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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Efficient and synergistic gene delivery mediated by a combined polymeric-based nanosystem

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Henrique Manuel dos Santos Faneca (Centro de Neurociências de Coimbra) e da Professora Doutora Paula Cristina Veríssimo

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2015

AGRADECIMENTOS

Gostaria de começar por agradecer ao Dr. Henrique Faneca por todo apoio e orientação prestados durante o trabalho e por todo o tempo e paciência a mim dedicados durante este ano. Estou muito grata pela oportunidade de trabalhar num tema tão empolgante.

Quero também deixar umas palavras de agradecimento à Professora Doutora Paula Veríssimo, por ter aceitado ser minha orientadora interna e pela competência e apoio que sempre dedicou aos alunos de Bioquímica.

Gostaria também de dedicar um agradecimento especial à Dina e à Rose por me terem acolhido, ensinado e ajudado em todos os momentos.

Aos amigos, a família que me acolheu em Coimbra, e que partilhou os maus e os bons, os muito maus e os muito bons momentos desta caminhada. Estarei sempre grata pela vossa paciência e por tudo o resto.

Por fim, um agradecimento a toda a minha família, por todo o apoio e dedicação e pela certeza que aqui não estaria sem eles. Dedico ainda um agradecimento especial à minha irmã e à minha mãe por acreditarem em mim em todas as situações.

Muito obrigada!

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ABSTRACT

Gene therapy is believed to have the necessary characteristics to become a frontline treatment in a variety of diseases, including cancer. To reach its full potential it is still necessary to develop suitable vectors for the transport and delivery of genetic material into target cells. Currently, the most used vectors are of viral type. However, non-viral strategies have been emerging in the last decades as promising alternatives, and the most prominent examples are liposomes and cationic polymers. These are advantageous especially due to the fact that they are safe and their properties are easy to manipulate. In spite of these advantages, most of the used non-viral systems still demonstrate low efficiencies in transfection.

The objective of this work was to investigate the gene delivery potential of novel non-viral vectors, constituted by different combinations of two polymers, to efficiently transport and delivery DNA into target cancer cells. In order to do so, their transfection activity was measured in different cell lines through luminescence, fluorescence microscopy and flow cytometry. Other parameters like their cytotoxicity, degree of DNA condensation, size and surface charge were also evaluated.

The obtained results have demonstrated that our formulations are good candidates for gene delivery, due to their potent transfection efficiencies when compared to a "golden standard" polymeric-based gene delivery system, branched polyethylenimine (bPEI). From the developed combined formulations, C_E-based polyplexes, prepared at the 100/1 N/P ratio, were selected as the best formulation, owing to their great transfection activity, even in the presence of serum, that is much higher than that obtained with polyplexes prepared with each one of the two polymers, and to their reduced levels of cytotoxicity. Furthermore, the other experimental studies revealed that the polyplexes designed with this combination of polymers present suitable

physicochemical properties for in vivo applications, namely a high level of DNA protection and a reduced mean diameter.

RESUMO

A terapia génica é considerada uma estratégia terapêutica com as características necessárias para se tornar um tratamento de primeira linha para uma grande variedade de doenças, incluindo o cancro. Apesar disso, para atingir o seu potencial ainda é necessário desenvolver vectores adequados para o transporte e entrega de material genético às células alvo. Actualmente, a maioria dos vectores utilizados são de tipo viral. No entanto, nas últimas décadas, as estratégias do tipo não-viral têm vindo a impor-se como alternativas promissoras, sendo os mais proeminentes exemplos os lipossomas e os polímeros catiónicos. Estes sistemas são vantajosos especialmente na medida em que são seguros e as suas propriedades são fáceis de manipular. Apesar destas vantagens, a maioria destes vectores ainda demonstra uma baixa eficiência de transfecção.

O objectivo deste trabalho foi investigar o potencial de novos vectores não-virais, constituídos por diferentes combinações de dois polímeros, para transportar e entregar o material genético de forma eficiente a células cancerígenas. Para tal, a eficiência de transfecção foi avaliada em diferentes linhas celulares através dos ensaios de luminescência, microscopia de fluorescência e citometria de fluxo. Foram também avaliados outros parâmetros como a sua citotoxicidade, capacidade de condensação do DNA, tamanho e carga superficial.

Os resultados obtidos mostraram que as nossas formulações são bons candidatos para entrega de material genético, devido à sua potente eficiência de transfecção quando comparada com a obtida com a referência padrão dos sistemas de base polimérica, polietilenimina ramificada (bPEI). Das formulações desenvolvidas envolvendo a combinação de polímeros, os poliplexos com base na combinação C_E preparados na razão N/P de 100/1 foram selecionados como a melhor formulação, devido à sua

elevada atividade de transfecção, mesmo na presença de soro, que é muito superior à obtida com os poliplexos preparados com cada um dos dois polímeros individualmente, e aos reduzidos níveis de citotoxicidade. Adicionalmente, os outros ensaios experimentais demonstraram que os poliplexos desenvolvidos com esta combinação de polímeros apresentam propriedades físico-químicas favoráveis à sua aplicação *in vivo*, nomeadamente elevada proteção de material genético e um diâmetro médio reduzido.

LIST OF ABBREVIATIONS

AAV – Adeno-associated virus

AdV - Adenovirus

CO_2 – Carbon dioxide

DLS – Dynamic light scattering

DMEM – Dulbecco's Modified Eagle's Medium

DMEM-HG - Dulbecco's Modified Eagle's Medium – high glucose

DNA – Deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

EtBr – Ethidium bromide

FBS – Fetal bovine serum

GFP – Green fluorescent protein

Luc - luciferase

$MgCl_2$ – Magnesium chloride

mRNA – Messenger ribonucleic acid

NP - nanoparticle

PAMAM - Polyamidoamine

PβAE - Poly(beta-amino ester)

PBS - Phosphate-buffered saline

PDI – Polydispersity index

pDNA – Plasmid of deoxyribonucleic acid

PEG - Polyethylene glycol

PEI – Polyethylenimine

bPEI – Branched polyethylenimine

PLL - Poly-L-lysine

RES - reticuloendothelial system

RNA - Ribonucleic acid

RPMI - Roswell Park Memorial Institute medium

SCID-X1 - X-linked severe combined immunodeficiency

siRNA – Small interfering ribonucleic acid

KEYWORDS

Gene therapy

Gene delivery

Polyplexes

Cationic polymers

1. Introduction

INTRODUCTION

1.1. Gene therapy

The first evidences of the existence of genes came to light with the work of Mendel, in the 19th century. Since then, new and groundbreaking findings, like the discovery of double stranded helix DNA structure and several key enzymes, have been reported and have marked a slow but steady path towards what we know today as genetic engineering and have allowed scientists to contemplate gene therapy for more than 30 years.[1,2]

Gene therapy can be defined as any procedure projected to treat or alleviate a disease through a genetic modification of the target cells of a patient.[3] This can be done by the introduction of DNA or RNA.

It can be directed either to germ cells or somatic cells. In germ cell therapy, the objective is the introduction of functional genes into the genome of sperm or egg cells. The changes made are passed on to following generations and it would be highly effective in counteracting genetic disease and hereditary disorders. In the other type of gene therapy, called somatic therapy, the target cells are the somatic cells of the patient. Any modifications will be restricted to the individual. The latter is a more viable alternative for the near future for technical and ethical reasons.[1,4]

The use of genetic material transfer protocols is being studied to treat a multitude of diseases, including inherited diseases, cancers and other acquired disorders that include but are not limited to cystic fibrosis, hemophilia, muscular dystrophy, sickle cell anemia, cancer, AIDS, heart disease, diabetes mellitus, arthritis and Alzheimer's.[1,5] The majority (64%) of gene therapy clinical trials to date have addressed cancer and more than 25% are directed to monogenic, cardiovascular and infectious diseases (Figure 1).

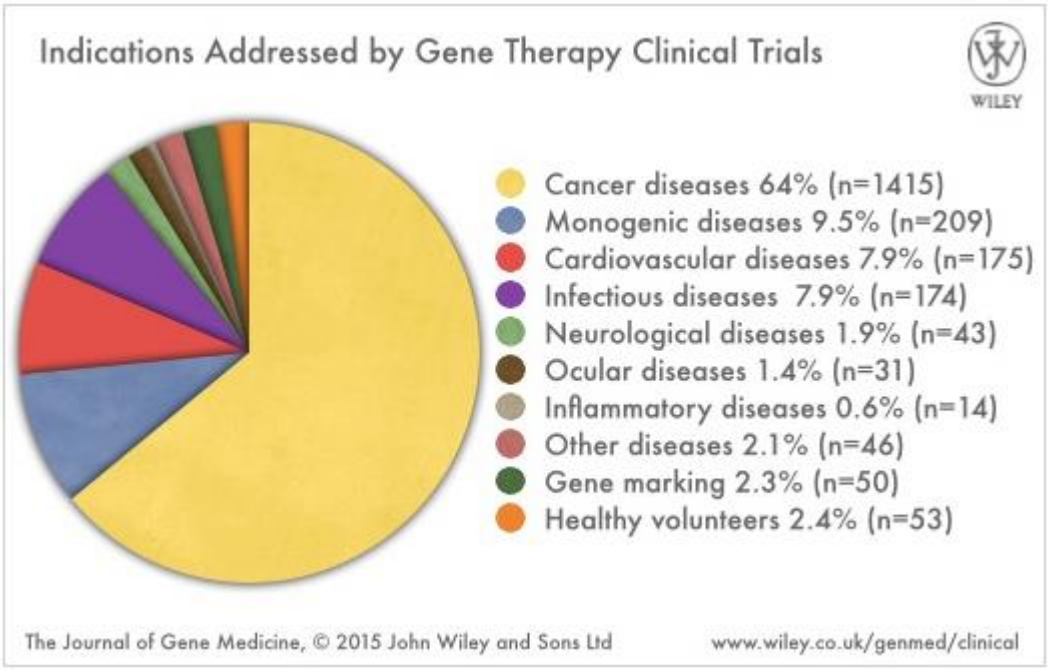

Figure 1. Indications addressed by gene therapy clinical trials up to 2015. The most commonly addressed types of diseases in gene therapy trials are cancer, monogenic, cardiovascular and infectious diseases, representing 89% of all trials. (Source: www.wiley.co.uk/genmed/clinical)

In 1990, the first clinical trial of gene therapy with a therapeutic purpose was initiated for adenosine deaminase deficiency. Since then, the number of clinical protocols initiated worldwide has increased greatly (Figure 2). Despite the great success reported in some of the cases, there were also severe adverse reactions reported. One of the most infamous examples took place in 2000, when a patient of a initially very successful trial to treat X-linked severe combined immunodeficiency (SCID-X1) developed T cell leukemia, as a result of insertional mutagenesis provoked by the retrovirus used as a vector.[5] Even though the confidence of the science community on gene therapy declines when confronted with results as such and the number of trials suffers a tendency to drop in years immediately following reports of severe adverse reactions, the overall trend is an increase or maintenance in the number of trials over the years (Figure 2).

