Minicircles [28, 29], MIDGE vectors [30, 31], pFARs [32] and optimized plasmids, such as pEPito, are some of the strategies used. The pEPito plasmid has a S/MARs sequence which allows the maintenance of the vector in a transcriptional active state where the plasmid is replicated during cell mitosis [33]. In "traditional" plasmids, the existence of unmethylated CpG motifs that can be methylated leads to a decrease in gene expression, so this plasmid has been optimized to contain a low CpG content [34], thus reducing silencing events.

In recent years, however advances have been made in the field of lipid- and polymer- strategies in an attempt to overcome these barriers.

3. Lipossome-based vectors

Liposomes are vesicular structures constituted by the assembly of cationic lipids with a hydrophilic tail and a positive hydrophobic head group, with similar structure to the cellular membrane [35]. Liposomes are also composed by neutral helper lipids such as dioleylphosphatidyl ethanolamine (DOPE) and dioleylphosphatidyl choline (DOPC) [36]. Due to the positive nature of liposomes, DNA can be complexed and constitute lipoplexes (cationic lipid/DNA complexes, figure 3). One of the earlier lipid-delivery system was based in a synthetic cationic lipid, N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA). Felgner *et al* showed that DOTMA can be more effective than calcium phosphate or DEAE-dextran in DNA delivery [37]. Since Felgner *et al* study, significant progress has been made in developing the perfect lipid-

based system. New lipids are more efficient than the earlier cationic lipids in gene delivery [2], and lipoplexes have been studied in clinical trials for the treatment of cancer [38] (Clinicaltrials.gov NCT00059605) and cystic fibrosis [39] (Clinicaltrials.gov NCT00004471).
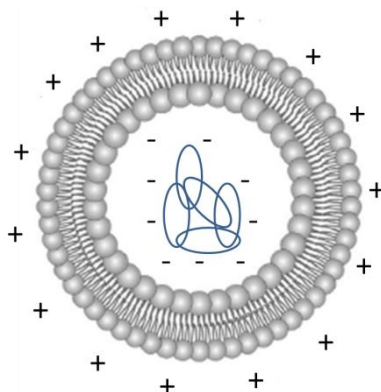
Figure 3 - Schematic of cationic lipids (grey)/DNA (blue) complexes, adapted from [35].

4. Polymers

Polymers are long chain structures composed by identical, several different monomers or two different monomers, so called homopolymers, heteropolymers and co-polymers, respectively. Cationic polymers have gained increasing importance through the years due to their capacity to form polyelectrolyte complexes with plasmid DNA due to the presence of primary, secondary, tertiary and quaternary amines in the polymer structure which interact electrostatically with the phosphates in the pDNA backbone (Figure 4).

Polymers can be sorted in to two main categories: natural, such as chitosan, alginate and dextran; and synthetic, such as polyethylenimine (PEI), poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) and dendrimers, such as polyamidoamine (PAMAM) and polypropylenimine (PPI) [1, 3, 40].
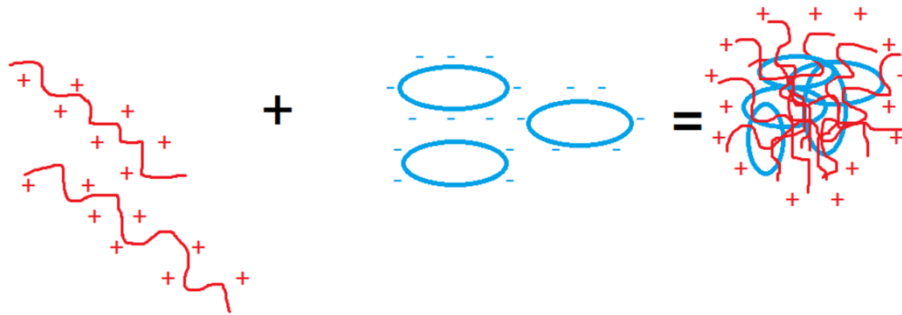
Figure 4 – Representative schematic of polyplex formation, electrostatical interaction between a cationic polymer (red) and a plasmid DNA (blue).

In an attempt to develop an efficient vector, Lehtinen *et al* used the polymer polyethylenimine (PEI) to complex DNA and then coated the complex with two lipids: DOPE and cholesteryl hemisuccinate (CHEMS). This system showed the ability to resist to extracellular polyanions, but revealed to be a poor transfectant compared with PEI, mainly due to a low cell uptake [41]. Another study developed a new system composed by PEI, DOTAP and cholesterol that show higher transfection efficiency than conventional lipid- and polymer- systems [42]. These studies showed that low doses of lipoplexes are safe when delivered locally. However, the low gene transfer efficiency *in vivo* has led the research into a more fundamental study: the molecular and cellular barriers to gene therapy, and the biological interaction between delivery systems and the host.

4.1 Chitosan

Chitosan is a natural carbohydrate polymer, derived from the deacetylation of chitin, formed by D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) β-(1-4)-linked (figure 5) [43, 44].

Figure 5- Chemical structure of chitosan, D-glucosamine and N-acetyl-D-glucosamine β--(1-4)-linked [44].

In the past few years, chitosan has been extensively studied for of gene delivery, due to its properties: biodegradability, biocompability and cationic nature [45, 46]. These characteristics make chitosan a very attractive polymer for gene therapy. Initial reports considered chitosan inefficient as a system deliver vector [35]. To overcome the low transfecti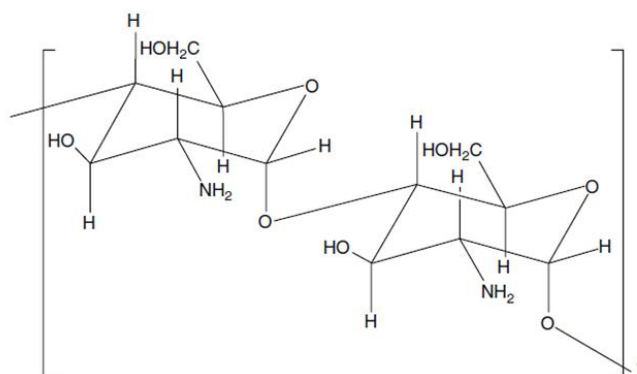on efficiency modifications in the molecular weight were studied. Huang *et al* demonstrated that the transfection efficiency was dependent on the chitosan molecular weight *i.e*, higher M_w yielded higher transfection efficiency (213 kDa > 98 kDa > 48 kDa > 17 kDa) [47]. Chemical modifications in order to increase the buffering capacity involved N-quartenization of chitosan terminal amines with the aim of increasing charge density, which resulted in improved transfection efficiency but with higher cytotoxicity [48, 49]. The conjugation with PEI is also a strategy to enhance the buffering capacity of chitosan. Zhao *et al* determined that chitosan/PEI/DNA complexes were capable of increased gene expression in HeLa cells compared with only chitosan. Additionally, the cytotoxicity of PEI was also reduced by the combination with chitosan [50, 51].

Other strategies used include coating chitosan with other polymers, such as polyethylene glycol (PEG), which showed promise due to efficient transfection of and low cytotoxicity in MCF-7 cells (human breast adenocarcinoma cell line) [52]. Chitosan/DNA complexes coated with poly(gamma-glutamic acid) can significantly enhance their cellular uptake via γ-glutamyl transpeptidase (GGT) present on cell membranes [53]. To achieve targeted delivery, chitosan was

conjugated with several cell-targeting ligands: for tumor cell-targeting, chitosan complexes were combined with folate [54], for hepatic cell-targeting, the sugars: galactose [55] and lactose [56] were used.

Over the years chitosan has been tested in many animal models of genetic diseases. Several studies showed that chitosan-based systems transfect the lungs [57], the cornea [58] and other tissues. All studies discussed here show that chitosan has a great potential to constitute a non-viral gene delivery alternative.

4.2 Polyethylenimine (PEI)

Polyethylenimine is considered by many as the gold standard of gene delivery since it, is a great example of cationic polymers capable of transfecting cells. Behr *et al* led the first successful PEI-mediated gene transfer in 1995 [10]. Since then PEI has been extensively modified in order to improve the physicochemical and biological properties of polyplexes. There are two main structures for PEI: linear and branched (figure 6). The degree of branching was found to have a great influence in the complexation and stability of polyplexes. For similar molecular weights, branched-PEI is more effective at condensing DNA than linear-PEI. This happens due to the fact that branched-PEI contains a higher charge density than linear PEI [59]. The effect of the molecular weight of PEI in the ability to transfect cells was studied by Godbey *et al*, who observed that PEI with higher molecular weights has higher transfection efficiency than PEI with lower molecular weights [60]. However, it was also determined that high molecular weight polymers have high cytotoxicity [61]. Aggregation and adhesion to the cell membrane is the cause for the cytotoxic effects of PEI [62]. For polyplex formation, the optimum molecular weight is between 5 and 25 kDa [63].
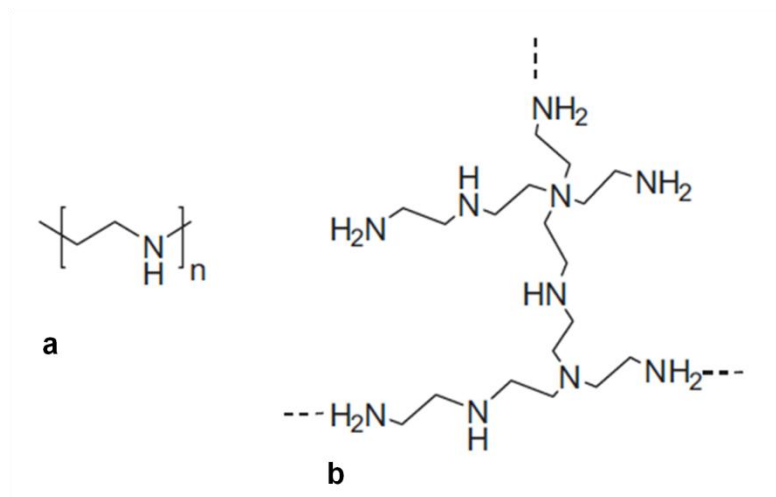
Figure 6- Schematic representation of PEI structure: linear (a) and branched (b). Adapted from [1].

An effort to improve the transfection efficiency and safety of the polymer has been made using an extensive set of chemical modifications. The most studied modification is probably PEGylation, which creates a hydrophilic barrier that reduces interactions between polyplexes and plasma proteins and erythrocytes. The transfection efficiency in vitro is influenced by the density and length of PEG chains conjugated to PEI: higher density with a shorter PEG is more effective in transfecting DNA [64, 65]. *In vivo* studies revealed that PEI-g-PEG polymers had reduced cytotoxicity and increased circulation time, but even with high doses of DNA no gene expression was detected [66].

In order to obtain a new type of polymers that is less toxic and more efficient in vivo, further studies on the relationship between structure and function of the polymer are necessary.

4.3 Poly(2-dimethylaminoethyl methacrylate) (PDMAEMA)

Poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) was first studied in 1996 by Hennink *et al,* which showed that this polymer had potential as a gene delivery system [67]. This polymer is very versatile, with defined molecular weights, well-defined chain ends, and in different macromolecular architectures (such as block, star, and graft chain structures) can be made using polymerization techniques like atom transfer radical polymerization (ATRP) [68] and reversible addition fragmentation transfer (RAFT) [69]. Initial reports on PDMAEMA (figure 7) showed high transfection efficiency with acceptable cytotoxic effects in COS-7 (an african green monkey kidney cell line) and OVCAR-3 (a human ovarian cancer cell line) [70]. Van de Wetering demonstrated that PDMAEMA is an efficient transfectant *in vitro* and *ex vivo* [71]. When PDMAEMA polyplexes were injected intraperitoneally in mice, no transfected cells were detected. The authors of this study hyphotesized that the negatively charge components of the ascites fluid presence in the peritoneal cavity hindered the *in vivo* transfection [71]. When OVCAR-3 cells were trasnfected *in vitro* in the presence of ascites fluid, the transfection efficiency decreased significantly [71].
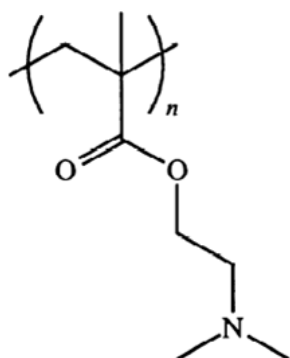

Figure 7- Schematic representation of PDMAEMA monomer [70].

For PDMAEMA, like most polymers, the transfection efficiency and toxicity are directly correlated to the molecular weight. High molecular weight PDMAEMA has higher transfection efficiency than low molecular weight. Subsequently, higher cytotoxicity was also observed for higher molecular PDMAEMA [70, 72, 73]. Therefore, an optimum molecular weight showing acceptable efficiency and toxicity is required.

To further improve the transfection efficiency of PDMAEMA, several modifications to his structure have been studied. Incorporation of an additional tertiary amino group in each monomer in order to promote the "proton sponge effect" have been tried by Hennink *et al* [74] and the results did not show a positive effect.

In order to reduce cytotoxic effects, PDMAEMA was grafted to poly(ethylene glycol) (PEG) and results showed that cytotoxicity decreased in 293T cells [75]. Another investigated strategy was the synthesis of a reducible PDMAEMA (rPDMAEMA), in which disulfide bonds were incorporated, so that this polymer can be degraded in the reducing intracellular environment. The results in B16F10 cells (a mouse melanoma cell line) showed that rPDMAEMA was less cytotoxic than PDMAEMA, and was also a more efficient as a delivery vector than the non-reducible polymer [76].

The mechanism by which PDMAEMA polyplexes enter the cell has been suggested by van der Aa *et al*. This author proposes that both clathrin- and caveolae- dependent pathways are involved in the cellular uptake of this polyplexes, with the caveolae dependent pathway appearing to be essential for effective gene delivery by PDMAEMA [77].