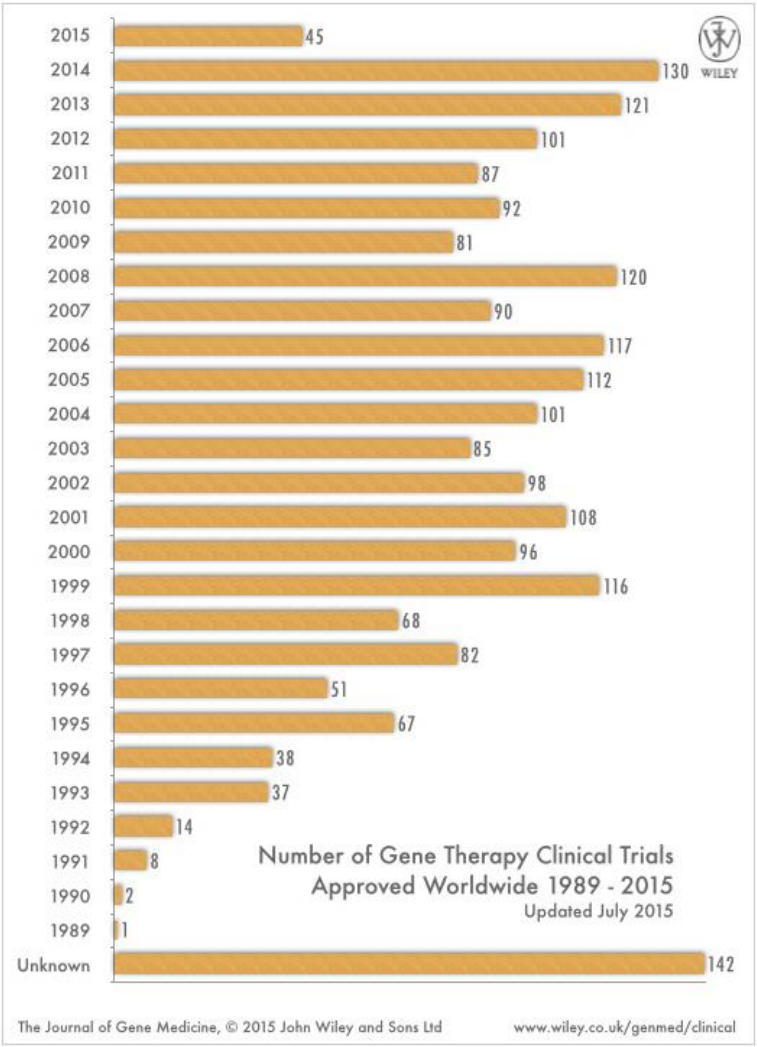

Figure 2. Number of gene therapy clinical trials approved worldwide up to 2015. The number of trials approved had a tendency to increase exponentially in the first 10 years and since then has been maintained at around 100 trials per year.

Although being generally accepted as a strategy with great potential, gene therapy has still some major limitations. In many situations the cells containing the therapeutic genetic material are short lived and unstable making for a transient expression of the gene, and consequently preventing it from being a permanent cure. Moreover, even though the current used gene therapies are best aimed for conditions that arise from a single gene, most common disorders are caused by the combined effects of variations in multiple genes.[1] On top of that, the vectors used to transport and deliver genetic material can provoke unwanted reactions, which will be further discussed in the next section.

The complexity of gene therapy remains a challenge due to the extensively investigation needed prior the development of the technique, including a comprehensive understanding of the disease, its link to genetic malfunction and the associated gene.

Before the fulfillment of these very ambitious goals, a number of ethic and social considerations should be taken into account and strict guidelines are essential to monitor, ensure safety and increase confidence in the gene therapy procedures.

1.2. Delivery vectors

The use of molecules like DNA or RNA as therapeutic material is specially challenging because they have to act intracellularly and are usually more fragile than smaller molecules. It is therefore essential both the protection against degradation and the enhancement of transgene expression provided by vectors[6-8]. These vectors, which can be of several types, can be divided into two major categories: viral and non-viral vectors. Their efficiency is usually defined based on their transduction/transfection efficiencies, respectively. These terms refer to all the course of the vector including entrance in the cell and subsequent gene expression.[2]

From administration up to reaching the target, there are a number of barriers that must be overcome, both extra and intracellularly (Figure 3).

The host immune response is one of the main limitation to the use of viral vectors since the immune system has always evolved to fight and eliminate pathogens, including virus.[9] However, immune system does not affect only vectors of viral type but can also be a problem for non-viral gene delivery namely through the innate immune system since the carriers can be eliminated through processes like complement-mediated clearance, opsonization and phagocytosis.[10]

Once in the systemic circulation, vectors must not only avoid an immune system reaction but also escape recognition by the reticuloendothelial system (RES), which

could translate into a rapid removal via phagocytosis. Smaller particles show less uptake via the RES.[11]

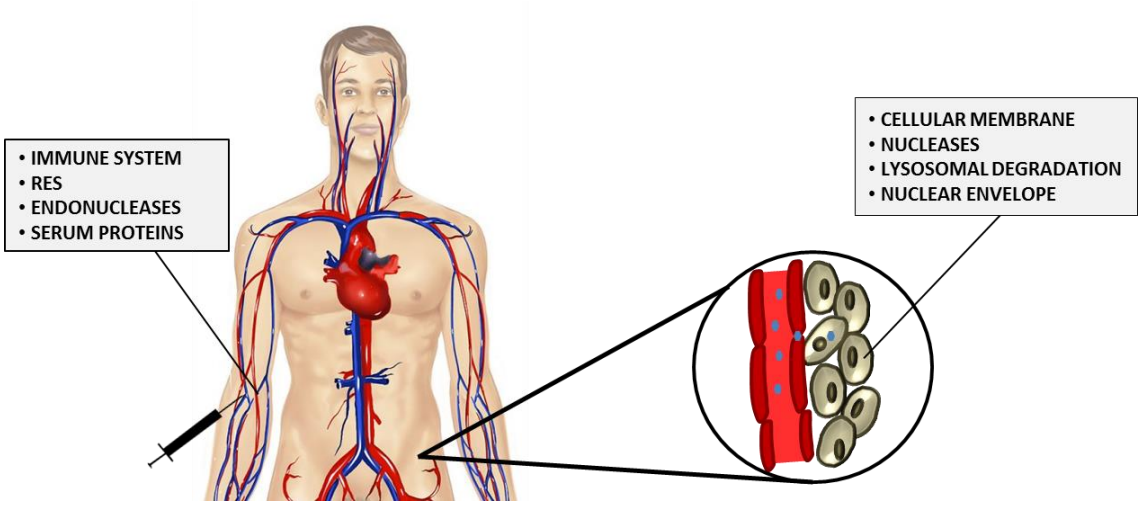

Figure 3. Extra and intracellular barriers to gene therapy. From administration up to reaching the nucleus of the host cell, gene delivery systems are confronted with many physical and chemical obstacles.

The extracellular matrix also contains components that can bind to the complexes like glycosaminoglycans, which can also be found on cell surface and whose presence influences gene delivery efficiency[12]. Other proteins of the serum are able to potentially form complexes with the polymeric systems, with special mention to albumin.[13] The aggregated formed with these proteins can cause not only a decrease in gene expression but also cytotoxicity, for example from lung embolization.[14] Moreover, there are endonucleases present in the serum, which can enzymatically degrade DNA, reducing the amount of DNA available and the possibilities to achieve desirable gene expression levels.[7,14]

Depending on the method of administration which can assume many forms, from intravenous injections, topical applications, oral delivery, amongst others, the vectors must pass several physical barriers until reaching the target, such as endothelial barriers and cellular and compartment membranes.[7]

In the case of delivery of DNA, it needs to reach the nucleus, i.e., to surpass the nuclear membrane, making the most slowly dividing cells harder to transfect and transduce because of the less frequent breakdown of the nuclear envelope.[14]

There is a variety of factors involved in the successful delivery of DNA, from the chemical and supramolecular structure of the genetic delivery system, to their interactions with the membrane and capacity to release of DNA.[15]

1.3. Viral vectors

Viruses usually bind to target cells and introduce their genetic materials into the host cell as part of their replication process. Taking advantage of this natural ability, genetic engineering is used to remove the infectious part of the virus and replace it with functional human genes.[16]

Even though this class of vectors has demonstrated to be highly effective in delivering genes, it presents some limitations, especially concerning safety. Side effects following the treatments have been reported, and toxicity, mutagenicity and immunogenicity have raised great concern.[1,3]

The different types of viruses used as gene therapy vectors include adenovirus (AdV), retrovirus, vaccinia virus, adeno-associated-virus (AAV), amongst others. (Figure 4)

Adenovirus is the most commonly used class of virus accounting for 22% of all vectors used in gene therapy clinical trials. Adenoviruses present some characteristics that from the genesis of gene therapy made them naturally attractive to use as vectors: their infectious properties and the natural delivery of the viral genome in the nucleus are crucial to their success.

As vectors they have advantages over other types of viruses like the ample space available (up to 37 Kb foreign genetic material can be inserted), which allows the

transport of larger therapeutic genes and they can be purified to high titers relatively easily, simplifying the process of scaling-up.[17-19]

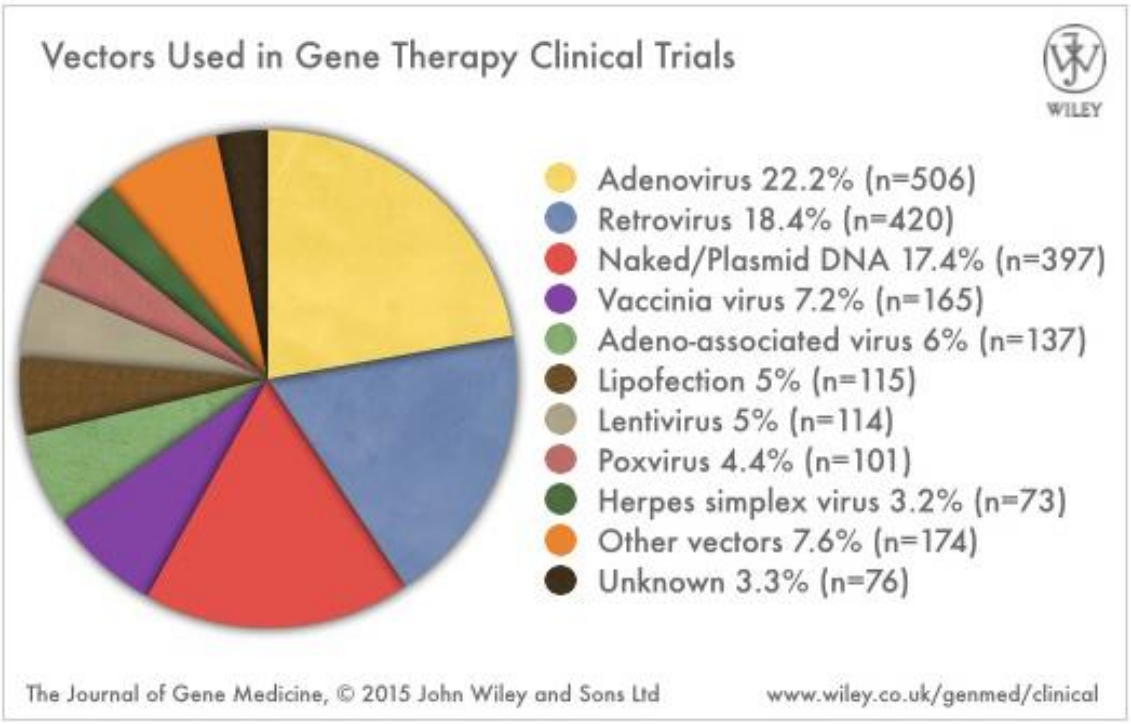

Figure 4. Vectors used in gene therapy clinical trials. The most commonly used vectors in gene therapy clinical trials are adenovirus and retrovirus. The most used non-viral approach is naked/plasmid DNA. (Source: www.wiley.co.uk/genmed/clinical)

The vector genome remains episomal, i.e., it is not integrated in the genome of the cell. This implies a transient expression. In gene therapy, the period of time for which the expression is needed depends on the disease being treated. Most genetic diseases require lifelong expression whereas acquires disorders like cancer may only require expression for a limited period of time. However, since the gene is not integrated in the genome of the host cell, there is no risk of potentially dangerous issues such as random activation of an oncogene or the alteration of the expression of an important endogenous gene (Figure 5).[16,17]

There are two major limitations to the use of adenoviral vectors. The first one is due to the fact that around 80% of healthy people have antibodies against one or more of the more than 40 serotypes of AdV which prevents their use. The second one is the severe toxicity provoked by high immunogenicity with immediate innate immune