5. Polymer synthesis: Reversible Addition-Fragmentation chain Transfer (RAFT)

Polymer synthesis is essential for the development of new polymers for biomedical applications, in particular gene therapy. One of the most commonly used polymerization techniques is reversible addition-fragmentation chain transfer. RAFT was first described by Chiefari *et al*, and it is a relatively, new controlled/"living", free radical polymerization technique [78].
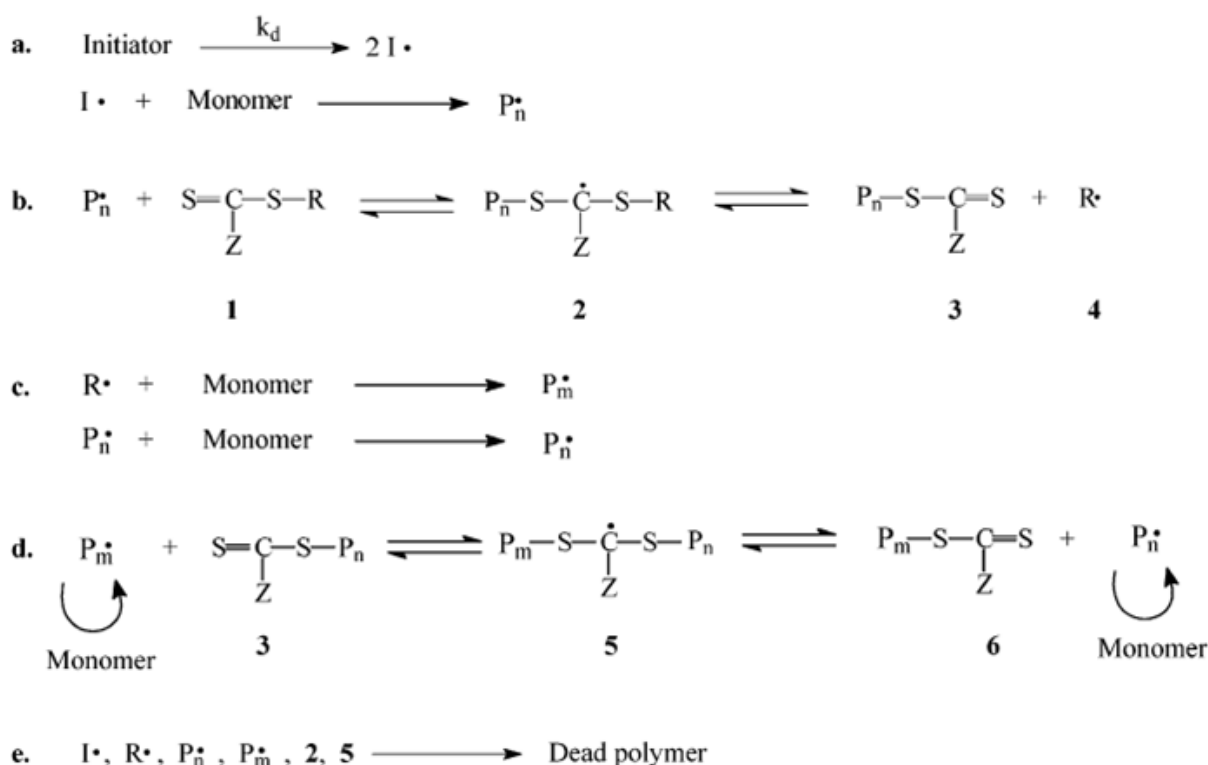
Figure 8- Proposed RAFT polymerization Mechanism as proposed by [79]. Briefly, initiation and propagation (**a**); RAFT pre-equilibrium (**b**); re-initiation (**c**); main RAFT equilibrium (**d**) and termination (**e**).

The reaction can be initiated by thermal, redox or γ-irradiation methods. The mechanism is composed of several steps: 1) **initiation**, where the reaction is started by a free-radical source such as AIBN (azobisibutyronitrile). The initiator decomposes to form two fragments (I•) which react with a single monomer molecule in order to propagate a polymeric radical (P_n•) (figure 8a); 2) **propagation**, where propagating radical chains of length n in radical form (P_n•), add to monomer (M) in order to form longer propagating radicals (P_{n+1}•) (figure 8a); 3) **RAFT pre-equilibrium**, where a polymeric radical with n monomer units

(P_n) reacts with the RAFT agent to form a RAFT adduct radical. This may undergo a fragmentation reaction in either direction to yield either the starting species or a radical (R•) and a polymeric RAFT agent. This is a reversible step in which the intermediate RAFT adduct radical is capable of losing either the R group (R•) or the polymeric species (P_n•) (figure 8b); 4) **re-initiation**, where the remaining radical group (R•) reacts with other monomer species, which starts another active chain (figure 8c); 5) **main RAFT equilibrium**, which is the most important step in RAFT, is a process of rapid interchange where the radicals present are shared among all species that have not yet undergone termination. The ideal situation is when the radicals are shared equally, causing chains to have equal opportunities for growth and therefore a narrow polydispersity index (PdI); and finally , 6) **termination**, where chains in their radical form react via a process known as bi-radical termination to form chains that cannot react further (P_n•+ P_m•), know as dead polymer [78, 79].

6. Therapeutic target

The objective of this work is to evaluate the synthetic polymer poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) as non-viral delivery system for retinal gene therapy. The eye is a very attractive organ for gene therapy due to its small size and low diffusion of agents into systemic circulation due to the blood-retinal-barrier (BRB). Therefore, to obtain a significant therapeutic effect, only a small amount of drug is required. The location of the eye allows an easy access for the administration of drugs, which obviates the need for systemic delivery [4, 80].

The retina is the sensory tissue of the eye, and it is constituted mainly by three cell types: the retinal pigment epithelium (RPE), several types of nerve cells and photoreceptors (figure 4). Several genetic diseases cause retinal degeneration by affecting these different cell types, and therefore gene therapy is a potentially efficient therapeutic strategy. In our lab, we aim to develop non-viral gene therapy vectors for retinal pathologies. Within this framework, the present study has the following aim.

7. Aim of the work

The objective of this is work to evaluate the synthetic polymer PDMAEMA as non-viral delivery system for retinal gene therapy.

The first part of the work was testing a previously synthetized linear PDMAEMA with M_w of 354 kDa). We prepared PDMAEMA-DNA polyplexes in a broad range of N/P ratios (5/7.5/10/12.5/20) and characterized these in terms of size, polydispersity (PdI) and surface charge (zeta potencial), and the ability to encapsulate and protect the pDNA. The stability of the polyplexes was also evaluated over time in storage (4ºC) and physiological (37ºC) conditions.

Next, we evaluated the polymer and polyplexes cytotoxicity in two different cell lines: a human embryonic kidney cell line (HEK293) and a human retinal pigment epithelial cell line (D407). In order to evaluate the transfection efficiency, both cell lines were transfected with polyplexes containing a plasmid which codified for the green fluorescent protein (GFP). The efficiency was assessed qualitatively by fluorescence microscopy and quantitatively by flow cytometry. Since, biocompability is a necessary requisite for an *in vivo* application, we evaluated the activation of the microglia by injecting PDMAEMA polyplexes in the eye of C57BL/6 mice.

Based on the previous results, the aim of the second part of this work was to synthetize a new linear polymer with an expected size of 200 kDa in order to determine if the transfection efficiency could be modulated by variation in the molecular weight of the polymer. The polymer was characterized by GPC, and polyplexes in same N/P ratios as above produced. We then selected the two best ratios, in terms of size, PdI and surface charge to continue further into the characterization. The ability to encapsulate, protect the pDNA and stability in the presence of serum proteins was assessed. Due to time constraints for the 200kDa polymer we only used D407 cells, to evaluate the polymer and polyplexes cytotoxicity, but in a larger range of concentrations than before. This study enabled a comparison between both M_w and the selection of the best two candidate condition for enhanced gene transfection.

Methods and Materials

1. Synthesis of PDMAEMA by RAFT

The polymer was synthesized by RAFT polymerization. Prior to the synthesis, DMAEMA was passed through a neutral alumina column (BDH) to remove the free radical inhibitor. DMAEMA (1 mL, $5,93 \times 10^{-3}$ mol), 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (4,65 $\times 10^{-6}$ mol, 279,38 g/mol Sigma-Aldrich) and 2,2'-azobisisobutyronitrile (AIBN) (1 mol % of the monomer, 0,0097 g) were added to tetrahydrofuran (THF, Panreac) (approximately 5 mL) in a Shleck flask. Previously to the polymerization, the reaction mixture was submitted to three freeze-pump-thaw cycles. The flask was then submerged in an oil bath preheated to 60 ºC and the polymerization was allowed to occur for 24 h under magnetic stirring. The resulting polymer was precipitated by pouring the mixture into hexane and centrifuged at 10 000 rpm for 10 min. Afterwards, the supernatant was discarded and the pellet was resuspend in THF, this process was repeated two more times for the pellet. The polymer was then dried under vacuum at 40 ºC for 72 h. To the neutral PDMAEMA product obtained 12 M HCl were added under stirring until the polymer completely dissolved, due to its conversion into a hydrochloride salt. The PDMAEMA•HCl polyelectrolyte was then precipitated with acetone, the mixture was cooled at -20 ºC for 10 min and then centrifuged at 10 000 rpm for 10 min. The white powder constituting the pellet was recovered and dried under vacuum at 60 ºC overnight.

The exact amount of polymer was dissolved in water to reach the final concentration of 1 mg/ml. The polymer was then stored at 4ºC.

2. Gel permeation chromatography

A triple detection GPC (SEC[3]) analysis was performed in a modular system constituted by a degasser, HPLC pump (K-1001) and RI detector (K-2300) are from Knauer; viscometer and RALLS from Viscotek (Trisec Dual Detector Model 270), using two PL aquagel-OH mixed 8 µm 300 x 7.5 mm columns and using two PL aquagel-OH mixed columns. Eluent was 0,5 M NaNO3 in pH 2 phosphate buffer (containing 0.1% sodium azide) at 1mL/min. The sample was dissolved at 10 mg/mL in 10^{-2} M HCl solution.

3. Preparation of PDMAEMA/pDNA polyplexes

In the first part of the work we used the plasmid pAAV2.1 CMV eGFP3 (5504bp) and for second part we used the plasmid pEPito-hCMV-eGFP (5245bp), both encoding an enhanced green fluorescent protein (GFP) to prepare the polyplexes with the 300 and 200 kDa PDMAEMA, respectively (figure 4). The pAAV-plasmid was amplified in *Escherichia coli* TOP10 competent cells and the pEPito-plasmid was amplified in *Escherichia coli* GT115 competent cells (Invivogen) in LB medium to sufficient quantities by using standard molecular biology techniques, including harvesting and purification via Quiagen's Maxi-Prep kit. pDNA concentration and quality were determined by A260/280 ratio (NanoDrop 2000, Thermo Scientific) and by agarose gel electrophoresis.
Despite the different plasmid backbone, the sizes are very similar and the reporter gene very similar.

The polyplexes were prepared in water in N/P (nitrogen/phosphorus) ratios of 5/1; 7.5/1; 10/1; 12.5/1; 20/1 for the 354 kDa polymer and in the ratios 5/1; 7.5/1; 10/1; 12.5/1; 20/1 for the new polymer (M_w 103.3kDa). To achieve the indicated N/P ratios, calculations were done according to equation below, where m_p is the mass of polymer, m_D is the mass of DNA, $M_{o,D}$ is the average repeat unit molecular weight of DNA, and $M_{o,p}$ is the repeat unit molecular weight of the

polymer. This equation assumes there is only one ionizable nitrogen group per repeat unit of the polymer.

$$\frac{N}{P} = \frac{m_{\mathrm{p}} M_{\mathrm{o,D}}}{2 m_{\mathrm{D}} M_{\mathrm{o,P}}}$$

4. Determination of size and surface charge of polyplexes

The determination of diameters of the polyplexes was perfomed by dynamic light scattering (DLS) using a Malvern Zetasizer Nanoseries instrument (Malvern Instruments, Malvern). Dynamic light scattering is also known as Photon Correlation Spectroscopy. This technique is one of the most popular methods used to determine the size of particles. Focusing a monochromatic light beam, such as a laser, a solution with spherical particles with Brownian motion causes a doppler shift when the light hits the moving particle, changing the wavelength of the incoming light. This change is related to the size of the particle. The Brownian motion is the random movement of the particles in a solution, where the smaller particles move faster than bigger particles [81, 82]. Scattered light was detected at 173° angle and a temperature of $25^{\circ}C$. The surface charge of the polyplexes was estimated by zeta potential measurements using laser Doppler velocimetry and phase analysis light scattering (M3-PALS) technology.

5. Morphologic analysis of PDMAEMA polyplexes by transmission electron microscopy

Samples were deposited on copper grids coated with Formvar® films, stained with 2% (w/v) phosphotungstic acid and washed with water. The analysis was performed using a transmission electron microscopy (TEM) (JEOL JEM-1011 electron microscope, Tokyo, Japan).

6. Stability assay over time

The polyplexes (354kDa PDMAEMA) in 5/1; 10/1; 20/1 N/P ratios were prepared in triplicate for each temperature condition (4ºC and 37ºC), as described before. The samples were measured in the day of preparation and every two weeks to determine variations in size, polydispersity index (PdI) and surface charge, as described in the previously section.

7. Electrophoretic mobility shift assay (EMSA)

DNA complexation with the 354kDa PDMAEMA and the newly synthesized PDMAEMA was confirmed by gel electrophoresis. PDMAEMA/pDNA complexes were prepared at N/P ratios 5/1, 7.5/1, 10/1, 12.5/1 and 20/1, as described in section 3. As control, 0.4 µg of DNA were mixed with Green Safe Direct Load® and loaded onto a 1% agarose (Ultrapure agarose, Invitrogen) gel in Tris-acetate-EDTA (TAE) buffer (pH 8.0). The gel was electrophoresed at 60V for 90 minutes in 1x TAE buffer and then imaged in a transiluminator (Alpha Innotech).

8. DNAse protection assay

PDMAEMA polyplexes were incubated with DNAse I to assess the ability of the polyplexes to protect the DNA from enzymatic degradation. The polyplexes were prepared as described above, to digest the DNA, 10x reaction buffer (200mM Tris-HCl, pH 8.3, 20 mM $MgCl_2$, Sigma-Aldrich) was added and then we used a proportion of 1U DNAse I (1unit/µl in 50% glycerol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, Sigma-Aldrich) per 1µg of plasmid. This mixture was incubated for 5 min at 37ºC for the PDMAEMA 354kDa and 15 min

at 37ºC for the new PDMAEMA, and after was stop with the addition of 50 mM EDTA (Sigma-Aldrich). The samples were mixed with Green Safe Direct Load® and loaded onto a 1% agarose (Ultrapure agarose, Invitrogen) gel in Tris-acetate-EDTA (TAE) buffer (pH 8.0).

The gels were run at 60V for 90 minutes in TAE buffer and imaged in a transiluminator (Alpha Innotech).

9. Stability assay in the presence of serum proteins

To assess the stability of the polyplexes in the presence serum, the polyplexes were incubated at 37ºC and 150 rpm in DMEM w/ 5% FBS in a ratio of 1/4 during 24h, 48h and 72h. To assess the stability, an electrophoretic mobility shift assay perfomed as described in section 7.

10. Cell lines and culture conditions

The HEK293 and D407 cell lines used in the *in vitro* experiments were maintained in DMEM (Sigma-Aldrich) cell culture medium supplemented with 10% fetal bovine serum (FBS, PAA), 1% Penicilyn/Streptomicyn (Sigma-Aldrich) and 1% Glutamine (Sigma-Aldrich), and 5% fetal bovine serum (FBS, Sigma), 1% Penicilyn/Streptomicyn (Sigma-Aldrich) and 1% Glutamine (Sigma-Aldrich), respectively. Cells were maintained at 37ºC in a humidified 5% CO_2 atmosphere.

11. Cell viability assay for cytotoxicity evaluation

The cytotoxicity of the polymer and polyplexes was evaluated up to 72 hours, with controls as follows: cells in standard cell culture conditions and cells treated with a latex solution, as positive (blank) and negative controls, respectively. Cell viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide, Sigma-Aldrich) assay as described by [83].

For these experiments, HEK293 and D407 cells were seeded at a density of $2x10^4$ cells/well (48-well plate). After 24h, cells were incubated with increasing concentrations of the free polymer (5, 7.5, 10, 12.5, 15 and 20 µg/mL). For the new polymer, D407 cells were incubated in a larger range of polymer concentrations (1, 3, 5, 7.5, 10, 12.5, 15, 20, 50, 100 µg/mL). To prepare each of the polymer solutions, 1 mg/ml polymer stock solution was diluted in cell culture medium and added to each well.

For the polyplexes formed with either the 354kDa PDMAEMA and the new PDMAEMA, we have tested three concentrations for each polymer:DNA ratio. In the cytotoxicity evaluation we have chosen the final concentration of 0,1 µg, 0,2 µg and 0,5 µg of DNA per $2x10^4$ cells to reflect future transfection conditions. The polyplexes were diluted in cell culture medium and added to each well.

At each time point (24, 48 and 72 hours), 25 µL MTT solution (5mg/mL in PBS) were added to each well and incubated for 4 hours. The solution was removed and replaced with 250µL of isopropanol-HCl (0.04N) to dissolve the blue formazan crystals produced and left to incubate further for 1 hour at 37ºC, 5% CO_2. Absorbance at 570nm and 630nm were measured for each well using an infiniteM200 (TECAN) microplate reader. The relative cell viability (%) was calculated by $[abs]_{sample}/[abs]_{control}$x100 [84], with cells from the positive control representing 100% viability.

12. Transfection efficiency assay

For the determination of the gene transfer efficiency of 354kDa PDMAEMA, cells were plated at $1x10^5$ cells/well in clear 6-well tissue culture plates. FuGENE HD® (Promega, USA) was used as the transfection control and according to the manufacturer instructions. PDMAEMA-DNA polyplexes were added to the cells at a ratio of 1μg of pDNA per $1x10^5$ cells and further incubated for 72 h. Cells cultured in standard culture conditions were used as a non transfection control.

Transfection efficiency was evaluated qualitatively by fluorescence microscopy and quantitatively by flow cytometry. Imaging was performed in an inverted fluorescence microscope (LEICA DMIL-LEICA DC500). Green fluorescent protein (GFP) positive cells were scored by flow cytometry (FACSCalibur, BD Biosciences, USA) using the FL-1H green channel. A total of $1x10^4$ events were counted for each sample and the percentage of positive events corresponds to the gated events minus the negative control, for transfection (non-transfected cells).

For the determination of the gene transfer efficiency of the newly synthetized PDMAEMA, cells were plated at $1,5x10^5$ cells/well in clear 6-well tissue culture plates. Cells were plated in higher number than before to ensure that we had enough cells to acquire a bigger number of events in the flow cytometry. FuGENE HD® (Promega, USA) was used as the transfection control and according to the manufacturer instructions. PDMAEMA: DNA polyplexes were added to the cells at a ratio of 1μg of pDNA per $1x10^5$ cells in two conditions: directly added (non-diluted) and diluted in 100 μL of medium without FBS. After 2 min complete medium was added and further incubated for 72 h. Cells cultured in standard culture conditions were used as a non transfection control.

Transfection efficiency was evaluated qualitatively by fluorescence microscopy and quantitatively by flow cytometry. Imaging was performed in an inverted fluorescence microscope (Axiovert 40 CFL, Zeiss). Green fluorescent protein (GFP) positive cells were scored by flow cytometry (FACScan, BD Biosciences, USA) using the FL-1H green channel. A total of $1x10^5$ events were counted for

each sample and the percentage of positive events corresponds to the gated events minus the negative control.

13. Animals

C57BL/6 mice (3 months old) were used as experimental animals, and housed in controlled temperature and 12 hour light/dark cycle with food and water *ad libitum*. All experimental procedures were carried out according to the Portuguese and European Union regulations (FELASA) for the use of animals and the Association for Research in Vision and Ophthalmology (ARVO) for the use of animals in ophthalmic and vision research. All procedures were performed under anesthesia with 2,2,2 tribromoethanol (Sigma-Aldrich) administered by intraperitoneal injection (250 mg/Kg dose). Animals were humanely sacrificed by anesthesia and death was confirmed by cervical dislocation.

14. Activation of microglia after intravitreous injection in C57/BL6 mice

In order to evaluate the compability of the PDMAEMA:DNA polyplexes *in vivo*, 3 month old C57BL/6 mice were injected with polyplexes with a N/P ratio of 12.5 by intravitreous injection in the right eye, under a stereomicroscope (Nikon Stereoscopic Microscope). The left eye was use as control. A injection procedure without administration of any vehicle was also performed (sham injection). The mice were sacrificed 14 days post-injection, the eyes enucleated and fixed in PFA 4%(Sigma-Aldrich), immersed in sucrose 30% (Sigma-Aldrich), and after 24h were included in Optimal Cutting Temperature (OCT) compound (VWR). To eliminate the autofluorescence of the retina, sections were first washed with PBS 0.1% TritonX for 5 minutes, incubated in 0.25%

KMnO$_4$ in PBS for 15 minutes, rinsed with PBS twice, and then incubated in 0.1% oxalic acid in PBS for 20 minutes at room temperature. Treated sections were washed in PBS 0.1% TritonX three times. An immunofluorescence assay was performed, using an antibody against Iba-1, a marker of microglia activation. The primary antibody (Iba-1, Wako Pure Chemical Industries) was incubated overnight at 4ºC at a dilution of 1/1000. A secondary antibody, Alexa Fluor 594 (1/2000, Invitrogen), was used for detection of the signal. The secondary antibody was incubated for 1h at room temperature, and the samples were visualized in an Axio Observer Z2 Fluorescence microscope (Zeiss).

15. Statistical analysis

Statistical analysis was performed between the control and the tested conditions by a one-way ANOVA followed by a post hoc Dunnett's Multiple Comparison Test for the cell viability and transfection assays, between the tested conditions an unpaired t-test was performed. For the polyplexes, analyses between different ratios were determined with one-way ANOVA followed by a post hoc Benferroni's Multiple Comparison Test.

Results and Discussion

This section is divided in two parts: in part one, we have characterized a previously synthesized PDMAEMA with 354kDa and in part two, we describe the synthesis and characterization of a smaller M_w PDMAEMA (that was found to have 103.3kDa).

Part one:

1. Characterization of 354kDa PDMAEMA-DNA polyplexes

1.1 PDMAEMA-DNA polyplexes have characteristics compatible with gene therapy

Characterization of the polyplexes included the determination of size, polydispersity and zeta potential, as shown in table I.

Ideally, polyplexes should present a size below 500 nm, an optimum polydispersity of 0.1, and a positive surface charge, which constitute favorable requisites for cellular uptake.

Table I: Characterization of polyplexes by size, PdI and zeta potencial

Formulation	Size (nm)	Polydispersity Index (PdI)	Surface Charge (+mV)
PDMAEMA 5	129.96±4.28	0.310±0.062	42.32±6.25
PDMAEMA 7.5	123.0±15.36	0.301±0.090	37.26±9.06
PDMAEMA 10	141.58±28.2	0.298±0.055	44.82±7.17
PDMAEMA 12.5	164.4±49.83	0.290±0.048	25.74±5.79
PDMAEMA 20	266.52±32.64	0.383±0.071	24.06±4.83

*There are only significant size differences between the ratio 20/1 and the other ratios

*The ratios of 5/1, 7.5/1 and 10/1 are significantly different from the ratios 12.5/1 and 20/1 in terms of zeta potential

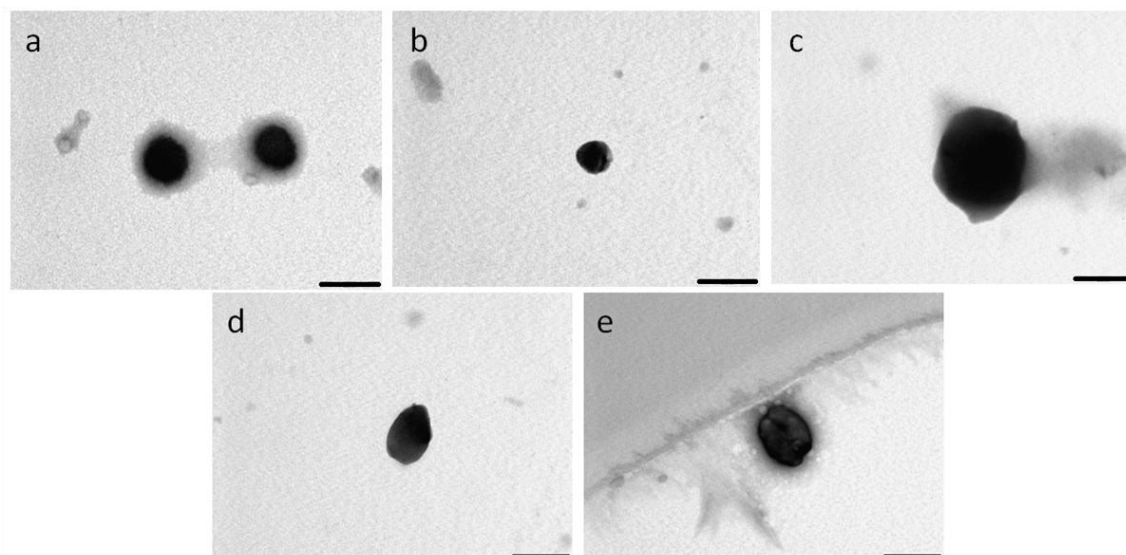*There is no significant difference in the polydispersity index

Figure 9- A analysis by transmission electron microscopy of PDMAEMA:DNA polyplexes at different ratios: 5**(a)**, 7.5**(b)**, 10**(c)**, 12.5**(d)** and 20**(e)**. Scale bar represents 200nm.

In table I it is possible to observe that the size of PDMAEMA/pDNA polyplexes increases with the increase in N/P ratio from 5 to 20, but all ratios have diameters smaller than 300 nm. This trend correlates well with the morphological analysis performed by transmission electron microscopy (TEM) (figure 9). The polydispersity index, which evaluates the homogeneity, is below 0.4, indicating a homogeneous population of particles, which is desirable for consistency of results regarding cellular penetration. As expected, the polyplexes have positive charge since there is an excess of polymer to DNA.

However, whilst there is a clear trend of increase in size with increasing N/P ratio, zeta potential values present quite a random variation. In the literature, two opposite trends in size with N/P ratio, depending on M_w, were observed for PDMAEMA. For a polymer with 915 kDa, a behavior similar to that presented by

our polymer was observed, with a random but not very significant variation in polyplex diameter for small N/P ratios, and a significant increase when going from N/P = 16 to N/P = 20 [73]. PDMAEMA with 43 kDa referred in that study presented a completely different behavior from one of comparable M_w (39 kDa) described in a different study [85]. The former showed a size reduction with the increase of the N/P ratio, and the latter a random variation in size and a decrease in zeta potential.

It seems, therefore, that size and surface charge are very sensitive to polymer molecular weight and, probably, to polyplex preparation conditions, rendering it difficult to establish a correlation between those parameters and the N/P ratio. In fact, there are indications that the polymer molecular weight may affect transfection more than the size of polyplexes [73].

1.2 PDMAEMA efficiently complexes pDNA

The electrophoretic mobility shift assay is based on the behavior of pDNA in an agarose gel, which migrates from the negative to the positive end. Upon complexation with a cationic polymer, the DNA is retained in the well, unable to migrate through the gel. As observed in figure 10, for all N/P ratios the PDMAEMA polyplexes were able to complex and retain the DNA, and this is evidenced by the absence of DNA bands in the gel lanes, with all DNA retained in the wells. As expected, free DNA (lane 2) migrates towards the positive pole and PDMAEMA polymer (lane 3) shows no signal in the gel.
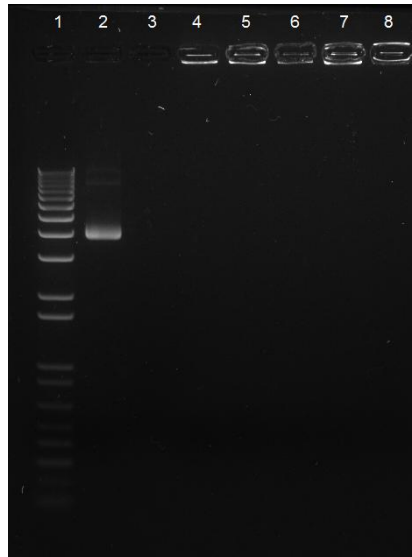
Figure 10- Electrophoretic mobility shift assay for polyplexes of different N/P ratios analyzed in a 1% agarose gel, with pDNA visualized by GreenSafe Direct Load: Lane 1: Marker 1kb; Lane 2: Free pDNA; Lane 3: Free PDMAEMA; Lane 4: NPs 5/1; Lane 5: NPs 7,5/1; Lane 6: NPs 10/1; Lane 7: NPs 12,5/1; Lane 8: NPs 20/1

1.3 PDMAEMA polyplexes protect DNA from DNAse degradation

In addition to the capacity of DNA complexation by PDMAEMA, it is also important to assess if the polyplexes can protect DNA from degradation, both in the extracellular and intracellular millieu. The presence of nucleases is an important barrier that the polyplexes must overcome. In order to evaluate protection of DNA from degrading enzymes, PDMAEMA polyplexes were incubated with DNase for 5 min and as shown in figure 11, able to protect DNA from degradation, as evidenced by the DNA retained in the wells when compared with free DNA subjected to the action of DNAse (lane 3), which has been totally degraded, as expected
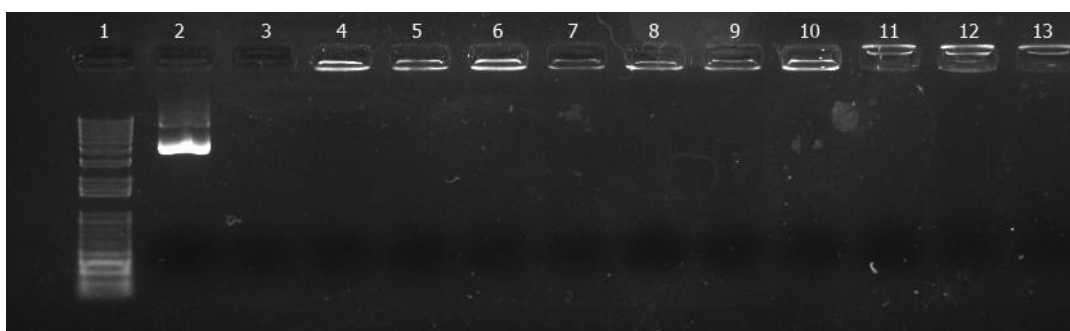
.