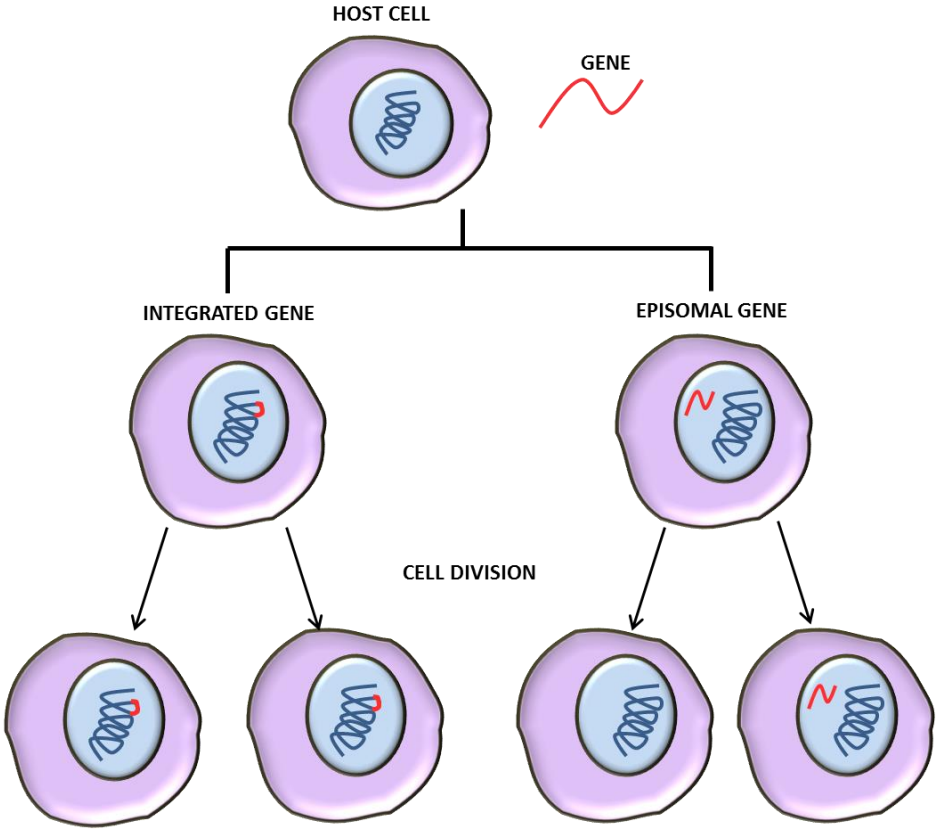

Figure 5. Influence of integrated or episomal gene insertion on gene expression in daughter cells. When genes are integrated into the host cell genome, the foreign genetic information is passed on to next generations,

response and a secondary antigen-dependent response that limits the number of administrations in the same patient.[17,18]

Retroviruses were the first viruses to be used as vectors in gene therapy and are the second most common in gene therapy clinical trials. Unlike adenovirus, retroviruses integrate their genome into host cell genome, leading to a permanent expression. This can be used as an advantage, but it also raises safety concerns: since the integration is mostly random, there is a high risk of insertional mutagenesis.[1,17,18]

Vectors based on adeno-associated viruses (AAV) have been increasingly popular in the last years.[20] These viruses have the particularity of needing co-infection with AdV or herpes simplex virus to complete its replication cycle. They constitute attractive vectors for gene therapy due to their low immunogenicity, long term gene expression

and the ability to infect both dividing and quiescent cells, but perhaps the main reason of the great interest in this class of virus is the fact that they insert genetic material on a specific site (chromosome 19). In spite of these properties, they also present a major limitation to their use in its limited packaging capacity, of only up to 4.1-4.9 Kb.[1,18,21]

Table 1. Summary of adenoviral, retroviral and adeno-associated viral vectors characteristics.

Vector subtype	Transduce non-dividing cells	Integration	Immune response	Payload	Production Scale-up
Adenovirus	Yes	No	High	High	Moderate
Retrovirus	No	Yes	Low	High	Complex
Adeno-associated virus	Yes	Yes	Low	Low	Complex

The future of viral-based vectors relies on the introduction of novel vectors or modifications on the already existing ones. Thanks to vector engineering, the most attractive characteristics for gene therapy of the wild-type viruses are maintained while alterations are made so that they become better vectors. These alterations may implicate a wide variety of features, for example, a better cell infection, the capacity of selectively targeting certain cellular receptors or molecular defects, or an increase in the packaging capacity.[3,22]

1.4. Non-viral vectors

For non-viral vectors, different approaches have been utilized, using physical or chemical modes of genetic material transfer. Generally, non-viral approaches are advantageous in terms of safety and easy modifiability, but present a lower transfection efficiency compared to viral vectors.[3,14]

1.4.1. Physical methods

Physical transfer of genetic material into target cells involves the injection of naked DNA. As simple and straightforward as this strategy may sound, its low transfection efficiency in most cells is a limiting factor. However, it does permit long-term expression in some types of cells and with the development of mechanical techniques like electroporation, the gene expression is reaching higher efficiencies.[8]

Gene gun is a technique based on the bombardment of microparticles (usually gold beads) into the target cell. The DNA is precipitated onto the particles and is released once it is inside the cell. This technology main application is genetic immunization.[23,24]

Most physical methods rely on the principle of destabilizing the cell membrane to enhance its permeability, allowing the entrance of exogenous molecules, such as DNA.[23] This disturbance can be achieved through the use of high intensity electrical pulses (electroporation)[25,26], ultrasounds (sonoporation)[27,28] or a hydrodynamic force (hydrodynamic delivery)[29,30].

Despite its limitations, the delivery of naked DNA assumes a prominent position in gene therapy clinical trials (Figure 6).

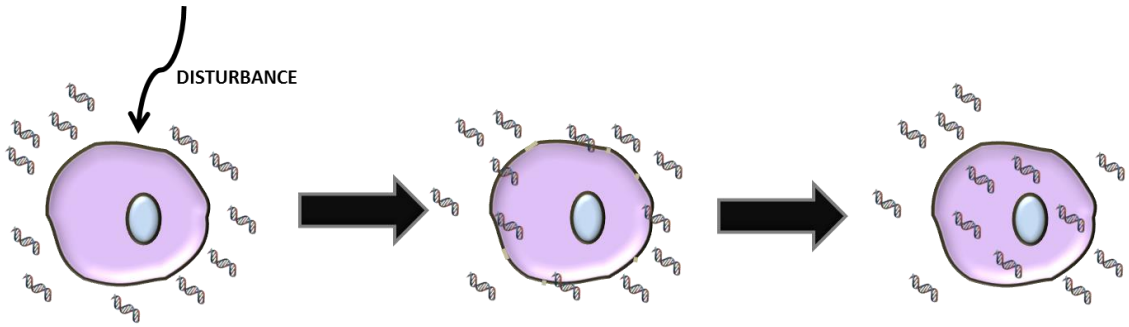

Figure 6. Physical methods to transfect genetic material. Methods like electroporation, sonoporation or hydrodynamic delivery are based on a disturbance on the cell membrane that permeabilizes it, allowing the entrance of genetic material. The membrane restores its integrity after some time.

1.4.2. Chemical methods

Nanoparticles are the most popular alternative to viral vectors. They are safer as they do not elicit a specific immune response to the vector, which ultimately allows for repeated administrations. They also present advantages such as the larger genetic packaging they allow and the ease to synthesize. The development of new and improved nanocarriers has been a vital field in medicine and health care.[6,14,31]

Nanocarriers may be polymer-based (e.g., polymeric nanoparticles, polymeric micelles, dendrimers), lipid-based (e.g., liposomes, solid lipid nanoparticles) and metal-based (e.g., gold, silica).[32] Increasing numbers of clinical trials, research reports, and approved drug products have placed liposomes and biodegradable polymeric NPs as the dominant classes of nanocarriers amongst all the non-viral approaches.[33] Both classes have advantages and limitations in terms of their physicochemical and biological properties.

Liposomes present themselves as good alternatives for gene therapy mostly because of their versatility, since the library of natural and synthetic lipids available is vast; and their safety, compared to viral counterparts. Since this class of molecules is highly present on humans, namely on membranes, lipids, mostly of natural sources, are usually biocompatible and biodegradable, with little to no toxicity and do not provoke severe immune reactions. Cationic lipids have the ability to effectively condense genetic material, and are usually used, many times along with a neutral helper lipid, as a vector to transport DNA.[34,35]

Even though they present promising features, liposomal vectors have some shortcomings such as the chemical and physical stability, problems with reproducibility from batch to batch and scaling-up.[33]

Generally, polymeric nanoparticles present a higher structural integrity compared to liposomes. They are also advantageous because of the greater variety of preparation methods, availability of various polymers and ease to manipulate their characteristics, which makes it easier to develop novel and improved delivery systems and also to develop systems in a rational manner.[32,33] One of the biggest shortcomings of polymeric nanoparticles is their low biocompatibility, resulting in cytotoxicity.[33,36]

These classes of nanoparticles can be used not only to carry genetic material, but also low molecular weight chemotherapeutics and proteins. They are also useful to overcome problems associated to the administration of drugs like the insufficient drug concentration on target tissue, due to poor absorption; rapid metabolism and elimination; poor drug solubility; and high fluctuation of plasma concentrations. These features will lead to a better bioavailability control, reduced off-target toxicity and lower administration frequency.[31,37,38]

Nanoparticles can be used as delivery systems for a diversity of therapeutic molecules such as anticancer[39] and antibacterial[40] agents, imaging and probing agents[41], hormones[42], proteins[43], vaccines[44] and genetic material[45,46]. These can be encapsulated inside the nanoparticles, adsorbed or covalently attached onto the carriers surface.[6]

The new non-viral vectors can be developed to be biodegradable, biocompatible, targeted and stimulus responsive.[31] Some of the most wanted characteristics in a nanocarrier are the ability to protect the material from premature degradation and premature interaction with the biological environment, the capacity to enhance the drug absorption into a selected tissue, the ability to control the pharmacokinetic and drug tissue distribution profile, and the capacity to improve intracellular penetration.[31,32,47]

1.4.3. Targeting molecules

One of the most important things looked for in a good carrier is the targeting capacity, i.e., the ability to specifically target the disease site. The importance of this process is easily understood when taken into account the numerous deviations the system can suffer, especially when it is not locally administered. The greatest advantages of this technique are the fewer side effects provoked and the highest concentration of material on target, leading to better therapeutic results.[38,47]

In order to target exclusively a specific tissue, cell or intracellular organelle, the use of molecules that specifically bind to antigens or receptors on the target cell is required. The use of materials like polymers to construct vectors provides an opportunity to do so, since they are readily functionalized.[36,45]

To fully take advantage of such approach, a long work is needed particularly to better understand the mechanisms of diseases and to construct competent vectors.

The strategy described above is considered active targeting, since an active effort is made to direct nanoparticles towards a specific target. Alternatively, physiological and particle clearance processes can direct a particle to a determined site and an understanding of these can be used to control the destination of the system; an approach usually known as passive targeting.[6]

The enhanced permeation and retention (EPR) effect is an example of these mechanisms, and can be found on tumor vessels. Tumor tissues exhibit a leaky vasculature and, unlike other tissues, the extravasation is slow and the molecules retained accumulate in the tumor interstitium for a long time.[48,49]

1.4.4. Co-delivery

The use of more than one therapeutic agent is a very common approach, especially when referring to complex diseases like cancer. It is denominated combinatorial drug delivery, and can prove itself more effective than single drug therapy, showing advantages like synergistic effects between the two transported compounds, reversal of drug resistance and improvement of target selectivity. However, clinical results are not as successful as *in vitro* studies mainly due to varying pharmacokinetics among different drugs. To achieve a more precise and controlled result, a variety of delivery systems can be used to transport and subsequently deliver simultaneously the multiple therapeutic agents into the site of action.[38,50] However, this task is not easy since it is necessary to create systems ready to transport two fundamentally different therapeutic agents, for example when transporting a drug and genetic material.[51]

The most common approaches to co-encapsulate multiple agents into a single carrier are to physically load the materials and/or to chemically conjugate to the particle surface. There are also examples of covalent linkage to the polymer backbone prior to nanoparticle preparation[52] or pre-conjugating the drugs covalently through hydrolysable linkers[53]. These strategies can be used to obtain a better control of the loading yield and release kinetics of the different agents.