Figure 11- The PDMAEMA polyplexes can effectively protect the pDNA against the DNAse I as analyzed in a 1% agarose gel, with pDNA visualized by GreenSafe Direct Load: Lane 1: Marker 1kb; Lane 2: Free pDNA; Lane 3: Free pDNA with DNAse I; Lane 4: NPs 5/1; Lane 5: NPs 5/1 with DNAse I; Lane 6: NPs 7,5/1; Lane 7: NPs 7,5/1 with DNAse I; Lane 8: NPs 10/1; Lane 9: NPs 10/1 with DNAse I; Lane 10: NPs 12,5/1; Lane 11: NPs 12,5/1 with DNAse I; Lane 12: NPs 20/1; Lane 13: NPs 20/1 with DNAse I.

1.4 Stability assay of polyplexes over time

In order to assess the stability in storage (4ºC) and physiological conditions (37ºC), the polyplexes (N/P 5/10/20) were measured in terms of size, PdI and zeta potencial every 14 days (2 weeks) (figure 12). For the NPs 5 ratio in terms of size, at 37ºC the size appears to be pretty stable until around 25 days, after that the size starts to increase. At 4ºC, the polyplexes were very stable until 125 days after preparation. The polyplexes are stable until 32 days at 37ºC, from this point the PdI increased. At 4ºC the polyplexes were stable up to 125 days. The zeta potential in both conditions remains stable.
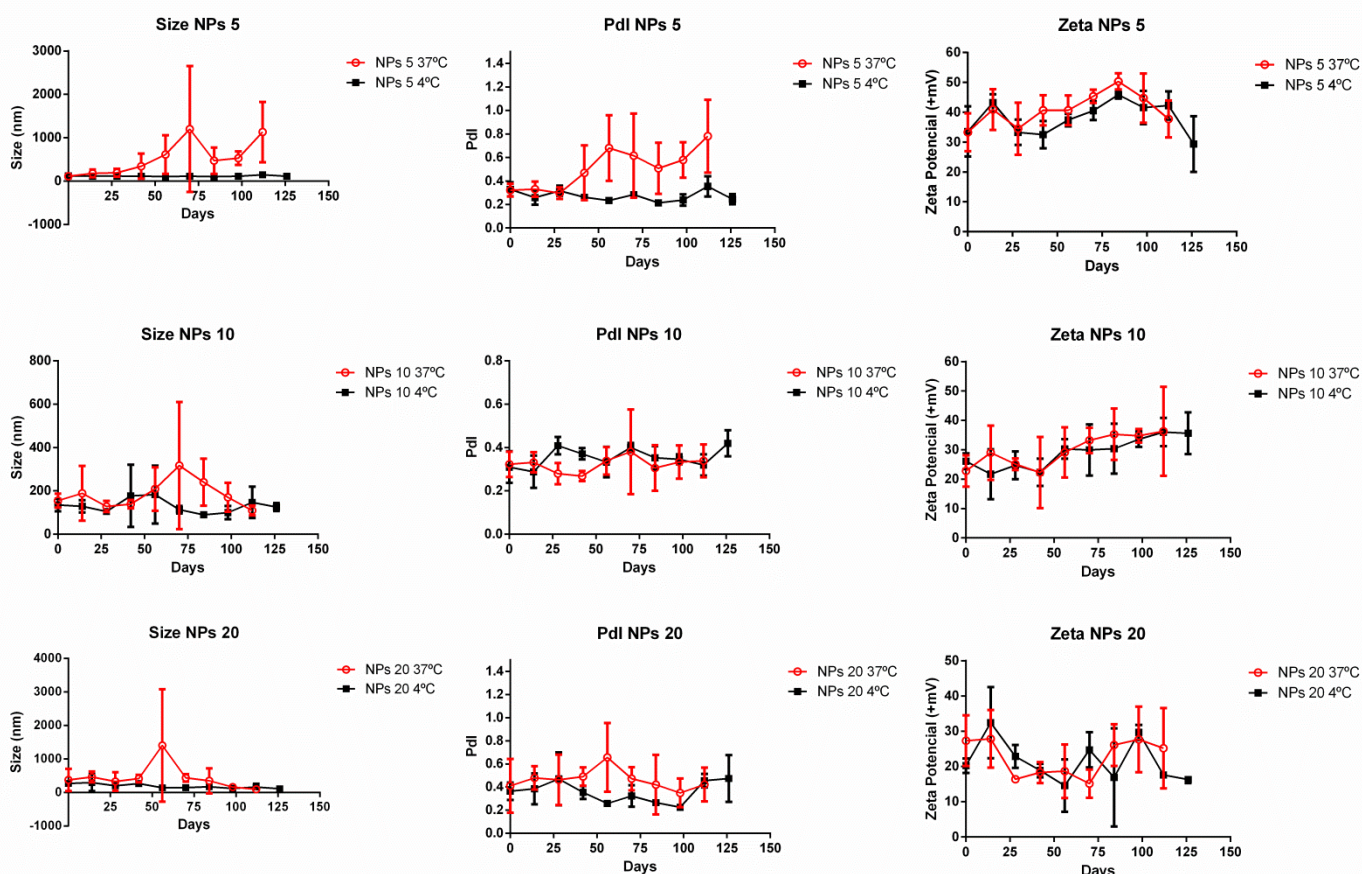

Figure 12- Measurements of size, PdI and zeta potencial of the stability assay of three polyplexes ratios (5/10/20). The polyplexes were aged in two conditions: 37ºC (red open circles) and 4ºC (black closed squares).

The increased size and PdI can be explained by the higher temperature, which increases the Brownian motion leading to the increased collision of the polyplexes, causing aggregation. This behavior was also observed with PDMAEMA polyplexes aged in 10% sucrose/aqueous solutions by Cherng *et al.* After 6 months of aging, the size of these polyplexes increased at 40ºC in comparison with lower temperatures of 4ºC and 20ºC [86]. This was also observed for other polymer, chitosan, that at a higher temperature (45ºC), its polyplexes also increased their size [87].

The ratios 10 and 20 were shown to maintain their size stable up to 125 days in both temperature conditions. In terms of PdI and zeta potential no major changes were observed great alterations. This could indicate that higher ratios of polymer/pDNA are more stable, which follows a logical trend that higher amounts of polymer to DNA can increase the complexation of DNA and thus promote stronger interaction and as consequence, resistance to temperature induced changes.

2. Evaluation of the cytotoxicity of the PDMAEMA polymer and PDMAEMA-DNA polyplexes

A critical feature in all materials to be used for biomedical applications, including gene therapy, is the biocompatibility of the material. The latter is first evaluated *in vitro* by testing the cytotoxicity on a cell culture setup. We have evaluated the cytotoxic profile of both the polymer and polymer-DNA complexes using two cell lines: a commonly used cell line for transfection studies - the HEK293 cells - and a retinal pigment epithelium cell line, D407, which is used as an in vitro model for evaluating potential gene therapy vectors for the retina.

We firstly evaluated the effect on cellular viability of the PDMAEMA-DNA polyplexes, as shown in figures 13 and 14. The results are presented in terms of mass of DNA, and for all the conditions tested (different polymer:DNA ratios and different quantities of polyplexes, there was no deleterious effect for cell viability both for HEK293 and D407 cells. Therefore, the polyplexes, even at concentrations more than two times higher than those to be used for transfection, are not cytotoxic for either cell line.
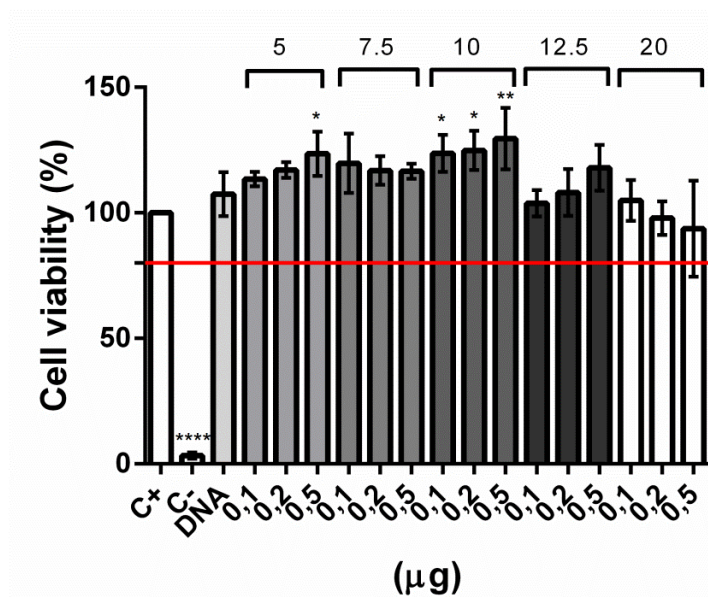

Figure 13- HEK293 cell viability upon challenge with PDMAEMA:DNA polyplexes at different concentrations, in function of mass of DNA, up to 72h. Lowest acceptable threshold of cell viability indicated by red bar (80% cell viability). Statistical significance was determined with one-way ANOVA followed by a post hoc Dunnett's Multiple Comparison Test and the statistical diference indicated by the star (*) symbol (* $p<0.05$, **$p<0.01$, *** $p<0.001$ and ****$p<0.0001$).

Figure 14- D407 cell viability upon challenge with PDMAEMA:DNA polyplexes at different concentrations, in function of mass of DNA, up to 72h. Lowest acceptable threshold of cell viability indicated by red bar (80% cell viability). Statistical significance was determined with one-way ANOVA followed by a post hoc Dunnett's Multiple Comparison Test and the statistical diference indicated by the star (*) symbol (* $p < 0.05$, **$p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$).
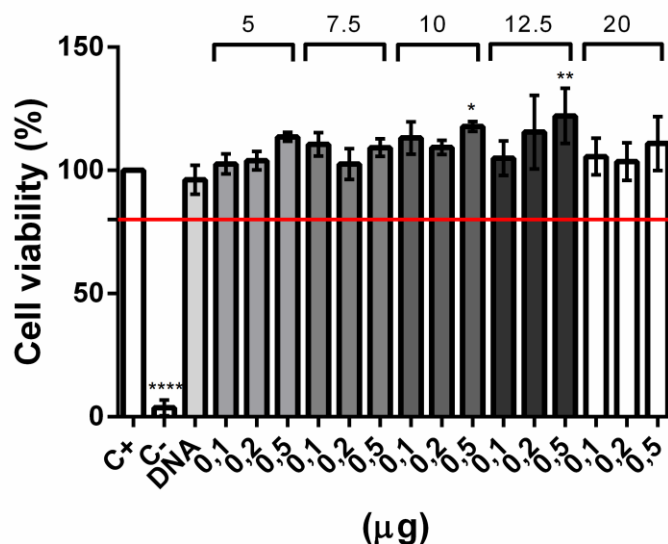
However, it is also important to test the cytotoxicity of the polymer after it releases its DNA load, since it is known that, for synthetic polymers such as PEI the free polymer shows toxicity towards cells [61].

Figures 15 and 16 show the results for the cytotoxicity profile of PDMAEMA, up to 72 hours, in HEK292 and D407 cells, respectively. For the HEK293 cells, concentrations of polymer higher than 10 μg/ml causes a significant decrease in cell viability, which is more pronounced after 48 hours. Concentrations below 10 μg/ml do not affect cell viability, with values similar to control for all time points (24, 48 and 72 hours).

For D407 cells, a similar pattern is observed to the one for HEK293 cells, but cellular viability values are higher than what is observed for HEK293 cells. Another difference is that at 72 h there is a significant increase in cell viability. This is most likely due to a cell density effect, since it is commonly known that cell density in a plate affects the proliferation rate of cells. For HEK293 cells the decrease in cellular viability was considerable, and the cell density in the plate

therefore did not permit a significant recovery of the proliferation, unlike what was observed for D407 cells, where the polymer affected much less the cellular viability.

In summary, in the concentration range to be used for transfection studies, which is bellow 10 µg/ml, the free polymer has not shown a significant cytotoxic effect over both cell lines.
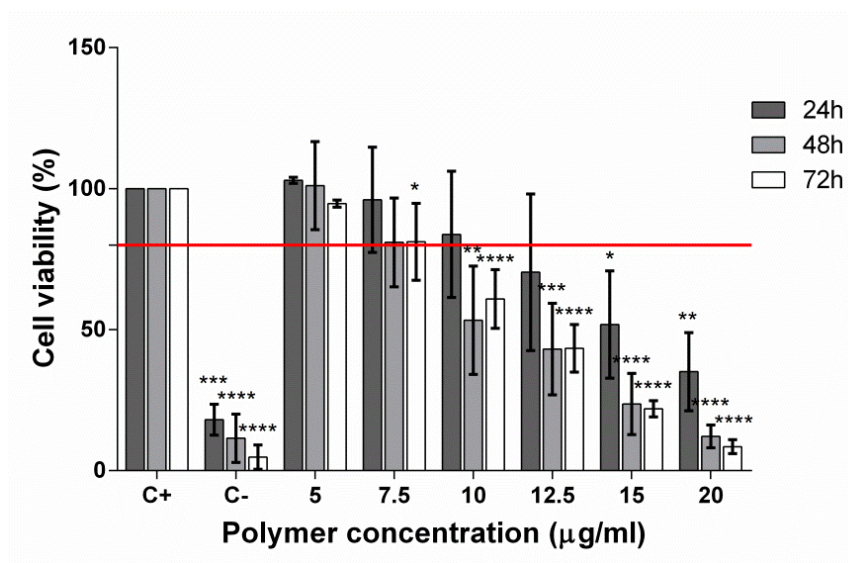

Figure 15- HEK293 cell viability upon challenge with different PDMAEMA concentrations (5, 7.5, 10, 12.5, 15 and 20 µg/ml) up to 72h. Lowest acceptable threshold of cell viability indicated by red bar (80% cell viability). Results are averaged from three independent experiments. Vertical bars correspond to standard deviation. Statistical significance was determined with one-way ANOVA followed by a post hoc Dunnett's Multiple Comparison Test and the statistical diference indicated by the star (*) symbol (* $p<0.05$, **$p<0.01$, *** $p<0.001$ and ****$p<0.0001$).

Figure 16- D407 cell viability upon challenge with different PDMAEMA concentrations (5, 7.5, 10, 12.5, 15 and 20 µg/ml) up to 72h. Lowest acceptable threshold of cell viability indicated by red bar (80% cell viability). Results are averaged from three independent experiments. Vertical bars correspond to standard deviation. Statistical significance was determined with one-way ANOVA followed by a post hoc Dunnett's Multiple Comparison Test and the statistical diference indicated by the star (*) symbol (* $p < 0.05$, **$p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$).
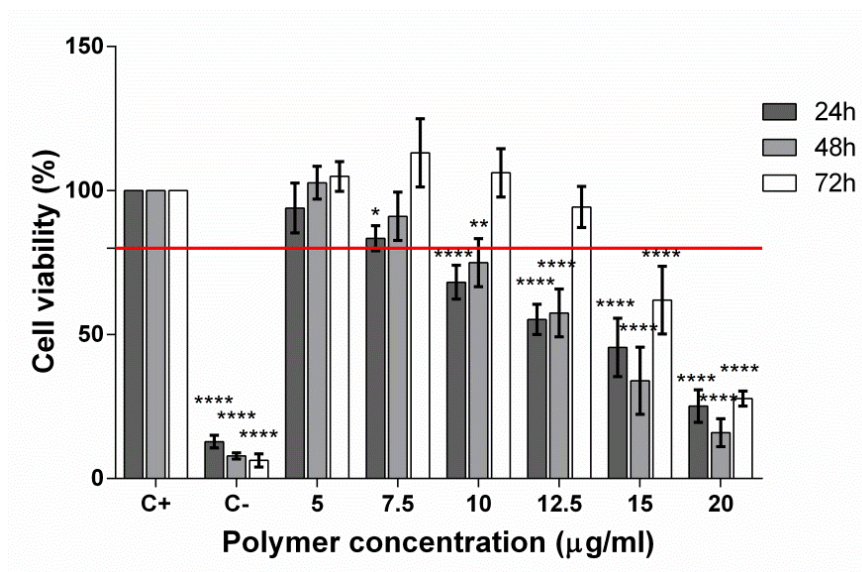
3. Transfection efficiency of PDMAEMA-DNA polyplexes

We have evaluated the transfection efficiency of the different PDMAEMA polyplexes in the two cell lines (HEK293 and D407 cell lines) qualitatively by fluorescence microscopy (figures 17 and 18) and quantitatively by flow cytometry (figures 19a and 19b). The results for the HEK293 cell line (figure 19a) show that the transfection efficiency of PDMAEMA polyplexes increases with the N/P ratio, with values for cell transfection ranging from 10% for the ratio of N/P 5 to 50% in the highest ratio, N/P 20.

The results for the retinal cell line, D407 (figure 19b), show that the transfection efficiency for this cell line follows the same trend as the one observed for HEK203 cells, but with lower values. This is consistent with what has been reported in the literature [88], as D407 are cells difficult to transfect even with

commercial reagents, as observed by the value obtained for FuGene HD® (40% compared with the 80% observed for HEK293 cells).

When compared with other reports in the literature, the transfection efficiency observed for PDMAEMA for other cells (CHO cells) follows a similar trend in terms of ratio [72] .

Figure 17- GFP expression, obtained by fluorescence microscopy at 72h post-transfection in HEK293, for positive control for transfection (FuGENE®) and the different polyplexes ratios (5/7.5/10/12.5/20). Scale bar represents 100 µm. Images are representative of three independent experiments.
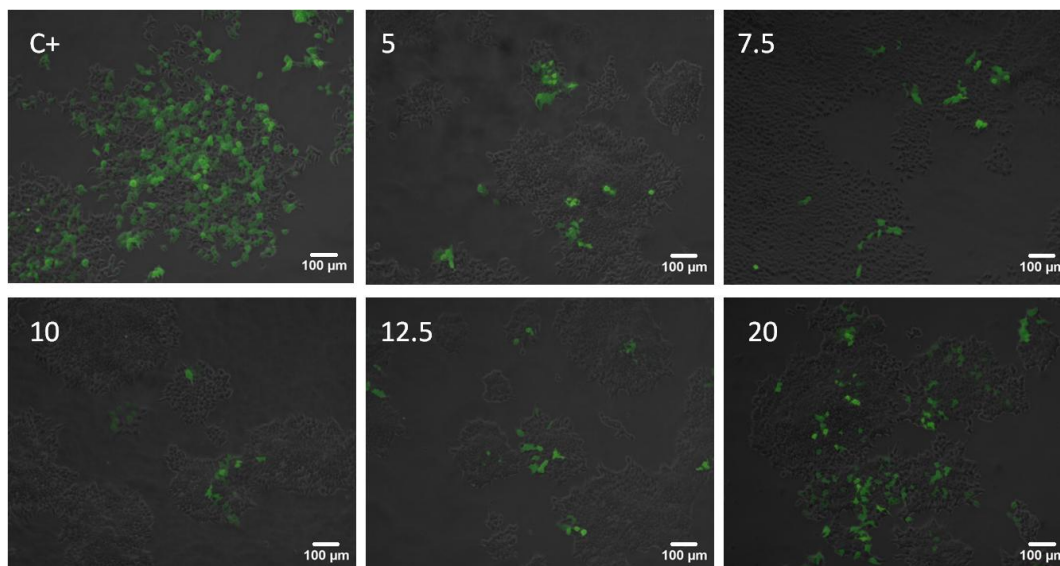

Figure 18- GFP expression, obtained by fluorescence microscopy at 72h post-transfection in D407 cells, for positive control for transfection (FuGENE®) and the different polyplexes ratios (5/7.5/10/12.5/20). Scale bar represents 100 µm. Images are representative of three independent experiments.
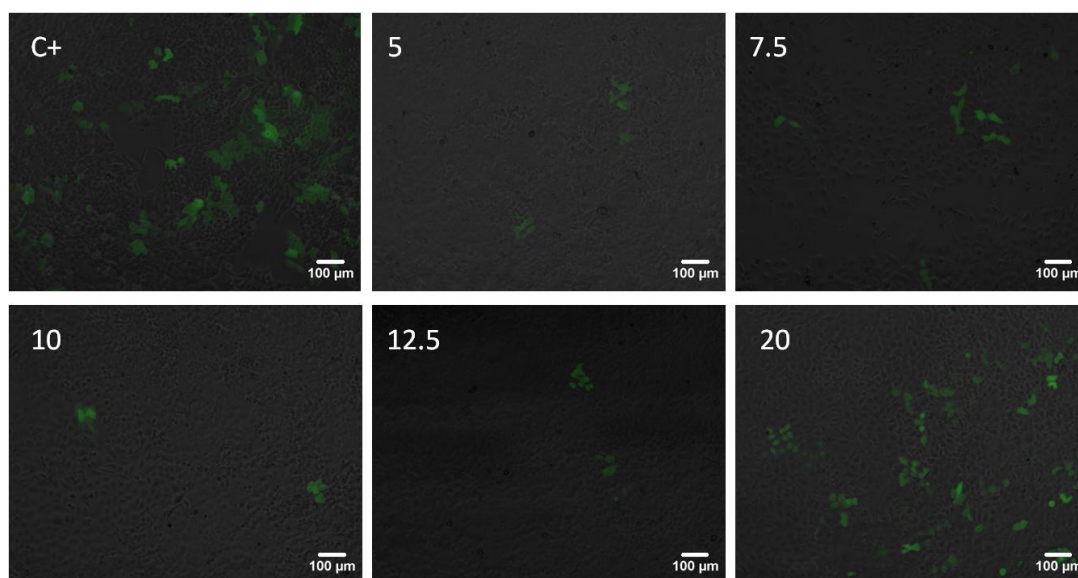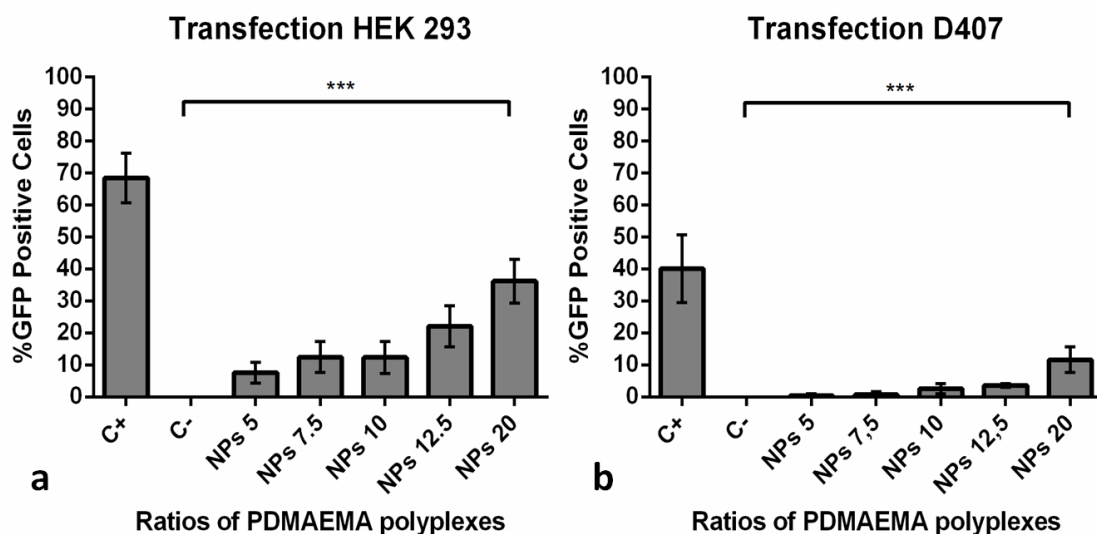
Figure 19- Transfection efficiency represented as GFP-positive cells as function of polyplexes ratios in **(a)** HEK293 and **(b)** D407 cell line. Results are averaged from three independent experiments. Vertical bars correspond to standard deviation. Statistical significance was determined with one-way ANOVA followed by a post hoc Dunnett's Multiple Comparison Test and the statistical diference indicated by the star (*) symbol (*** $p < 0.001$)

It has been previously observed that larger PDMAEMA polyplexes have lower transfection efficiencies [89]. In the present work, our polyplexes have sizes in the range of 100 to 300 nm, sizes below the maximum threshold acceptable for transfection, so it is unlikely that this can explain the different observations for transfection efficiencies. Compared with other PDMAEMA-based approaches found in the literature, our polymer has a size which has been reported to be more efficient transfecting cells, but much more toxic [73]. In this work, we have found that despite the fact that our polymer had a size around 300KDa, its cytotoxicity is reduced.

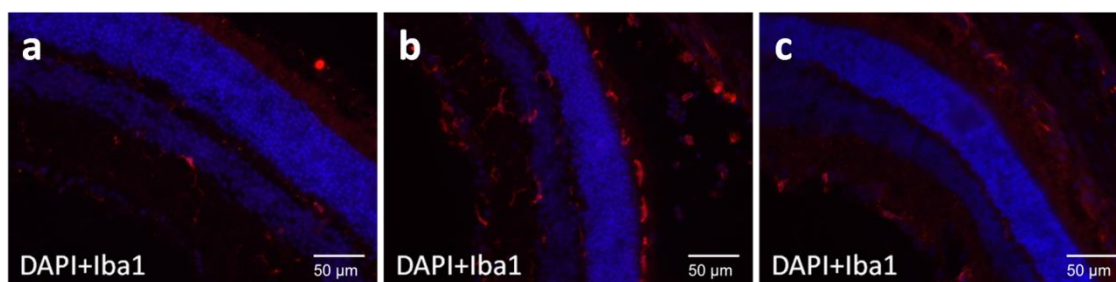# 4. Activation of microglia after intravitreous injection in mice C57BL/6

Figure 20- Immunohistochemistry for Iba1, a marker of active microglia. **(a)** Non-injected eye **(b)** PDMAEMA injected eye; **(c)** Sham injected eye. Red color: Iba-1; blue colour:DAPI. Scale bar represents 50 µm

In order to assess the in vivo compatibility of the PDMAEMA polyplexes, we have injected these polyplexes intravitreously in the eyes of C57BL/6 wild type mice and analyzed the activation of the microglia as a signal for inflammation. We have performed three conditions: non-injected eyes (control, figure 20a), injection procedure without administration of any vehicle (sham injection, figure 20c) and injection of polyplexes (figure 20b).

Two weeks post-injection, we have observed that, as expected, there is activation of the microglia in the retina, which is a sign of inflammation, in the eyes injected with the polyplexes. However, the degree of microglia activation is similar with the one observed for sham injected eyes, which indicates that the inflammation is mostly due to the injection procedure rather than the polyplexes.

In this work we describe the synthesis by RAFT and evaluation of PDMAEMA as a vector for retinal gene therapy. Our results show that a 300KDa polymer is capable of forming nanosized, stable polyplexes in the presence of DNA. These polyplexes have size, charge and morphology adequate for the transfection of retinal cells in vitro. In vivo administration has shown no further inflammation than the one caused by the administration procedure. Further studies regarding the capacity of in vivo transfection will determine the suitability of this system as a vehicle for gene therapy for retinal pathologies.

Part two:

1. Synthesis and structural characterization of a 200kDa PDMAEMA

For the synthesis of PDMAEMA, RAFT was chosen from the several polymerization methodologies, due to its versatility regarding monomers and reaction conditions, as well as the synthesis of polymers with controlled molecular weight and narrow polydispersities [78]. 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (figure 21) was selected as chain transfer agent (CTA), due to its commercial availability and good performance with a number of monomers [90]. A $[M]_0/[CTA]_0$ ration of 1500 was used, which would lead to a polymer with M_n = 200 kDa.
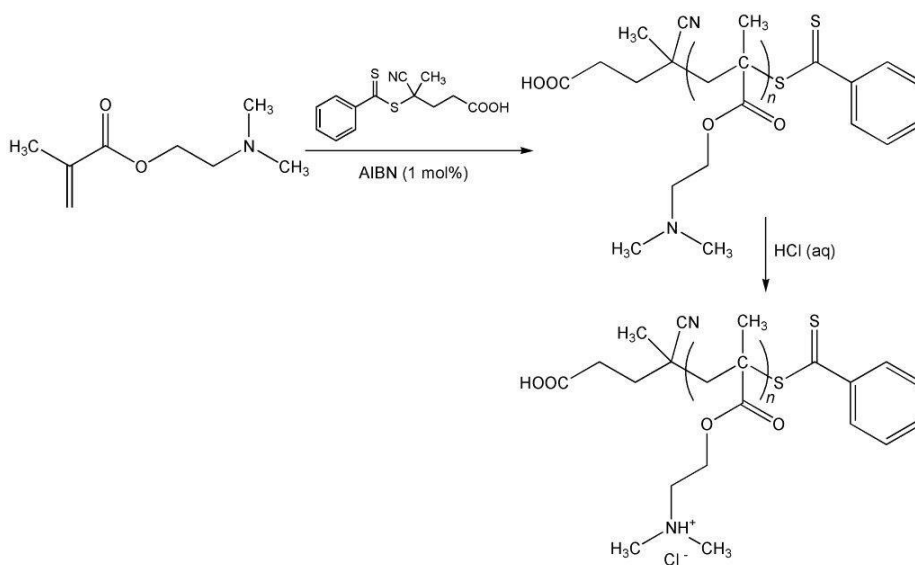

Figure 21- Scheme of chemical synthesis of PDMAEMA.