1.5. Cationic polymers

Polymers are viable transporters of materials *in vivo*, as was demonstrated several times[54,55], and present many advantages towards the more common carriers. As discussed above, polymeric nanoparticles present a high structural integrity, stability during storage and controlled release capability. In addition, they allow for a greater

control and higher range of properties like size and charge, in part due to the different polymers available and the different preparation methods that can be used.[32,33]

However, they also present some limitations that include use of toxic organic solvents in the production process, poor drug encapsulation for hydrophilic drugs, drug leakage before reaching target issues, polymer cytotoxicity, polymer degradation, and scale-up issues.[33]

Cationic polymers are an attractive alternative for the delivery of DNA, the positive surface charge is generally preferable for transfection since it is required for efficient binding and uptake by the cells. Cationic polymers can combine with DNA, to molecules of a relatively small size, forming complexes denominated polyplexes.[2,15]

Cationic polymers have, however, several limitations to their use in vivo, namely their cytotoxicity and non-specific interactions with serum proteins. These result in unwanted side effects and possible rapid elimination of the complexes from the blood, leading to low therapeutic effects.[56]

A popular approach to deal with these shortcomings is the conjugation with polyethylene glycol (PEG) (Figure 7). This polymer enhances the circulation half-life of the coated vectors, increases stability in serum and reduces cytotoxicity.[57,58]

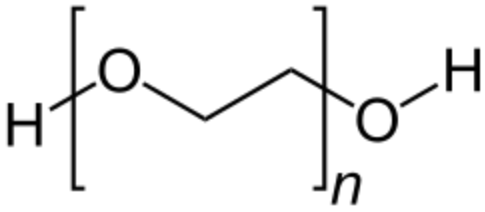

Figure 7. Structure of polyethylene glycol (PEG). PEG is used to coat poplyplexes in order to enhance their properties.

The polymers cytotoxicity and biodistribution problems can also be addressed through the manipulation of its characteristics like its molecular weight – small changes may have a strong impact on polyplexes properties, such as their size or surface charge.[59] Generally, the particles cannot be too large because they accumulate in the

liver and spleen and result in lower accumulations in tumors. However, the particles should be large enough to prevent their rapid leakage into blood capillaries.[60]

The surface charge of the particle has influence on the performance of the gene delivery systems for various reasons. At complex neutrality, there is tendency to aggregate with each other. An excess of polycation must then be used to condensate the DNA properly and to obtain smaller particles, which implicates a net positive charge. However, positively charged particles have a tendency to interact with serum proteins, causing loss of bioavailability. On the other hand, positive charge promotes the association of the nanoparticles with cellular membranes, facilitating the intracellular delivery. This effect can be negative, depending on the zeta potential, because the nanoparticles can also have untargeted cytotoxicity by disassembling cell membranes.[57,59,61]

Another advantage of cationic polymers is the possibility of using polymers with a responsive nature, i.e., polymers that can undergo physical or chemical changes in response to stimulus, usually releasing their cargo only when under their influence. These stimuli can be temperature and pH, but also ultrasound, ionic strength, redox potential, electromagnetic radiation, and chemical and biochemical agents.[43,62]

A great variety of cationic polymers have been explored for the transport and delivery of genetic material. The most commonly used are polyethylenimines (PEIs), poly-L-lysines (PLLs), polyarginines, chitosan and cationic PAMAM dendrimers as a result of their ability to form stable complexes via electrostatic interaction under physiological conditions.

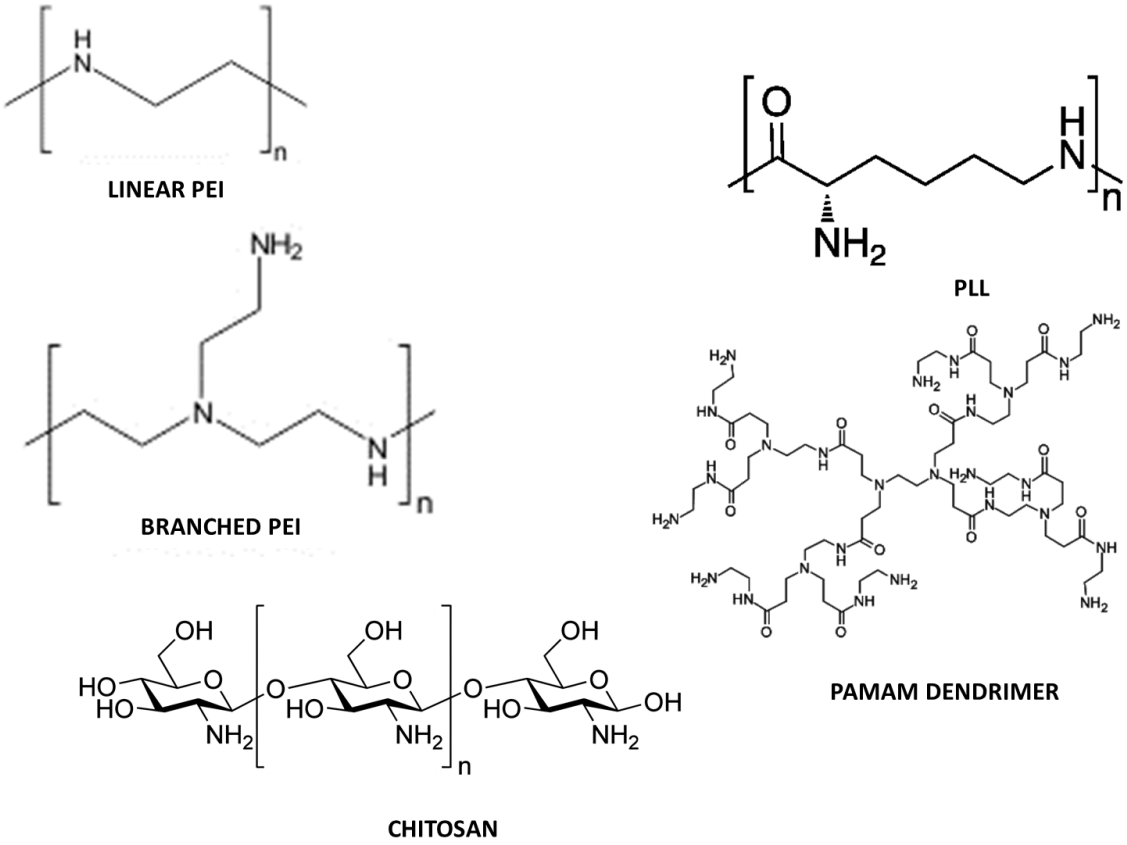

Figure 8. Strutcure of the most commonly used polymers for gene delivery.

PLL was one of the first cationic polymers to be considered to deliver genetic material due to its capacity to condensate DNA.[63] Although presenting good transfection characteristics, its high cytotoxicity is a great limitation that has been avoided by modifications of the PLL properties[64] and combination with other polymers like PEG[65].

Polyethylenimine (PEI) is an organic molecule with a high cationic charge density, and has been proven to be a highly efficient vector to deliver DNA both in vitro and in vivo.[66] To further increase the success of this polymer, additional modifications have been introduced to add target specificity and improve the biocompatibility for in vivo applications.[59] All of these characteristics make PEI and its variants one of the most studied vector for non-viral gene delivery and it is considered an example of a successful vector for non-viral transfection.[14,67]

In spite of the widespread use of PLL and PEI, these still present several limitations compared to viral vectors, especially regarding transfection efficiency. Hence, there is still room to search for new polymers for gene delivery.

Poly(β-amino ester)s (PβAE) are a class of polymers that has been recently studied for its DNA condensation and transfection capacities. These have been demonstrated to be effective transfection vectors and to have a very low cytotoxicity. As a single modification in the structure can have substantial effects on the delivery capacity, numerous modifications of PβAE have been explored, especially concerning end-modifications[68], and many of them have been studied through high-throughput screenings to better understand the relation between structure and function.[69]

On the other hand, chitosan is a derivative of a natural cationic polysaccharide and is a good candidate to gene delivery since it is able to form homogenous polyelectrolyte complexes with DNA. Chitosan is also easily chemically modified which can lead to a more efficient and target-specific vector.[70,71]

A popular strategy to attempt to overcome the limitations associated to each polymer is to create copolymers combining different polymers, for example chitosan and PEI[72], which many times demonstrate better characteristics than the individual polymers.

1.5.1. Cellular uptake and endosomal escape

For efficient transfection to occur, a multistep process has to be mediated by the delivery vector which includes DNA condensation, uptake into the cell, endosomal release, migration through the cytoplasm, uptake into the nucleus, and release of the DNA from the polymer.

The uptake into the cell is mainly attributed to the interaction between the positively charged particle and the negatively charged cell membrane, which is followed

by entrance on the cell by endocytosis. Depending on the some variables, mainly size and cell type, the internalization may occur via , clathrin-dependent pathways, caveolae-dependent pathways or micropinocytosis (particles smaller than 200 nm are associated with clathrin-dependent pathways, whereas larger particles are associated with micropinocytosis).[73]

It is known that the polyplexes that follow the endolysosomal pathway are able to escape, however the mechanisms of it are not yet fully clear.[15] One of the hypothesis for the polyplexes escape from the endosomes is the effect of the endosome low pH in increasing the proportion of protonated nitrogens on the polymers which will then generate a charge gradient that induces a Cl^- influx, which in turn induces a water influx, and, ultimately, endosome swelling and rupture. The vesicle rupture is also attributed to the possible interactions established between the polyplexes and the endosomal membrane.[15,67]

Following escape from endosomes, polyplexes need to approach and enter into the nucleus, as well as dissociate themselves, allowing the release of the genetic material. A high affinity of polycations for DNA may be a limiting step in successful transfection, because of the difficulty in the separation of the DNA from the carrier. In this regard, strategies that can facilitate the release of DNA have been developed, like the utilization of polymers that can be degraded by the intracellular environment.[74]

1.6. Aims

The aims of this project were to investigate the capacity of several combinations of two polymers, P1 and P2, to form polyplexes with pDNA and to study the physico-chemical characteristics of these polyplexes (size, surface charge and condensation), as well as their transfection capacity and cytotoxicity in order to find competent, biocompatible non-viral vectors for gene delivery into cancer cells.

2. Material and Methods

MATERIALS AND METHODS

2.1. Materials

The polymers were produced by Michael addition reaction and atom transfer radical polymerization.

2.2. Cell lines and culture conditions

2.2.1. COS-7, HeLa and MDA-MB-231 cell lines

The COS-7 cell line was derived from the kidney of the African Green Monkey, *Cercopithecus aethiops*. These cells resemble human fibroblast cells and are often used for transfections in cell biology experiments. The MDA-MB-231 cell line was isolated from pleural effusions of a Caucasian breast cancer patient. The HeLa cell line is a human epithelial cell line and is one of the most common cell lines used in research.

The COS-7 and the HeLa cells were maintained in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Sigma-Aldrich, MO, USA), supplemented with 10% (V/V heat-inactivated fetal bovine serum (FBS)) (Sigma–Aldrich, MO, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37ºC, under 5% CO_2. The cells grew in 75 cm^2 Corning® flasks up to 80% confluency and were split twice a week, according to the following protocol: firstly the medium on the flask where the cells were being maintained was poured off into waste, and they were washed with PBS. Then, 1.5 mL of trypsin was added and they were kept in the incubator for no more than 10 minutes until the cells are brought into suspension. After that, 8.5 mL of medium was added to inhibit the trypsin action and then the COS-7 and HeLa cell lines were split 1:10 and 1:20, respectively.

The MDA-MB-231 cell line was maintained in RPMI, in the same conditions as previously described for COS-7 and HeLa cells. It was split 1:5 twice a week, but instead of using trypsin to bring the cells to suspension, 3 mL of dissociation medium was used.

2.3. Biological Activity

2.3.1. *In vitro* transfection activity – luminescence assay

To evaluate transfection activity mediated by the different combinations of polymers, a plasmid containing the luciferase gene was used to prepare the polyplexes. Polymers were dissolved in milli-Q water, mixed with 1 µg of pCMV-Luc, at the desired polymer/DNA (N/P, +/-) charge ratio, and then the mixture was incubated for 15 min at room temperature. The polymer/DNA complexes (polyplexes) were prepared immediately before being used.

The seeding of the COS-7 cell line for the biological activity studies was made when the cells were 70-80% confluent, so they were still in the optimal phase of growth. Firstly, the cells were brought into suspension and the cell density in the suspension was determined by using a haemocytomer. For evaluation of luciferase expression, 3.5×10^4 cells/well of COS-7 cells; 2.5×10^4 cells/well of HeLa cells and 6×10^4 cells/well of MDA-MB-231 cells were seeded in 48-well culture plates. 24 hours later, cells were rinsed with PBS and then covered with 0.3 ml of DMEM-HG (with or without serum). Following the gentle addition of the polyplexes, there was a period of 4 h incubation (5% CO_2 at 37ºC) after which the transfection medium was replaced with DMEM-HG containing 10% (v/v) FBS and antibiotics, and the cells were further incubated for 48 h to allow gene expression.

At that time, cells were washed twice with phosphate-buffered saline solution (PBS) and 100 µL of lysis buffer [1 mM dithiothreitol; 1 mM EDTA; 25 mM Tris-phosphate (pH 7.8); 8 mM $MgCl_2$; 15% glycerol; 1% v/v Triton^{TM} X-100] were added to each well. The quantification of luciferase expression in cell lysates was evaluated by measuring light production by luciferase in a Lmax II384 luminometer (Molecular Devices). The protein content of the lysates was measured by the DC TM Protein Assay reagent (Biorad) using bovine serum albumin as a standard. The data was expressed as relative light units (RLU) of luciferase per mg of total cell protein.

2.3.2. *In vitro* transfection efficiency – flow citometry

Transfection efficiency was evaluated through flow cytometry, by analyzing green fluorescent protein (GFP) expression, a protein found in many marine animals and firstly isolated from jellyfishes. It emits green light (peak at 509 nm) when excited by blue light[75] and is usually used as a reporter gene in a wide variety if biotechnological studies.[76-78] The detection of fluorescent cells is possible using flow cytometry, a laser-based technique that distinguishes particles or cells according to their characteristics, for example the expression of a fluorescent chromophore like GFP.[79]

1.4 x 10^5 cells/well of COS-7 cells and 1.0 x 10^5 cells/well of HeLa cells were seeded in 12-well culture plates and, after 24 hours, polyplexes containing 4 µg of pCMV-GFP were added to cells previously covered with 1 ml of DMEM-HG (with or without serum). After 4 hours incubation (5% CO_2 at 37°C), the transfection medium was replaced with DMEM-HG, containing 10% (v/v) FBS and antibiotics, and cells were further incubated for 48 hours. Cells were then washed twice with PBS and detached with trypsin (5 minutes at 37°C). Thereafter, cells were washed and resuspended in PBS, and immediately analyzed in a FACSCalibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Live cells were gated by forward/side scattering from a total of 20000 events, and data were analyzed using CellQuest™ software.

2.3.3. *In vitro* transfection efficiency – fluorescence microscopy

The analysis of GFP expression was also evaluated through fluorescence microscopy.

1.9 x 10^5 cells/well of COS-7 cells and 1.3 x 10^5 cells/well of HeLa cells were seeded in 12-well culture plates (previously covered with a coverslip) and after 24 hours polyplexes containing 4 μg of pCMV-GFP were added to the cells. After 4 hours incubation (5% CO_2 at 37°C), the transfection medium was replaced with DMEM-HG, containing 10% (v/v) FBS and antibiotics, and cells were further incubated for 48 hours. Cells were then washed twice with PB and mounted in MowiolR mounting medium (Sigma-Aldrich Co.). The images (original magnification: ×20) were obtained on an Axioskop 2 Plus microscope (Zeiss, Munich, Germany) using an AxioCam HRc camera (Zeiss).

2.3.4. Cell viability assay

Cell viability under the different experimental conditions was assessed, in parallel experiments, by a modified Alamar Blue Assay.[80] This assay measures the redox capacity of the cells due to the production of metabolites as a result of cell growth and allows determination of viability over the culture period without the detachment of adherent cells.

Briefly, 47h post-transfection cells were incubated with 0.3 ml of 10% (v/v) Alamar Blue dye in complete DMEM-HG medium, prepared from a 0.1 mg/mL stock solution of Alamar Blue. After 1h incubation at 37ºC, 180 μL of the supernatant were collected from each well and transferred to 96-well plates. The absorbance at 570 and

600 nm was measured in a SPECTRAmax PLUS 384 spectrophotometer (Molecular Devices). Cell viability (as a percentage of untreated control cells) was calculated according to the formula: $\frac{(\text{A570}-\text{A600})\text{ of treated cells}}{(\text{A570}-\text{A600})\text{ of control cells}}$ x 100.

2.4. Physico-chemical characterisation of the polyplexes

2.4.1 Dynamic Light Scattering and Zeta Potential Analysis

Dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). This technique uses the autocorrelation spectroscopy of scattered laser light to determine its time-dependent fluctuations resulting from the Brownian motion of particles in suspension.

The particle size distribution (in intensity), average hydrodynamic particle size average (z-average), and polydispersity index (PDI) were determined with Zetasizer 6.20 software. Measurements were made at 25ºC and at a backward scattering angle of 173º.

Zeta-potential measurements were performed using a Zetasizer Nano-ZS (Malvern Instruments Ltd.,), coupled to laser Doppler electrophoresis and determined using a Smoluchovski model.

Polymers were dissolved in milli-Q water and mixed with 4 µg of pCMV-Luc at the desired polymer/DNA (N/P, +/-) charge ratio. The mixture was incubated for 15 min at room temperature. The polyplexes were prepared immediately before analysis and two independent experiments were performed in triplicate for size and zeta potential measurements.

2.4.2. Ethidium bromide intercalation assay

The accessibility to the DNA on the polyplexes was analyzed using an ethidium bromide (EtBr) intercalation assay.

Polyplexes were prepared as described above and 50 µL of each sample was transferred into a black 96-well plate (Costar, Cambridge, CA, USA). Then, 50 µL of EtBr solution was added to achieve a final EtBr concentration of 400 nM. Following 10 min incubation, fluorescence was measured in a SpectraMax Gemini EM fluorometer (Molecular Devices, Sunnyvale, CA, USA) at λ_{exc} = 518 nm, λ_{em} = 605 nm.

The fluorescence scale was calibrated such that the initial fluorescence of EtBr (50 µL of EtBr solution were added to 50 µL of Milli-Q water to achieve a final EtBr of 400 nM) was set at residual fluorescence. The value of fluorescence obtained with 1 µg of naked DNA (control) was set as 100%. The amount of DNA available to interact with the probe was calculated by subtracting the values of residual fluorescence from those obtained for the samples and expressed as the percentage of the control.

2.4.3. Agarose gel electrophoresis assay

To further evaluate the condensation of the DNA in the polyplexes an electrophoresis in agarose gel was performed.

Polyplexes were prepared as described above and 20 µL of each sample was added to 5 µL of loading buffer (15% v/v Ficoll 400, 0.05% w/v bromophenol blue, 1% w/v SDS, 0.1 M EDTA, pH 7.8). 20 µL of each blend were transferred to a 1% agarose gel prepared in TBE solution (89 mM Tris-buffer (pH 8.6), 89 mM boric acid, and 2.5 mM EDTA) and containing 1 µg/mL of EtBr. The electrophoresis was set to 30 min at 100 mV. Sample visualization took place in a GelDoc (BioRad, USA) system using the QuantityOne program.

3. Results and Discussion

RESULTS AND DISCUSSION

3.1. Biological Activity

3.1.1. *In vitro* transfection efficiency – luminescence assay

To evaluate transfection capacity, polyplexes were prepared with a DNA plasmid containing the gene that encodes luciferase. The luciferase proteins are found on organisms that naturally emit light (bioluminescence) like fireflies. They are often chosen as reporters genes because their presence will lead to the emission of light easily quantified after the following reaction[81]:

$$\underline{\text{Luciferin}} + \underline{\text{ATP}} + \underline{O_2} \rightarrow \text{Oxyluciferin} + CO_2 + \underline{\text{AMP}} + \text{PPi} + \text{light}$$

In the presence of ATP, luciferin can be converted to oxyluciferin and some of the energy released by this reaction is in the form of light. However, this reaction only occurs in the presence of the enzyme luciferase making possible to correlate the quantity of emitted light with the amount of luciferase present in the sample. This technique is very sensitive to small changes, allowing the comparison of samples with slight differences. This procedure quantifies the amount of transgene that is being expressed by the cells rather than the quantity of cells that have been transfected.

The first experiment performed was an initial screening of the transfection capacity, in COS-7 cells, of polyplexes prepared with each of the two synthetized polymers (P_1 or P_2), individually, and with five different combinations of these two polymers, from C_A to C_E, being the C_A the combination with the biggest proportion of P_1 and the C_E the combination with the largest proportion of P_2. All the developed polyplexes were prepared and tested at four different N/P ratios (10/1; 50/1; 100/1; 150/1). The broad choice of ratios studied was made to determine the best ratios to be

used in further procedures. Polyplexes prepared with bPEI, at 5 different ratios, were used as a control, since bPEI is considered the "gold standard" of the polymer-based gene delivery systems.[82]

As illustrated in Figure 9, the ability of the different polyplexes to successfully deliver DNA plasmid into the COS-7 cell line is dependent on their composition and N/P ratios. Regarding the polyplexes prepared with each of the individual polymers (P_1 or P_2), those containing P_1 show a much greater transfection capacity than those prepared with P_2, for all the tested N/P ratios, being their biological activity similar to that observed with the bPEI-based polyplexes prepared at the best N/P ratio (25/1). On the other hand, the luciferase gene expression obtained with most of the polyplexes prepared with the different combinations of P_1 and P_2 is much higher than that observed with polyplexes prepared with bPEI or the individual polymers (P_1 or P_2), at the best N/P ratios, being in some cases 30-fold higher than the best results of bPEI-based polyplexes. These data show a clear synergistic effect in the biological activity when combining the two synthesized polymers. Surprisingly, given the relatively poor transfection ability of the P_2-based polyplexes, the nanosystems prepared with the mixtures C_D and C_E, which contain a higher relative amount of P_2, present the highest transfection activities.

A dependence on the N/P ratio can also be observed. Generally, polyplexes prepared at the 50/1 and 100/1 N/P ratios are the best formulations for all the generated combinations (C_A, C_B, C_C, C_D and C_E), whereas for P_1-based polyplexes the transfection activity observed with those prepared at the N/P ratios of 50/1, 100/1 and 150/1 is similar.

Taking into account the obtained data, the best formulation was chosen and a similar transfection assay was performed, in the presence and in the absence of serum.