GPC analysis showed a monomodal molecular weight distribution (figure 22), with a number-average molecular weight (M_n) of 98,63 kDa, a weight average molecular weight (M_w) of 103,3 kDa, and a PdI (Polydispersity Index) (M_w/M_n) of 1,047. For the calculations, a value of $dn/dC = 0.149$ mL/g was used [73].

The polymer has a lower M_n value than expected, and also a low value of PDI indicating, that the chosen CTA produces polymers with a narrow chain distribution. In fact, PDMAEMA has recently been synthesized by RAFT and the same CTA and PdIs (below 1.20) were obtained, which are in agreement with our results, although for a different polymer size range (below 40 kDa) [85]. Due to some limitations of the GPC analysis equipment that did not allow an accurate analysis, these results will be further analyzed by other technologies such as ^{1}HNMR.
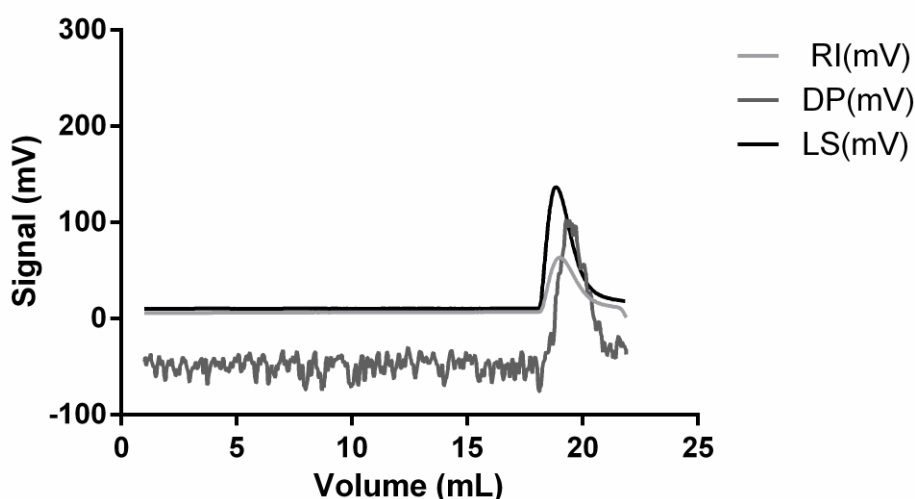

Figure 22- SEC[3] chromatogram of PDMAEMA (RI – refractometer, DP – viscometer, and LS – light scattering traces).

2. The 103.3kDa PDMAEMA is able to form PDMAEMA-DNA polyplexes

2.1 PDMAEMA-DNA polyplexes have characteristics compatible with gene therapy

Characterization of the polyplexes, performed by photon correlation spectroscopy and laser Doppler anemometry, included the determination of size, polydispersity and zeta potential, as shown in table II.

As stated before, polyplexes should ideally present a size below 500 nm, an optimum polydispersity of 0.1, and a positive zeta potential, favorable requisites for cellular uptake.

Table II: Characterization of polyplexes by size, PdI and zeta potencial

Formulation	Size (nm)	Polydispersity Index (PdI)	Surface charge (+mV)
PDMAEMA 5	159.73±8.27	0.237±0.061	39.2±4.73
PDMAEMA 7,5	148.47±19.94	0.266±0.016	43±7.18
PDMAEMA 10	307.87±181.7	0.401±0.129	27.6±7.45
PDMAEMA 12,5	323.13±171.73	0.441±0.055	27.27±9.24
PDMAEMA 20	587.7±193.64	0.582±0.059	24.57±9.87

In table II it is possible to observe that the size of PDMAEMA/pDNA polyplexes increases with the increase in N/P ratio from 5 to 20, but only the ratios 5 and 7,5 have diameters smaller than 500 nm. The polydispersity index is below 0.4 in the lower ratios (5 and 7.5), indicating a homogeneous population of particles, which is desirable for consistency of results regarding cellular penetration. As expected, the polyplexes have positive charge. By TEM analysis we observed that the polyplexes have a spherical morphology (figure 23). As expected, the polyplexes have positive charge. Based on these results, we have selected the ratios 5 and 7,5 for further studies.
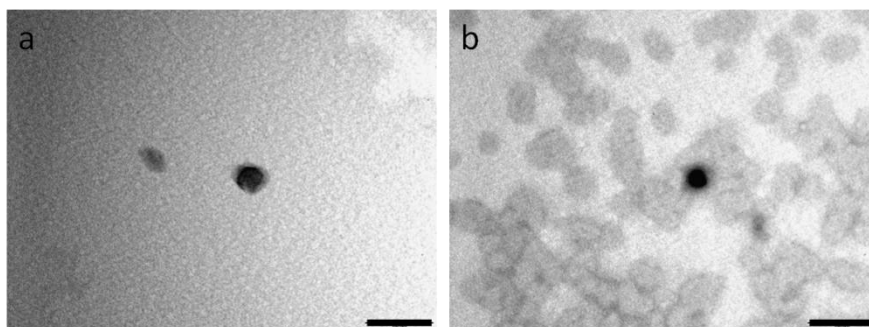
Figure 23- Analysis by transmission electron microscopy of PDMAEMA-DNA polyplexes at ratios, 5**(a)** and 7.5**(b)**. Polyplexes have sizes smaller than 200nm. Scale bar represents 200nm.

2.2 PDMAEMA efficiently complexes pDNA

As observed in figure 24, for all N/P ratios the PDMAEMA polyplexes were able to complex and retain the DNA, and this is evidenced by the absence of DNA bands in the gel lanes, lanes 4 (NPs 5) and 5 (NPs 7,5) with all DNA retained in the wells. As expected, free DNA (lane 2) migrates towards the positive pole and PDMAEMA polymer (lane 3) shows no signal in the gel, as expected. PDMAEMA efficiently complexes pDNA, which is retained in the well.

Figure 24- Electrophoretic mobility shift assay for polyplexes of different N/P ratios analyzed in a 1% agarose gel, with pDNA visualized by GreenSafe Direct Load: Lane 1: Marker 1kb; Lane 2: Free pDNA; Lane 3: Free PDMAEMA; Lane 4: NPs 5/1; Lane 5: NPs 7,5/1.

2.3 PDMAEMA polyplexes protect DNA from DNAse degradation
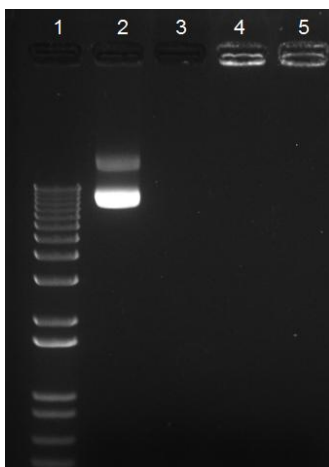
In addition to the capacity of DNA complexation by PDMAEMA, it is also important to assess if the polyplexes formed with this smaller Mw polymer can protect DNA from degradation, both in the extracellular and intracellular millieu. In order to evaluate DNA protection from degrading enzymes, PDMAEMA polyplexes were incubated with DNase for a longer time period than the 354kDa polymer and as shown in figure 25, able to protect DNA after 15 min of exposure to DNAse I, evidenced by the DNA retained in the wells when compared with free DNA subjected to the action of DNAse I (lane 7 and 9), which has been fully degraded.

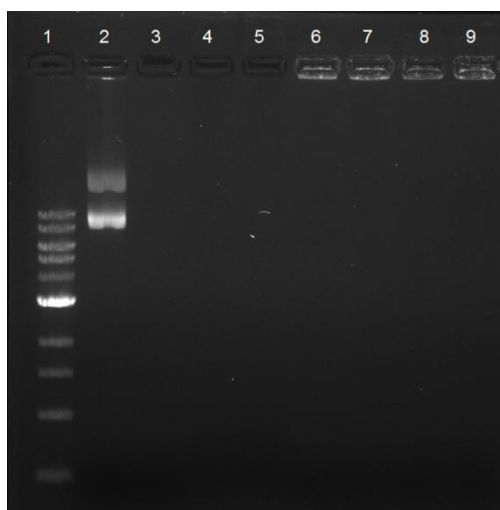
Figure 25- The PDMAEMA polyplexes can effectively protect the pDNA against the DNAse I as analyzed in a 1% agarose gel, with pDNA visualized by GreenSafe Direct Load: Lane 1: Marker 1kb; Lane 2: Free pDNA; Lane 3: Free pDNA with DNAse I; Lane 4: Free PDMAEMA; Lane 5: Free PDMAEMA with DNAse I; Lane 6: NPs 5/1; Lane 7: NPs 5/1 with DNAse I; Lane 8: NPs 7,5/1; Lane 9: NPs 7,5/1 with DNAse I.

2.4 PDMAEMA-DNA polyplexes are stable in *in vitro* culture conditions

In order to evaluate the behavior in culture conditions, the polyplexes were incubated in DMEM medium containing 5% FBS for different time periods: 24h, 48h and 72h. After the incubation time the stability of the polyplexes was evaluated by the electrophoretic mobility shift assay (figure 26).
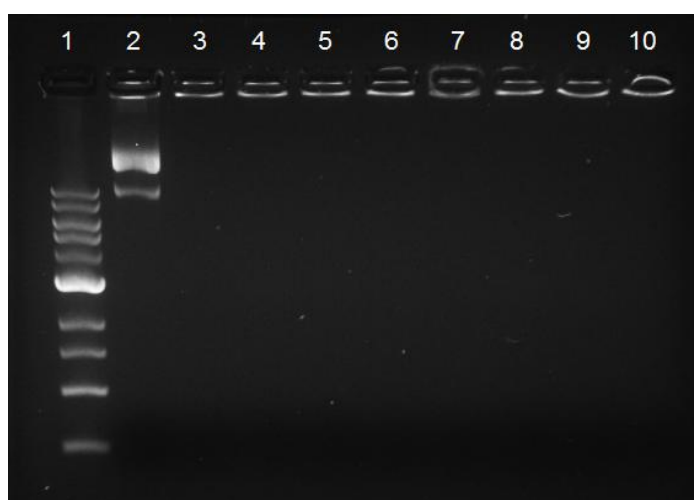
Figure 26- PDMAEMA polyplexes are stable in the presence of serum proteins up to 72h as analyzed in a 1% agarose gel, with pDNA visualized by GreenSafe Direct Load: Lane 1: Marker 1kb; Lane 2: Free pDNA; Lane 3: NPs 5/1; Lane 4: NPs 5/1 + 24h DMEM w/serum proteins; Lane 5: NPs 5/1 + 48h DMEM w/serum proteins; Lane 6: NPs 5/1 + 72h DMEM w/serum proteins; Lane 7: NPs 7,5/1; Lane 8: NPs 7,5/1 + 24h DMEM w/serum proteins; Lane 9: NPs 7,5/1 + 48h DMEM w/serum proteins; Lane 10: NPs 7,5/1 + 72h DMEM w/serum proteins.

Lanes 3 and 7 are corresponding to polyplexes with ratios of NPs 5/1 and NPs 7,5/1 respectively, without incubation with serum proteins. This assay demonstrates, the stability of these polyplexes in the presence of serum proteins up to 72h, since no pDNA migration was observed in the lanes 4, 5 and 6 for the NPs 5/1 and the lanes 8, 9 and 10 for the NPs 7,5/1. This stability can be an advantage in a systemic delivery since it will not release its load while in circulation, but can also be a problem for pDNA release inside the cells.

3. Cytotoxicity of the PDMAEMA polymer and PDMAEMA-DNA polyplexes is concentration-dependent

In this part of the work we have evaluated the cytotoxic profile of both the polymer and polymer-DNA complexes using only one cell line: a retinal pigment epithelium cell line, D407, which is used as an in vitro model for evaluating potential gene therapy vectors for the retina.

We firstly evaluated the effect on cellular viability of the PDMAEMA:DNA polyplexes, as shown in figure 27. The results are presented in terms of mass of DNA, and for all the conditions tested (different polymer:DNA ratios and different quantities of polyplexes), there was no deleterious effect on cell viability for D407 cells. Therefore, the polyplexes, even at concentrations more than two times higher than those used for transfection.
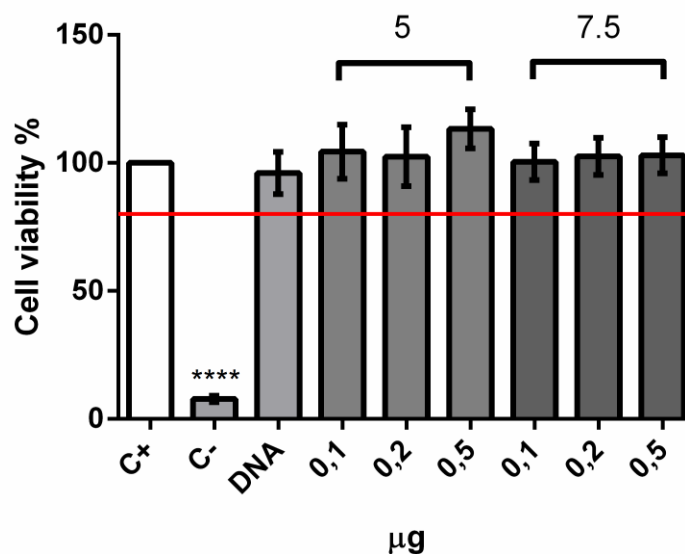
Figure 27- D407 cell viability upon challenge with PDMAEMA:DNA polyplexes at diferent concentrations, in function of mass of DNA, up to 72h. Lowest acceptable threshold of cell viability indicated by red bar (80% cell viability). Statistical significance was determined with one-way ANOVA followed by a post hoc Dunnett's Multiple Comparison Test and the statistical diference indicated by the star (*) symbol (* $p<0.05$, **$p<0.01$, *** $p<0.001$ and ****$p<0.0001$).
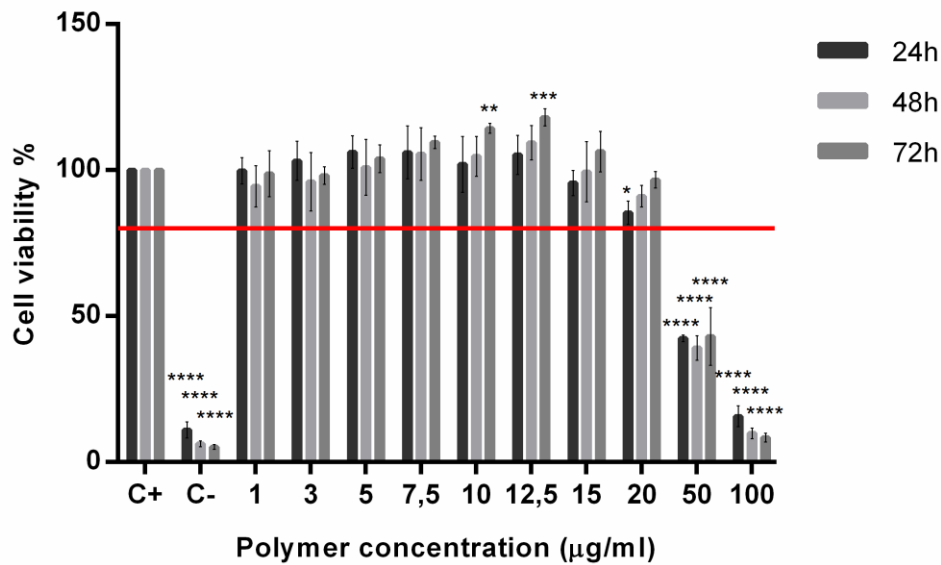
Figure 28- D407 cell viability upon challenge with different PDMAEMA concentrations (1, 3, 5, 7.5, 10, 12.5, 15, 20, 50 and 100 µg/ml) up to 72h. Lowest acceptable threshold of cell viability indicated by red bar (80% cell viability). Results are averaged from three independent experiments. Vertical bars correspond to standard deviation. Statistical significance was determined with one-way ANOVA followed by a post hoc Dunnett's Multiple Comparison Test and the statistical diference indicated by the star (*) symbol (* $p<0.05$, **$p<0.01$, *** $p<0.001$ and ****$p<0.0001$).

However, it is also important to test the cytotoxicity of the polymer after it releases its DNA load, since it is known that as mentioned before, for synthetic polymers such as PEI, the free polymer shows toxicity towards cells [61]. So the free PDMAEMA polymer was tested in a wider range of concentrations than the polymer described in part one of this results section (1, 3, 5, 7.5, 10, 12.5, 15, 20, 50 and 100 µg/ml).

Figures 28 show the results for the cytotoxicity profile of PDMAEMA, up to 72 hours, in D407 cells. Concentrations of polymer higher than 20 µg/ml cause a significant decrease in cell viability, which is more pronounced after 48 hours. Only with a higher PDMAEMA concentration of 50 µg/ml we observed a decreased cell viability of about 50% for all time points. With the highest concentration of 100 µg/ml, the cell viability only reaches around 10-15%. Concentrations below 20 µg/ml do not affect cell viability, with values similar to control for all time points (24, 48 and 72 hours).

Comparing the results of 103.3kDa PDMAEMA with the previously studied 354kDa PDMAEMA, at the same concentrations of 5, 7.5, 10, 12.5 and 20 µg/ml, the 103.3 kDa polymer showed to be less cytotoxic. Some reports showed that this reduction in cytotoxicity can be explained due to the molecular weight of the polymer [70, 73].

In summary, in the concentration range to be used for the transfection studies, which is bellow 20 µg/ml, the free polymer has not shown a significant cytotoxic effect over D407 cells.

4. 103.3kDa PDMAEMA-DNA polyplexes transfect D407 cells

For the 103.3kDa polymer, we only evaluated the transfection efficiency of the different ratios of PDMAEMA polyplexes in the D407 cell line. This was done qualitatively by fluorescence microscopy (figure 29) and quantitatively by flow cytometry (figure 30). We also evaluated if the administration method had influence on the transfection efficiency, by testing two different conditions: directly added to the cells (non-diluited) and diluited in 100 µL of medium without FBS.

The results obtained in D407 (figure 30), showed that the transfection efficiency for this cell line follows the same trend as observed with the PDMAEMA 354kDa for HEK203 and D407 cells, but with lower values. This difference in values for HEK293 and D407 is consistent with what has been reported in the literature [88], as D407 are cells difficult to transfect even with commercial reagents, as observed by the value obtained for Fugene HD®. In terms of comparison of transfection values between the 354 and 103.3kDa polymers, it is also consistent with what has been described in the literature, that higher Mw PDMAEMA has higher transfection efficiency. Regarding the administration method, the results showed that there is no significant difference between the two administration conditions.

The ratio 7.5 showed to have significantly higher transfection efficiency than the ratio 5 in either administration condition (figure 30). When compared with other reports in the literature, the transfection efficiency observed for PDMAEMA for other cells (CHO cells) follows a similar trend in terms of ratio [72].
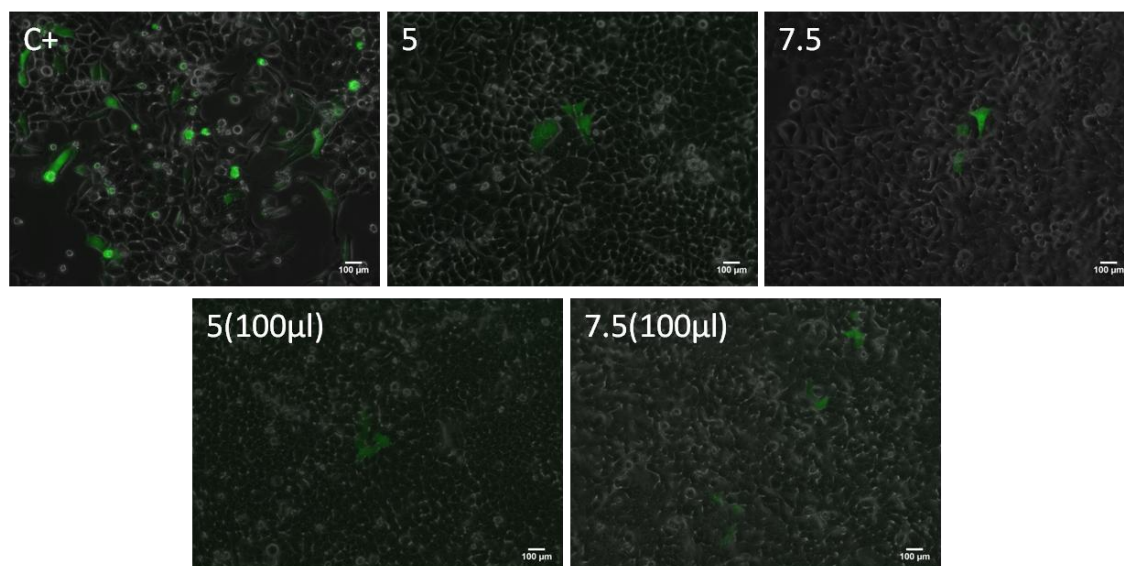
Figure 29- GFP expression, obtained by fluorescence microscopy at 72h post-transfection in D407 cells, for positive control for transfection (FuGENE®), the two polyplexes ratios (5/ 7.5) and the two administration conditions: non-diluted (5/1 and 7.5/1) and diluted (5/1 100µl and 7.5/1 100µl). Scale bar represents 100 µm. Images are representative of three independent experiments.

It has been previously observed that larger PDMAEMA polyplexes have lower transfection efficiencies [89]. In the present work, our polyplexes have sizes in the range of 100 to 200 nm, sizes below the maximum threshold acceptable for transfection, so it is unlikely that this can explain the different observations for transfection efficiencies. Compared with other PDMAEMA-based approaches found in the literature, our polymer has a size which has been reported to be more efficient transfecting cells, but much more toxic [73]. In this work, we have found that our polymer with size around 100KDa compared with previously synthesized PDMAEMA with 354kDa, has reduced cytotoxicity. The low transfection efficiency can be the result of the high positive charge of the polymer, which does not allow the dissociation of the polyplexes and release of the pDNA within the cellular environment. Another possible explanation is that

the polyplexes are unable to enter the nucleus and therefore gene expression does not occur.
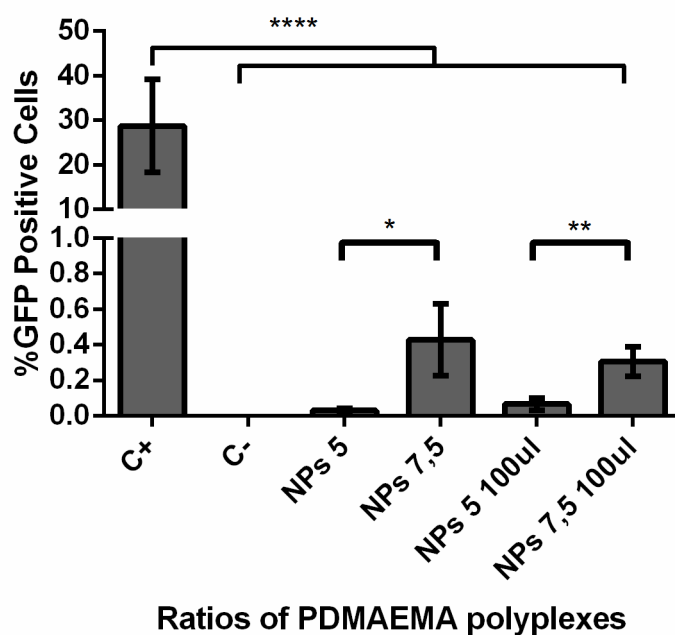
Figure 30- Transfection efficiency represented as GFP-positive cells as function of polyplexes ratios in D407 cell line. Results are averaged from three independent experiments. Vertical bars correspond to standard deviation. Statistical significance was determined with one-way ANOVA followed by a post hoc Dunnett's Multiple Comparison Test to compare the positive control with the tested conditions, unpaired t test was used to compare between conditions and the statistical diference indicated by the star (*) symbol (* $p < 0.05$, **$p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$).

Conclusions

The aim of the present study was two-fold: 1) characterize a previously synthesized PDMAEMA with a molecular weight of 354kDa as carrier for gene delivery in the eye and 2) synthetize a new PDMAEMA polymer by RAFT, with an M_w of 200kDa, to test if a variation in M_w could cause a variation in the gene efficiency.

Our results show that the 354kDa polymer is capable of forming nanosized, stable polyplexes in the presence of DNA. These polyplexes have size, charge and morphology adequate for the transfection of cells. The cytotoxicity assays showed that the polymer, in the concentrations used in the transfection studies, is not cytotoxic. We found the transfection efficiency to be dependent of the polyplex molecular ratio, since higher ratios transfected better than lower ratios. The transfection efficiency was also dependent of the cell line. HEK293 cell line was more transfectable than D407 cell line. In vivo administration has shown no further inflammation than the one caused by the administration procedure.

Based on these results a new polymer was synthesized by RAFT with a theoretical molecular weight of 200 kDa. What was obtained was a polymer with 103.3kDa that was also able to form nanosized polyplexes, which are stable in the presence of serum proteins. These polyplexes have a size, charge and morphology suitable for transfection, but in D407 cells, this polymer had less cytotoxic effects than the PDMAEMA 354kDa. The transfection studies for the 103.3kDa PDMAEMA showed that the polyplexes were able to transfect cells, despite the lower transfection efficiency than the one observed for the 354kDa polymers for the smaller ratios. Therefore, from this study we can conclude that higher Mw PDMAEMA has more potential for retinal gene therapy.

Future work

For future work, we plan to evaluate the *in vivo* biocompability of the 103.3 kDa PDMAEMA by evaluation of the activation of microglia. In order to extensively evaluate this polymer, we will produce polyplexes of higher N/P ratios (10/12.5/20) because these are the ones that were more efficient in transfecting cells in the 354kDa PDMAEMA.

In addition, we plan to conduct a series of experiments to elucidate the causes of low transfection efficiencies. For this purpose, we will evaluate what the rate limiting step in transfection is, by:

- Inhibiting endocytosis;
- Fluorescently tag the polymer and assess its intracellular path using endosomal markers;
- Conjugate pDNA with a tagging kit for DNA and tracking its nuclear penetration.

Althogether, these results will enable to identify the bottleneck in transfection and further optimize this polymer for improved gene delivery.

References

1. Sharon Y. Wong, J.M.P., David Putman, *Polymer systems for gene delivery—Past, present, and future.* Progress in Polymer Science, 2007. **32**(Issues 8–9): p. Pages 799–837.

2. Li, S. and L. Huang, *Nonviral gene therapy: promises and challenges.* Gene therapy, 2000. **7**(1): p. 31-4.

3. Manjila, S.B., et al., *Novel gene delivery systems.* Int J Pharm Investig, 2013. **3**(1): p. 1-7.

4. Borras, T., *Recent developments in ocular gene therapy.* Exp Eye Res, 2003. **76**(6): p. 643-52.

5. Witlox, M.A., et al., *Evolving gene therapy approaches for osteosarcoma using viral vectors: Review.* Bone, 2007. **40**(4): p. 797-812.

6. Melchiorri, D., et al., *Regulatory evaluation of Glybera in Europe - two committees, one mission.* Nat Rev Drug Discov, 2013. **12**(9): p. 719.

7. Niidome, T. and L. Huang, *Gene therapy progress and prospects: nonviral vectors.* Gene therapy, 2002. **9**(24): p. 1647-52.

8. Aied, A., et al., *Polymer gene delivery: overcoming the obstacles.* Drug Discov Today, 2013.

9. Conner, S.D. and S.L. Schmid, *Regulated portals of entry into the cell.* Nature, 2003. **422**(6927): p. 37-44.

10. Boussif, O., et al., *A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(16): p. 7297-301.

11. Zhou, J., et al., *PAMAM dendrimers for efficient siRNA delivery and potent gene silencing.* Chem Commun (Camb), 2006(22): p. 2362-4.

12. Liang, W.a.L., J (2012). Endosomal Escape Pathways for Non-Viral Nucleic Acid Delivery Systems, Molecular Regulation of Endocytosis, Dr. Brian Ceresa (Ed.), ISBN: 978-953-51-0662-3, InTech, DOI: 10.5772/46006.

13. Kurisawa, M., M. Yokoyama, and T. Okano, *Gene expression control by temperature with thermo-responsive polymeric gene carriers.* J Control Release, 2000. **69**(1): p. 127-37.

14. Cheng, N., et al., *A study of thermoresponsive poly(N-isopropylacrylamide)/polyarginine bioconjugate non-viral transgene vectors.* Biomaterials, 2006. **27**(28): p. 4984-92.