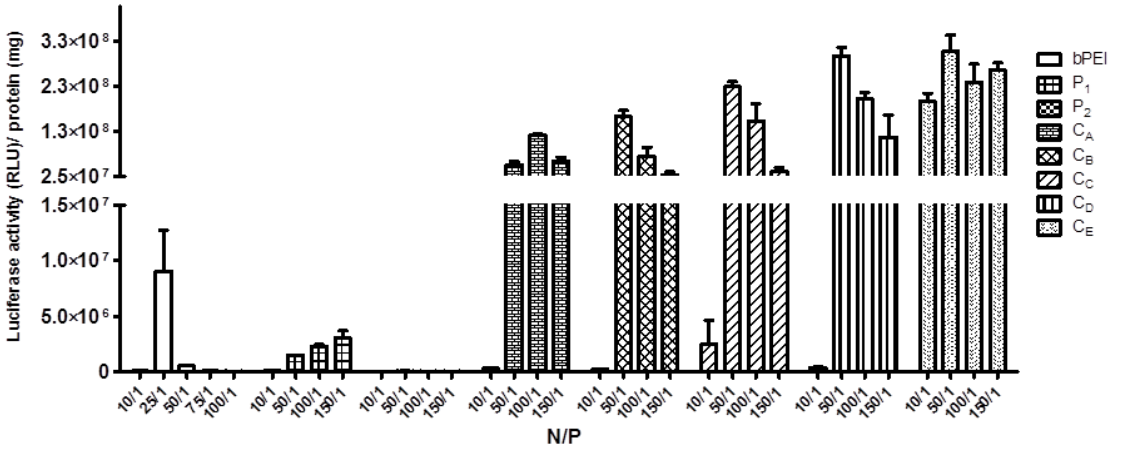

Figure 9. Effect of the N/P ratio and composition of polyplexes on luciferase gene expression in COS-7 cells. bPEI (N/P ratios 10/1; 25/1; 50/1; 75/1; 100/1), and P_1, P_2, C_A, C_B, C_C, C_D and C_E (N/P ratios 10/1; 50/1; 100/1; 150/1) polymers were complexed with 1µg of pCMV-Luc at the indicated N/P ratios. Cells were covered with 0.3 ml of serum-free medium and the polyplexes were added. After an incubation for 4 h, the medium was replaced with DMEM-HG containing 10% FBS and the cells were further incubated for 48 h. The level of gene expression was evaluated as described in 'Materials and methods'. The data are expressed as RLU of luciferase per mg of total cell protein (mean ± SEM, obtained from triplicates). The results are representative of at least two independent experiments.

Only five formulations were studied: bPEI-based polyplexes at the 25/1 N/P ratio, as a positive control; P_1- and P_2-based polyplexes prepared at the 50/1 N/P ratio; and C_E-based polyplexes at the N/P ratios of 50/1 and 100/1. C_E-based polyplexes were chosen over the other combinations to perform further studies because they presented the best results of biological activity, obtained in the luminescence assays, as well as low levels of cytotoxicity (data shown in the next section).

The results present in Figure 10a show that there are major differences between the two sets of results. When serum is absent, in agreement with Figure 9, the activity of C_E-based polyplexes is 30-fold higher than that of bPEI-based polyplexes and P_1-based polyplexes show a relatively low transfection activity while the transfection activity with P_2-based polyplexes is almost negligible. Moreover, in the absence of serum the results for the 50/1 and 100/1 N/P ratios are relatively alike. In the presence of serum, however, the data doesn't present itself in a similar manner.

It is clear that the transfection properties of bPEI-based polyplexes are negatively affected by the presence of serum since the luciferase gene expression decreases more

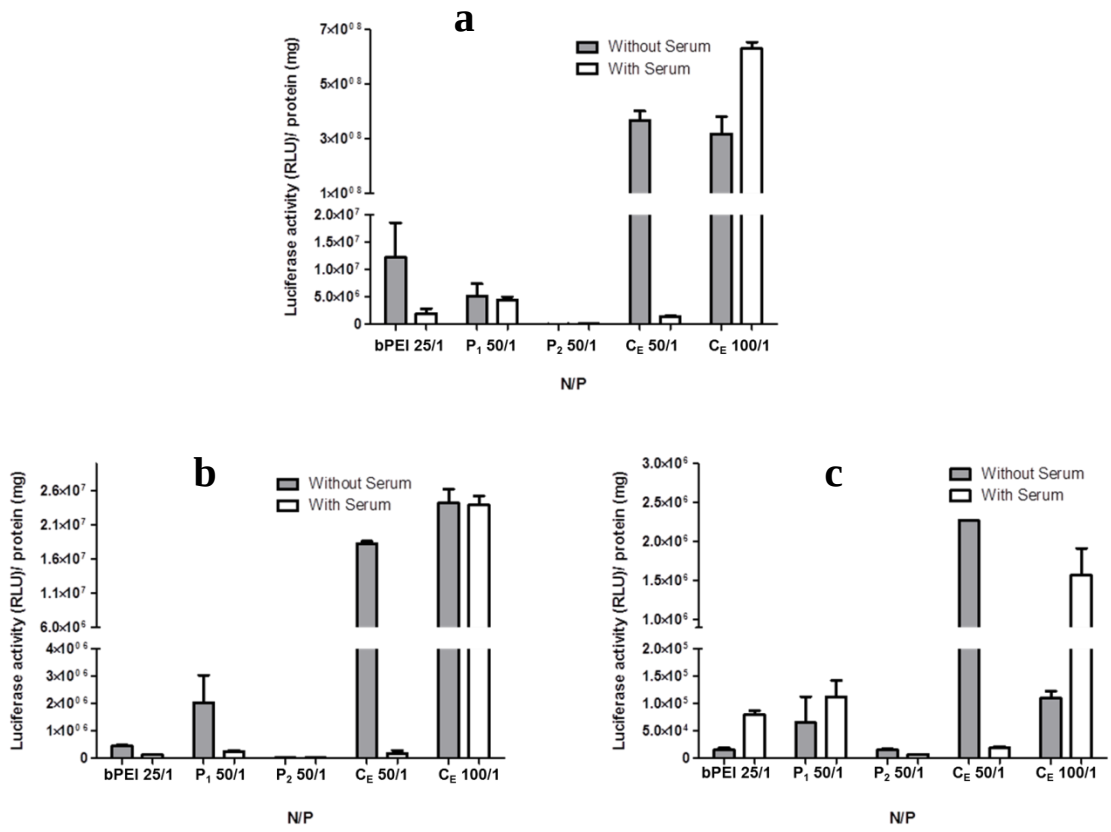

Figure 10. Effect of the presence of serum on luciferase gene expression for the different polyplexes in (a) COS-7, (b) HeLa and (c) MDA-MB-231 cells. bPEI (N/P ratio 25/1), P_1 and P_2 (N/P ratio 50/1), and C_E (N/P ratios 50/1; 100/1) polymers were complexed with 1µg of pCMV-Luc at the indicated N/P ratios. Cells were covered with 0.3 ml of serum-free medium or medium containing 10% FBS and the polyplexes were added. After an incubation for 4 h, the medium was replaced with DMEM-HG containing 10% FBS and the cells were further incubated for 48 h. The level of gene expression was evaluated as described in 'Materials and methods'. The data are expressed as RLU of luciferase per mg of total cell protein (mean ± SEM, obtained from triplicates). The results are representative of at least two independent experiments.

than six times. It is frequent to observe a decrease of the transfection activity in the presence of serum, namely due to the binding of serum components, such as serum proteins, to the polyplexes. These interactions established with the serum proteins can implicate a less successful internalization of polyplexes, because these interactions might prevent the binding of polyplexes to the cell membrane and/or their cellular internalization through endocytic pathway, due to a negative surface charge or to a size increase of polyplexes, respectively.[83,84] However, the addition of serum to the transfection medium mimics better the *in vivo* applications, and the fact that polyplex-mediated transfection is compromised in its presence presents a limitation to their use.

In spite of the common decrease in presence of serum, the luciferase activity of P_1 and P_2 and most of the combinations activity increases under this condition. In the case of C_E-based polyplexes there is a big difference between the two conditions (50/1 and 100/1 N/P ratios), for the 50/1 N/P ratio the luciferase activity obtained in the presence of serum is 100-fold lower than that observed without serum. In spite of this dramatic decrease, the value of luciferase activity for this experimental condition is still comparable to that of bPEI in the same condition.

The fact that higher N/P ratios behave better in the presence of serum is probably owed to the neutralization provoked by the serum proteins. As previously discussed, these proteins neutralize the cationic polyplexes, and difficult cell entrance. However, in the cases of higher N/P ratios, the excess of polymer and positive charges may have the capacity to overcome this neutralization.[84,85] The raise in activity can also be attributed in part to the fact that the presence of some serum components can stimulate endocytosis[85], which may contribute to a higher cellular uptake, leading in turn to a greater quantity of DNA reaching the target.

It is known and well reported that each type of cell has a distinct response to transfection efforts. Therefore, the biological activity of polyplexes was tested in two other cell lines, HeLa and MDA-MB-231 cells (Figure 10b and 10c, respectively), both in the presence and absence of serum.

In HeLa cells (Figure 10b), the results are not considerably different from those in COS-7 cells in the absence of serum. C_E-based polyplexes at the N/P ratio 100/1 are the ones with the better transfection activity, with values 54-fold higher than those of bPEI-based polyplexes. The two tested N/P ratios for these polyplexes (50/1 and 100/1) have a similar transfection activity and once again the P_1-based polyplexes present a much

higher luciferase activity than P_2-based polyplexes, although still lower than that of the combination of the two, represented by C_E-based polyplexes.

In MDA-MB-231 cells (Figure 10c), without serum, the same analysis apply, but this time C_E-based polyplexes at N/P ratio 50/1 have a much better transfection activity than those prepared at 100/1 N/P ratio. It is the best condition of all tested, representing a luciferase activity 140-fold higher than that observed for bPEI-based polyplexes.

When serum is present, it is observable in both cell lines a big difference in results for each formulation. Both in HeLa and MDA-MB-231 cells, the synergistic effect in the transfection activity of polyplexes, when combining the two polymers, is evident, in the absence and in the presence of serum. The biological activity levels of C_E-based polyplexes are much better than those obtained with polyplexes prepared with each one of the two polymers individually.

In MDA-MB-231 cells, in the presence of serum, unlike what happens in HeLa (Figure 2b) and COS-7 (Figure 2a) cells, the result for bPEI-based polyplexes is higher than in the absence of serum. This could be due to higher cell viability or a different effect of the serum in the internalization of these polyplexes in these cells.

In all three cell lines the results are expressive: firstly, C_E-based polyplexes prepared at the 50/1 N/P ratio present a much lower transfection activity than those prepared at the 100/1 N/P ratio, in the presence of serum. It is already been discussed the importance of the serum components to this effect, and these data endorse it, confirming, once again, C_E-based polyplexes at 100/1 N/P ratio are our best formulation. The results are also strongly suggestive that our best formulation (CE at 100/1 N/P ratio) is much a stronger transfecting agent in presence of serum than our control, with luciferase activities 319-, 186- and 19-fold higher than those obtained with

the complexes prepared with bPEI, for COS-7, HeLa and MDA-MB-231 cells, respectively.

3.1.2. *In vitro* transfection efficiency – flow cytometry and fluorescence microscopy

Flow cytometry studies were performed with the intent of collecting more information on the transfection efficiency of the developed formulations.

Cells were transfected with polyplexes prepared with a DNA plasmid containing the gene that encodes the green fluorescent protein (GFP), a fluorescent protein that emits green light and is found in many marine animals. Flow cytometry was used to count the cells that expressing GFP and the information given represents the percentage of cells that were transfected.

Four formulations were studied: bPEI-based polyplexes at the 25/1 N/P ratio, as a positive control; P_1- and P_2-based polyplexes prepared at the 50/1 N/P ratio; and C_E-based polyplexes at the N/P ratio of 100/1. This latter formulation was chosen, because it presents the best results of biological activity, obtained in the luminescence assays, and low levels of cytotoxicity (data shown in the next section). The flow cytometry studies were performed in COS-7 and HeLa cells and the results are displayed on Figures 11a and 12a.

In agreement with the results obtained in terms of luciferase gene expression levels, there is a great synergistic effect, on the percentage of transfected cells, observed after cells transfection with C_E-based polyplexes prepared at the 100/1 N/P ratio. This formulation transfects a higher percentage of cells than that observed with P_1- and P_2-based polyplexes or bPEI-based polyplexes in both cell lines.

These results show that our new polyplex formulation is a much better transfection agent than bPEI-based polyplexes, not only because it results in greater

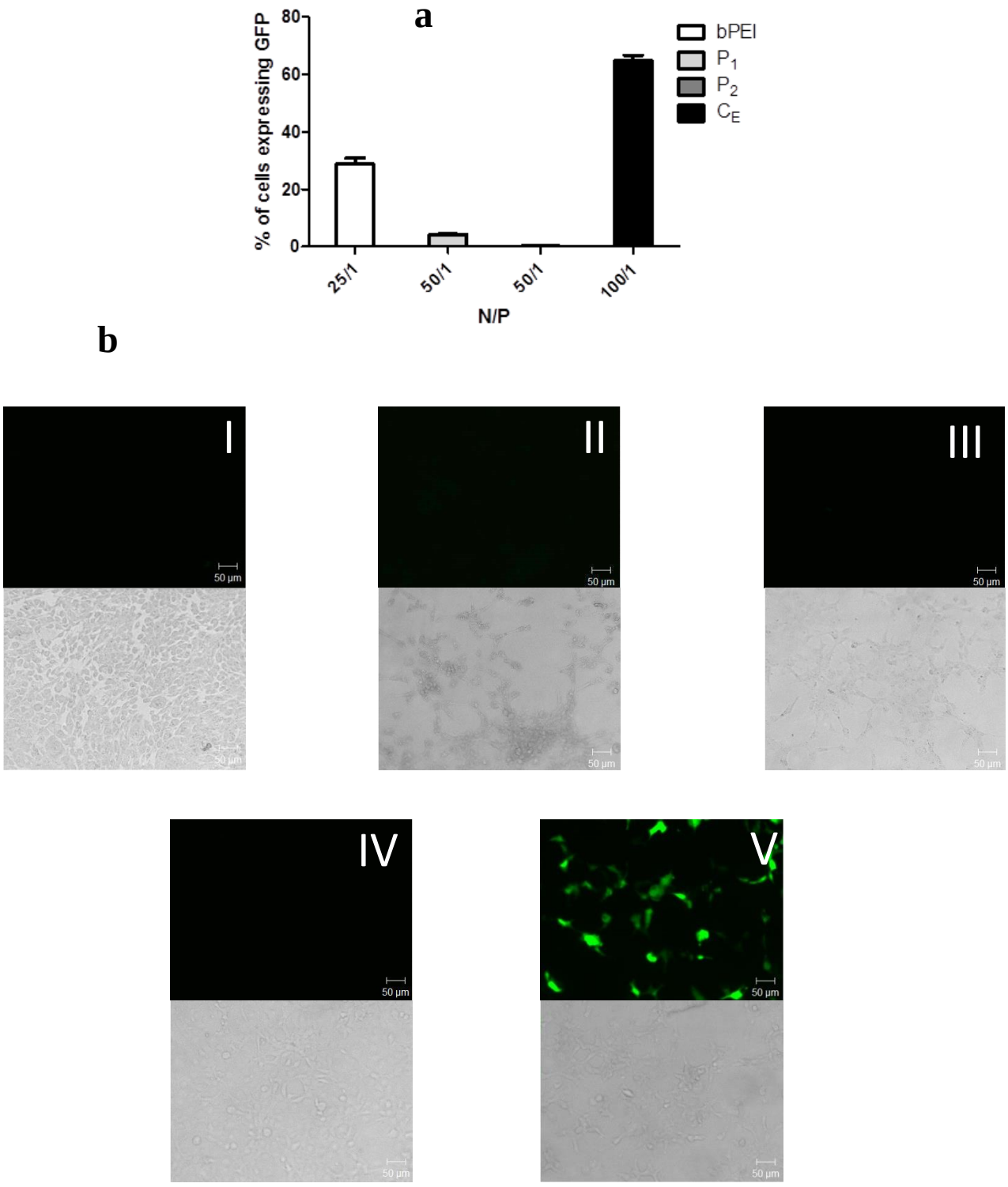

Figure 11. Effect of the composition of polyplexes on green fluorescent protein gene expression in the presence of serum in COS-7 cells evaluated by flow cytometry (a) and fluorescence microscopy (b). bPEI (N/P ratio 25/1), P_1 and P_2 (N/P ratio 50/1), and C_E (N/P ratio 100/1) polymers were complexed with 4 µg of pCMV-GFP at the indicated N/P ratios. Cells were covered with 1 ml of medium containing 10% FBS and the polyplexes were added. After an incubation for 4 h, the medium was replaced with DMEM-HG containing 10% FBS and the cells were further incubated for 48 h. (a) The data are expressed in percentage of transfected cells. (b) fluorescence microscopy images (panels): (I) control; (II) bPEI-; (III) P_1-; (IV) P_2-; (V) C_E-based polyplexes.

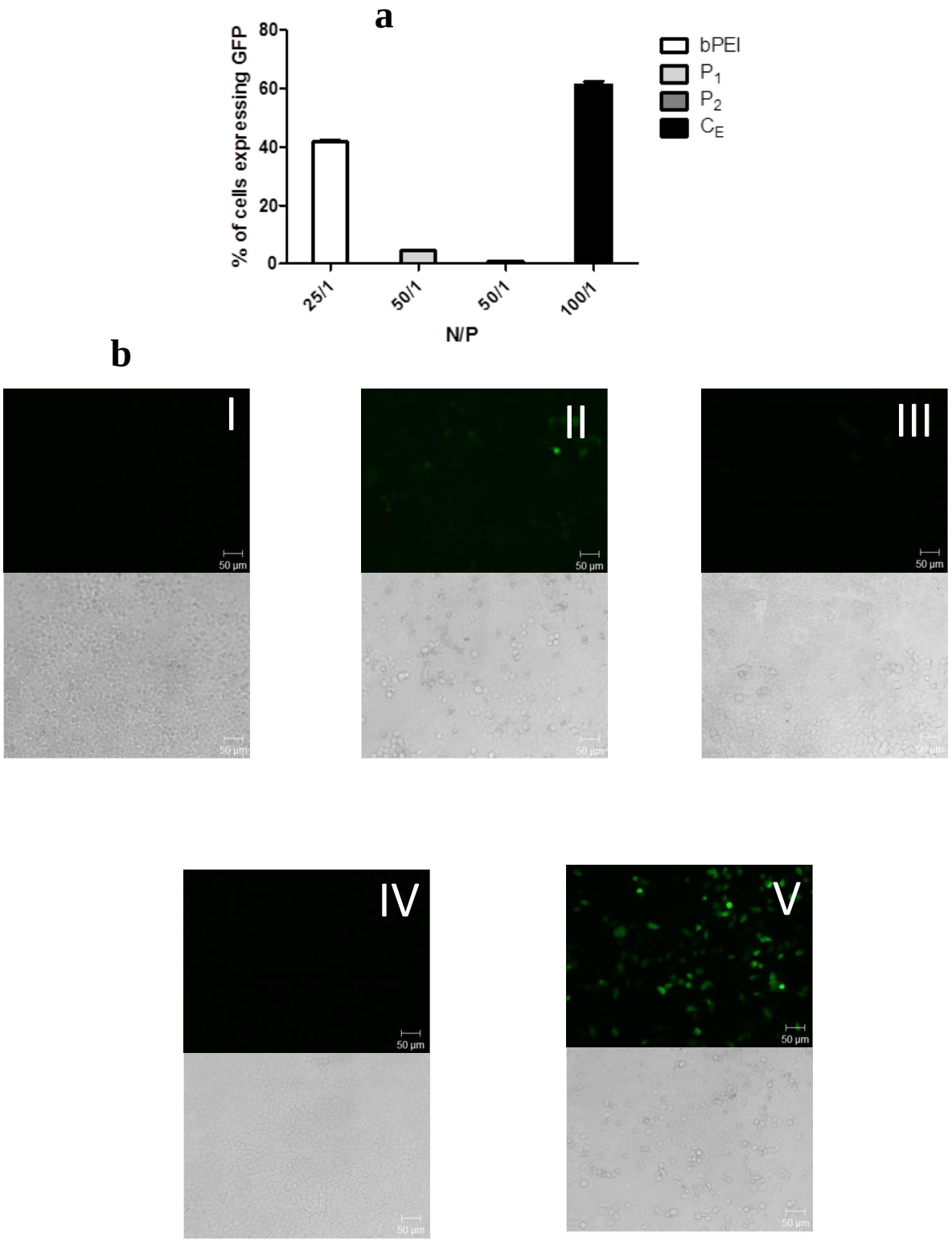

Figure 12. Effect of the composition of polyplexes on green fluorescent protein gene expression in the presence of serum in HeLa cells evaluated by flow cytometry (a) and fluorescence microscopy (b). bPEI (N/P ratio 25/1), P_1 and P_2 (N/P ratio 50/1), and C_E (N/P ratio 100/1) polymers were complexed with 4 µg of pCMV-GFP at the indicated N/P ratios. Cells were covered with 1 ml of medium containing 10% FBS and the polyplexes were added. After an incubation for 4 h, the medium was replaced with DMEM-HG containing 10% FBS and the cells were further incubated for 48 h. (a) The data are expressed in percentage of transfected cells. (b) fluorescence microscopy images (panels): (I) control; (II) bPEI-; (III) P_1-; (IV) P_2-; (V) C_E-based polyplexes.

levels of transgene expression, but also because it has the ability to transfect a higher number of cells.

In parallel with the flow cytometry studies, experimental assays of fluorescence microscopy were also performed. Once again the fluorescent properties of GFP were used to get data about the transfection capacity of the polyplexes constituted by bPEI, P_1, P_2 and C_E polymers. The study was also performed on COS-7 and HeLa cells and the results are exposed in Figures 11b and 12b, respectively, as representative images (phase contrast and fluorescence) for the control and for each formulation.

The results obtained in this assay show a direct correlation with the previously presented transfection data. The panels I of Figures 11b and 12b represent the untreated control cells that do not present fluorescence. In the other panels, corresponding to cells treated with the different polyplex formulations, the amount of expressed GFP by transfected cells is detected in the subsequent order: P_2<P_1<bPEI<C_E-based polyplexes. The difference between the transfection levels mediated by the C_E-based poplyplexes (panels V) and bPEI-based polyplexes (panels II) is absolutely remarkable, showing that C_E-based poplyplexes present a much higher transfection efficiency, which is observed not only in terms of number of transfected cells but also in terms of degree of fluorescence intensity, that is greater in panels V than in panels II, for both COS-7 and HeLa cell lines.

These results are aligned with the data obtained in the other transfection studies, demonstrating that C_E-based polyplexes exhibit a much larger transfection capacity than P_1- and P_2-based polyplexes, showing that the combinations of these two polymers result in a strong synergist increase in the biological activity; and than bPEI-based polyplexes, which are considered the “gold standard” of polymer-based gene delivery

systems. This greater efficacy of C_E-based polyplexes is translated in a higher percentage of transfected cells and, more notably, in a much bigger amount of transgene expression. To our knowledge, this is the first study showing that the combination of these two polymers (P_1 and P_2) can result in such an increase in polyplex-mediated gene delivery efficiency.

3.1.3. Cell viability assay

The evaluation of cell viability after treatment with polyplexes is crucial, since the high cytotoxicity is one of the main limitations usually associated to the use of cationic polymers.