15. Saito, G., J.A. Swanson, and K.D. Lee, *Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities.* Adv Drug Deliv Rev, 2003. **55**(2): p. 199-215.

16. Read, M.L., et al., *A versatile reducible polycation-based system for efficient delivery of a broad range of nucleic acids.* Nucleic acids research, 2005. **33**(9): p. e86.

17. Wang, Y., P. Chen, and J. Shen, *The development and characterization of a glutathione-sensitive cross-linked polyethylenimine gene vector.* Biomaterials, 2006. **27**(30): p. 5292-8.

18. Liu, X., J.W. Yang, and D.M. Lynn, *Addition of "charge-shifting" side chains to linear poly(ethyleneimine) enhances cell transfection efficiency.* Biomacromolecules, 2008. **9**(7): p. 2063-71.

19. Zhong, Z., et al., *A versatile family of degradable non-viral gene carriers based on hyperbranched poly(ester amine)s.* J Control Release, 2005. **109**(1-3): p. 317-29.

20. Li, Z. and L. Huang, *Sustained delivery and expression of plasmid DNA based on biodegradable polyester, poly(D,L-lactide-co-4-hydroxy-L-proline).* J Control Release, 2004. **98**(3): p. 437-46.

21. Forrest, M.L., J.T. Koerber, and D.W. Pack, *A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery.* Bioconjug Chem, 2003. **14**(5): p. 934-40.

22. Lechardeur, D. and G.L. Lukacs, *Nucleocytoplasmic transport of plasmid DNA: a perilous journey from the cytoplasm to the nucleus.* Human gene therapy, 2006. **17**(9): p. 882-9.

23. Dauty, E. and A.S. Verkman, *Actin cytoskeleton as the principal determinant of size-dependent DNA mobility in cytoplasm: a new barrier for non-viral gene delivery.* The Journal of biological chemistry, 2005. **280**(9): p. 7823-8.

24. Pante, N. and M. Kann, *Nuclear pore complex is able to transport macromolecules with diameters of about 39 nm.* Mol Biol Cell, 2002. **13**(2): p. 425-34.

25. Cartier, R. and R. Reszka, *Utilization of synthetic peptides containing nuclear localization signals for nonviral gene transfer systems.* Gene therapy, 2002. **9**(3): p. 157-67.

26. Kichler, A., et al., *Efficient DNA transfection mediated by the C-terminal domain of human immunodeficiency virus type 1 viral protein R.* Journal of virology, 2000. **74**(12): p. 5424-31.

27. Moffatt, S., S. Wiehle, and R.J. Cristiano, *A multifunctional PEI-based cationic polyplex for enhanced systemic p53-mediated gene therapy.* Gene therapy, 2006. **13**(21): p. 1512-23.

28. Darquet, A.M., et al., *A new DNA vehicle for nonviral gene delivery: supercoiled minicircle.* Gene therapy, 1997. **4**(12): p. 1341-9.

29. Riu, E., et al., *Histone modifications are associated with the persistence or silencing of vector-mediated transgene expression in vivo.* Molecular therapy : the journal of the American Society of Gene Therapy, 2007. **15**(7): p. 1348-55.

30. Machelska, H., et al., *Peripheral non-viral MIDGE vector-driven delivery of beta-endorphin in inflammatory pain.* Mol Pain, 2009. **5**: p. 72.

31. Schakowski, F., et al., *A novel minimal-size vector (MIDGE) improves transgene expression in colon carcinoma cells and avoids transfection of undesired DNA.* Molecular therapy : the journal of the American Society of Gene Therapy, 2001. **3**(5 Pt 1): p. 793-800.

32. Marie, C., et al., *pFARs, plasmids free of antibiotic resistance markers, display high-level transgene expression in muscle, skin and tumour cells.* J Gene Med, 2010. **12**(4): p. 323-32.

33. Wong, S.P., et al., *Non-viral S/MAR vectors replicate episomally in vivo when provided with a selective advantage.* Gene therapy, 2011. **18**(1): p. 82-7.

34. Haase, R., et al., *pEPito: a significantly improved non-viral episomal expression vector for mammalian cells.* BMC Biotechnol, 2010. **10**: p. 20.

35. Koirala, A., S.M. Conley, and M.I. Naash, *A review of therapeutic prospects of non-viral gene therapy in the retinal pigment epithelium.* Biomaterials, 2013. **34**(29): p. 7158-67.

36. Zuhorn, I.S., et al., *Nonbilayer phase of lipoplex-membrane mixture determines endosomal escape of genetic cargo and transfection efficiency.* Molecular therapy : the journal of the American Society of Gene Therapy, 2005. **11**(5): p. 801-10.

37. Felgner, P.L., et al., *Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure.* Proceedings of the National Academy of Sciences of the United States of America, 1987. **84**(21): p. 7413-7.

38. Nabel, G.J., et al., *Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(23): p. 11307-11.

39.   Caplen, N.J., et al., *Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis.* Nature medicine, 1995. **1**(1): p. 39-46.

40.   He, C.X., Y. Tabata, and J.Q. Gao, *Non-viral gene delivery carrier and its three-dimensional transfection system.* Int J Pharm, 2010. **386**(1-2): p. 232-42.

41.   Lehtinen, J., et al., *Glycosaminoglycan-resistant and pH-sensitive lipid-coated DNA complexes produced by detergent removal method.* J Control Release, 2008. **131**(2): p. 145-9.

42.   Garcia, L., et al., *Serum-resistant lipopolyplexes for gene delivery to liver tumour cells.* Eur J Pharm Biopharm, 2007. **67**(1): p. 58-66.

43.   Kiang, T., et al., *The effect of the degree of chitosan deacetylation on the efficiency of gene transfection.* Biomaterials, 2004. **25**(22): p. 5293-301.

44.   Templeton, N., *Gene and Cell Therapy-Therapeutic Mechanisms and Strategies, 3rd edition. Florida, U.S.A.* 2008.

45.   Hirano, S., H. Tsuchida, and N. Nagao, *N-acetylation in chitosan and the rate of its enzymic hydrolysis.* Biomaterials, 1989. **10**(8): p. 574-6.

46.   Borchard, G., *Chitosans for gene delivery.* Adv Drug Deliv Rev, 2001. **52**(2): p. 145-50.

47.   Huang, M., et al., *Transfection efficiency of chitosan vectors: effect of polymer molecular weight and degree of deacetylation.* J Control Release, 2005. **106**(3): p. 391-406.

48.   Thanou, M., et al., *Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines.* Biomaterials, 2002. **23**(1): p. 153-9.

49.   Kean, T., S. Roth, and M. Thanou, *Trimethylated chitosans as non-viral gene delivery vectors: cytotoxicity and transfection efficiency.* J Control Release, 2005. **103**(3): p. 643-53.

50.   Zhao, Q.Q., et al., *Combination of poly(ethylenimine) and chitosan induces high gene transfection efficiency and low cytotoxicity.* J Biosci Bioeng, 2008. **105**(1): p. 65-8.

51.   Wong, K., et al., *PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo.* Bioconjug Chem, 2006. **17**(1): p. 152-8.

52.   Saka, O.M. and A. Bozkir, *Formulation and in vitro characterization of PEGylated chitosan and polyethylene imine polymers with thrombospondin-I gene bearing pDNA.* J Biomed Mater Res B Appl Biomater, 2012. **100**(4): p. 984-92.

53.   Liao, Z.X., et al., *Mechanistic study of transfection of chitosan/DNA complexes coated by anionic poly(gamma-glutamic acid).* Biomaterials, 2012. **33**(11): p. 3306-15.

54.   Chan, P., et al., *Synthesis and characterization of chitosan-g-poly(ethylene glycol)-folate as a non-viral carrier for tumor-targeted gene delivery.* Biomaterials, 2007. **28**(3): p. 540-9.

55.   Gao, S., et al., *Galactosylated low molecular weight chitosan as DNA carrier for hepatocyte-targeting.* Int J Pharm, 2003. **255**(1-2): p. 57-68.

56.   Hashimoto, M., et al., *Lactosylated chitosan for DNA delivery into hepatocytes: the effect of lactosylation on the physicochemical properties and intracellular trafficking of pDNA/chitosan complexes.* Bioconjug Chem, 2006. **17**(2): p. 309-16.

57.   Mohammadi, Z., et al., *In vivo transfection study of chitosan-DNA-FAP-B nanoparticles as a new non viral vector for gene delivery to the lung.* Int J Pharm, 2011. **421**(1): p. 183-8.

58.   Klausner, E.A., et al., *Corneal gene delivery: chitosan oligomer as a carrier of CpG rich, CpG free or S/MAR plasmid DNA.* J Gene Med, 2012. **14**(2): p. 100-8.

59.   Dunlap, D.D., et al., *Nanoscopic structure of DNA condensed for gene delivery.* Nucleic acids research, 1997. **25**(15): p. 3095-101.

60.   Godbey, W.T., K.K. Wu, and A.G. Mikos, *Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle.* J Biomed Mater Res, 1999. **45**(3): p. 268-75.

61.   Fischer, D., et al., *In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis.* Biomaterials, 2003. **24**(7): p. 1121-31.

62.   Fischer, D., et al., *A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity.* Pharm Res, 1999. **16**(8): p. 1273-9.

63.   Neu, M., D. Fischer, and T. Kissel, *Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives.* J Gene Med, 2005. **7**(8): p. 992-1009.

64.   Brus, C., et al., *Physicochemical and biological characterization of polyethylenimine-graft-poly(ethylene glycol) block copolymers as a delivery system for oligonucleotides and ribozymes.* Bioconjug Chem, 2004. **15**(4): p. 677-84.

65.   Petersen, H., et al., *Polyethylenimine-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system.* Bioconjug Chem, 2002. **13**(4): p. 845-54.

66.   Merdan, T., et al., *PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice.* Bioconjug Chem, 2005. **16**(4): p. 785-92.

67.   Cherng, J.Y., et al., *Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles.* Pharm Res, 1996. **13**(7): p. 1038-42.

68.   Plamper, F., Schmalz, A, Penott-Chang, E, Drechsler, M, Jusufi, A, Ballauff, M, and Müller, A *Synthesis and Characterization of Star-Shaped Poly(N,N-dimethylaminoethyl methacrylate) and Its Quaternized Ammonium Salts.* Macromolecules, 2007. **40**(16): p. 5689-5697.

69.   Sahnoun, M., Charreyre, M, Veron, L, Delair, T, D'Agosto, F, *Synthetic and characterization aspects of dimethylaminoethyl methacrylate reversible addition fragmentation chain transfer (RAFT) polymerization.* Journal of Polymer Science Part A: Polymer Chemistry, 2005. **43**(16): p. 3551–3565.

70.   van de Wetering, P., Cherng, J, Talsma, H, Hennink, W, *Relation between transfection efficiency and cytotoxicity of poly(2-(dimethylamino)ethyl methacrylate) /plasmid complexes.* Journal of Controlled Release, 1997. **49**(1): p. 59-69.

71.   van de Wetering, P., et al., *Comparative transfection studies of human ovarian carcinoma cells in vitro, ex vivo and in vivo with poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes.* J Gene Med, 1999. **1**(3): p. 156-65.

72.   Synatschke, C.V., et al., *Influence of polymer architecture and molecular weight of poly(2-(dimethylamino)ethyl methacrylate) polycations on transfection efficiency and cell viability in gene delivery.* Biomacromolecules, 2011. **12**(12): p. 4247-55.

73.   Layman, J.M., et al., *Influence of polycation molecular weight on poly(2-dimethylaminoethyl methacrylate)-mediated DNA delivery in vitro.* Biomacromolecules, 2009. **10**(5): p. 1244-52.

74.   Funhoff, A.M., et al., *Endosomal escape of polymeric gene delivery complexes is not always enhanced by polymers buffering at low pH.* Biomacromolecules, 2004. **5**(1): p. 32-9.

75.   Lin, S., et al., *An acid-labile block copolymer of PDMAEMA and PEG as potential carrier for intelligent gene delivery systems.* Biomacromolecules, 2008. **9**(1): p. 109-15.

76.   You, Y.Z., et al., *Reducible poly(2-dimethylaminoethyl methacrylate): synthesis, cytotoxicity, and gene delivery activity.* J Control Release, 2007. **122**(3): p. 217-25.

77.   van der Aa, M.A., et al., *Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells.* Pharm Res, 2007. **24**(8): p. 1590-8.

78. Chiefari, J., et al., *Living free-radical polymerization by reversible addition-fragmentation chain transfer: The RAFT process.* Macromolecules, 1998. **31**(16): p. 5559-5562.

79. McCormick, C.L. and A.B. Lowe, *Aqueous RAFT polymerization: recent developments in synthesis of functional water-soluble (co)polymers with controlled structures.* Accounts of chemical research, 2004. **37**(5): p. 312-25.

80. Bainbridge, J.W., M.H. Tan, and R.R. Ali, *Gene therapy progress and prospects: the eye.* Gene therapy, 2006. **13**(16): p. 1191-7.

81. Wu, C, Gong, X, *Laser Light Scattering: Some Recent Developments.* Encyclopedia Of Polymer Science and Technology., 2010.

82. Clark, N., Lunacek, J, Benedek, G, *A study of Brownian Motion Using Light Scaterring.* American Journal of Physics, 1970. **38**(3).

83. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.

84. Pathak, A., et al., *Engineered polyallylamine nanoparticles for efficient in vitro transfection.* Pharm Res, 2007. **24**(8): p. 1427-40.

85. Samsonova, O., et al., *Low Molecular Weight pDMAEMA-block-pHEMA Block-Copolymers Synthesized via RAFT-Polymerization: Potential Non-Viral Gene Delivery Agents?* Polymers, 2011. **3**(2): p. 693-718.

86. Cherng, J.Y., et al., *Long term stability of poly((2-dimethylamino)ethyl methacrylate)-based gene delivery systems.* Pharm Res, 1999. **16**(9): p. 1417-23.

87. Romoren, K., et al., *Long-term stability of chitosan-based polyplexes.* Pharm Res, 2004. **21**(12): p. 2340-6.

88. Oliveira, A.V., et al., *Transfection efficiency of chitosan and thiolated chitosan in retinal pigment epithelium cells: A comparative study.* J Pharm Bioallied Sci, 2013. **5**(2): p. 111-8.

89. Zuidam, N.J., et al., *Effects of physicochemical characteristics of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes on cellular association and internalization.* J Drug Target, 2000. **8**(1): p. 51-66.

90. Xin, X.Q., Y.M. Wang, and W. Liu, *Synthesis of zwitterionic block copolymers via RAFT polymerization.* European Polymer Journal, 2005. **41**(7): p. 1539-1545.