The most common approach to measure the *in vitro* cytotoxicity is the use of colorimetric reagents.[86] For this work, the reagent chosen was Alamar Blue, which is a blue dye that is reduced by mitochondrial and cytoplasmic enzymes, present in metabolically active cells, by accepting electrons, and consequently changing into a fluorescent pink state. It is non-toxic and it allows the continuation of the assays after the assessment of the cell viability that is proportional to the measured absorbance.[87]

All polyplexes have an almost total absence of toxicity in COS-7 cells when prepared at the 10/1 N/P ratio (Figure 13). Increasing the polymer proportion brings a higher cytotoxicity in all cases, except for P_2-based polyplexes, showing that cell viability is dependent on the polyplex N/P ratios. This fact is not surprising, since higher N/P ratios means that there is a higher amount of polymer that could cause a greater cytotoxicity, probably due to an increased cationic surface charge of polyplexes, which could be more aggressive to cellular membranes since the electrostatic interaction between them could be stronger, and/or to a larger amount of unbound polymer to DNA, which could be more toxic to the cells.[82]

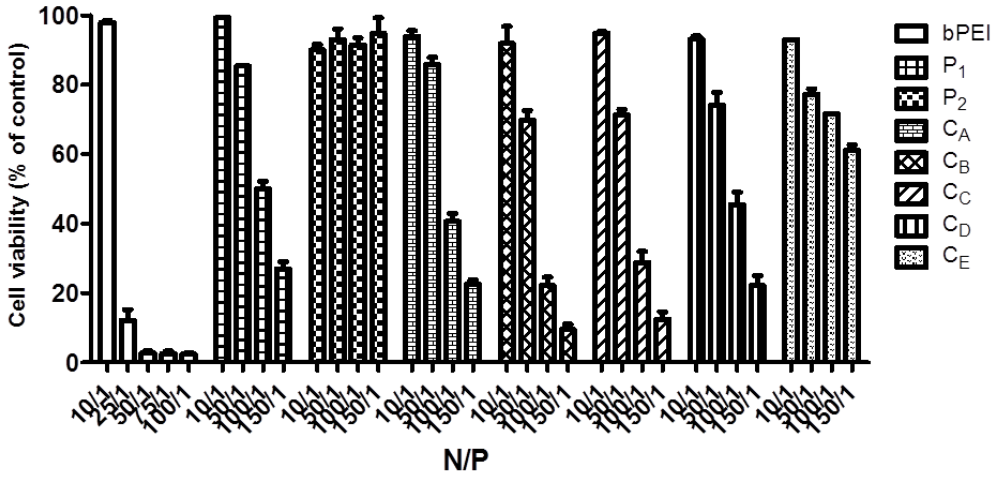

Figure 13. Effect of the N/P ratio and composition of polyplexes on the viability of COS-7 cells. bPEI (N/P ratios 10/1; 25/1; 50/1; 75/1; 100/1), and P_1, P_2, C_A, C_B, C_C, C_D and C_E (N/P ratios 10/1; 50/1; 100/1; 150/1) polymers were complexed with 1µg of pCMV-Luc at the indicated N/P ratios. Cells were covered with 0.3 ml of serum-free medium and the polyplexes were added. After an incubation for 4 h, the medium was replaced with DMEM-HG containing 10% FBS and the cells were further incubated for 48 h. Cell viability was measured by an Alamar blue assay as described in 'Materials and Methods' and it is expressed as a percentage of untreated control cells (mean ± SEM, obtained from triplicates). The results are representative of at least two independent experiments.

Polyplexes prepared by each one of the two polymers (P_1 and P_2) or by their different combinations are, nevertheless, less aggressive to cells than the polyplexes composed by bPEI, which in the 25/1 N/P ratio (the ones with the better transfection activity) results in a cell viability of approximately 12%. In the higher N/P ratios used to test our polyplexes, bPEI-based polyplexes induce a cell death of almost 100%.

P_2-based polyplexes are highly biocompatible in the tested conditions, since no significant cytotoxicity is observed for all the studied N/P ratios, opposed to P_1-based polyplexes, whose cytotoxicity increases up to 75% in the highest N/P ratio used. The cytotoxicity of polyplexes is, at least in part, attributed to the electrostatic interactions established between the cationic polymer and the negatively charged cell membranes. These interactions are mainly dependent on two aspects: the number of cationic charges (the increase of cationic charges density will result in a higher cytotoxicity) and the polymer and polyplex structures.[61] These two properties might justify the cell viability

differences observed after treatment with P_2-based polyplexes and P_1-based polyplexes. P_2 cationic polymer probably has a more rigid structure, making it more difficult to interact with the cell membranes, and a different three-dimensional arrangement of cationic residues, with more space between the amino groups, consequently resulting in less cytotoxicity.

Regarding the polyplexes prepared with the different P_1 and P_2 combinations, it is not surprising the observed increase in cell viability with a higher proportion of P_2 (C_A has the lowest and C_E the highest amount of P_2), this being particularly evident with C_E-based polyplexes prepared at the 100/1 and 150/1 N/P ratios. The lowest cytotoxicity showed by C_E-based polyplexes prepared at the 100/1 N/P ratio (approximately 25%), compared to the other polyplexes at the same N/P ratio, also contributed to the choice of these C_E-based polyplexes as the best formulation and the one used in further studies, like the microscopy and flow cytometry studies already presented.

In Figure 14, the cell viability observed after incubation of COS-7 (a) HeLa (b) and MDA-MB-231 (c) cells with C_E-based polyplexes, prepared at the 50/1 and 100/1 N/P ratios, and control polyplexes, in the presence of serum, is shown.

Compared to the results of Figure 13 for COS-7 cells, in the presence of serum the percentage of viable cells, observed after treatment with any of the tested formulations, is higher. This increase in cell viability can be explained by the better conditions of cell growth and by the possible toxicity reduction of some polyplexes formulations promoted by their interaction with serum components. This latter observation is particularly evident for bPEI-based polyplexes, which are much less toxic and even less efficient (Figures 9 and 10a) in the presence of serum, showing that most probably these polyplexes strongly interact with serum components that reduce their ability to binding

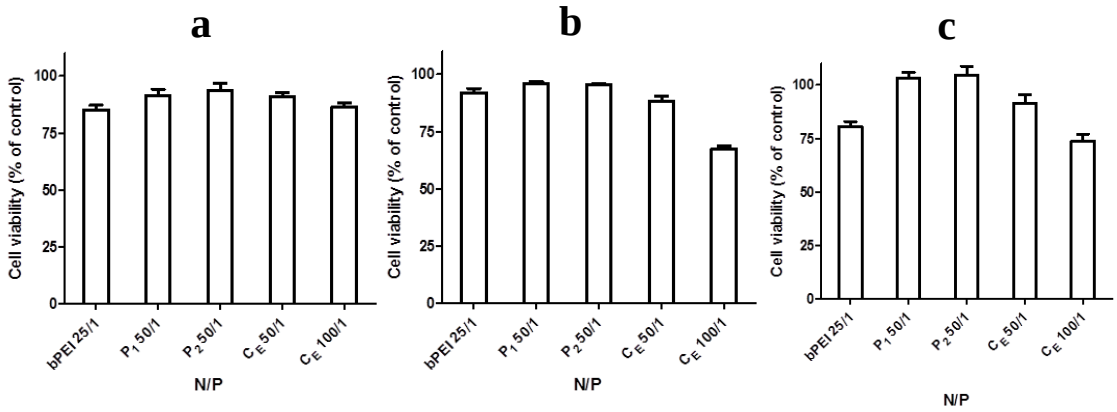

Figure 14. Effect of the presence of serum on viability of COS-7 (a), HeLa (b) and MDA-MB-231 (c) cells after treatment with different polyplexes. bPEI (N/P ratio 25/1), P_1 and P_2 (N/P ratio 50/1), and C_E (N/P ratios 50/1; 100/1) polymers were complexed with 1µg of pCMV-Luc at the indicated N/P ratios. Cells were covered with 0.3 ml of medium containing 10% FBS and the polyplexes were added. After an incubation for 4 h, the medium was replaced with DMEM-HG containing 10% FBS and the cells were further incubated for 48 h. Cell viability was measured by an Alamar blue assay as described in 'Materials and Methods' and it is expressed as a percentage of untreated control cells (mean ± SEM, obtained from triplicates). The results are representative of at least two independent experiments.

and/or to be internalized by the target cells, consequently reducing both their cytotoxicity and their transfection activity.

Regarding our best formulation, C_E-based polyplexes prepared at the 100/1 N/P ratio, it shows a slightly higher cytotoxicity than P_1-, P_2- or C_E-based polyplexes prepared at the 50/1 N/P ratio, which are not toxic in the presence of serum, nevertheless, in these experimental conditions, it presents an even more potent transfection capacity than the other polyplexes formulations, including the bPEI-based polyplexes, in all the tested cell lines (Figures 9 and 10).

3.2. Physicochemical characterization of the polyplexes

3.2.1. Dynamic Light Scattering and Zeta Potential Analysis

The analysis of the physicochemical characteristics of nanoparticles is very important as it evaluates essential parameters to their *in vitro* and *in vivo* success. Investigating the size and surface charge of the polyplexes is crucial to correlate their

physicochemical properties with their transfection activity, and consequently to design new and efficient gene delivery nanosystems.

The size of nanoparticles has a direct influence both on their accumulation on the targeted tissue, helping to profit from the EPR effect in the case of cancer-targeted therapeutics, and on their internalization by target cells (both *in vitro* and *in vivo*).[48]

The particle size can be determined through dynamic light scattering, a technique that is based on the analysis of the scattering of light promoted by the particles.[88]

The particle size of all the developed polyplexes is below 200 nm (Figure 15a), which allows their endocytic internalization by cells via the clathrin-dependent pathway[73]. Almost all the polyplexes prepared at the 100/1 N/P ratio present a mean diameter smaller than 150 nm and are slightly smaller than the respective formulations prepared at the 50/1 N/P ratio. This is most probably due to the DNA condensation induced by the polymers, since the increase in the amount of polymer could results in a higher genetic material condensation, consequently forming smaller polyplexes.

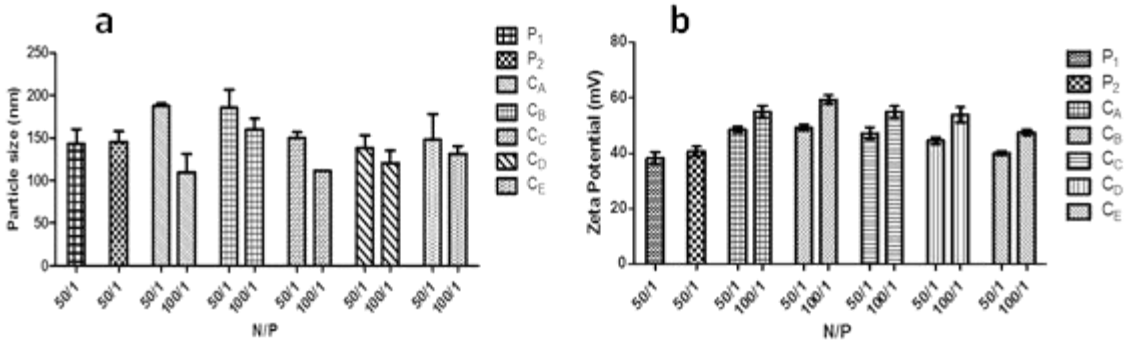

Figure 15. Particle size (a) and zeta potential (b) of the different polyplexes. The polyplexes were prepared with 1µg of pCMV.Luc at the indicated polymer/DNA N/P ratios. Polydispersity index is between 0.3 and 0.4 in all formulations. The data are expressed as particle size in nanometers (mean ± SEM, n=6) and zeta potential in mV (mean ± SEM, n=6). Two independent experiments were realized in triplicate.

The polymers P_1 and P_2 generate polyplexes with identical sizes, approximately 140 nm, which means that their different levels of cytotoxicity and transfection activity are not related to their size. Between the different combinations of polymers there isn't a noticeable trend, however it is possible to see that our best formulation, the C_E-based

polyplexes prepared at the 100/1 N/P ratio, presents a mean diameter of approximately 130 nm, which is a suitable particle size for *in vivo* applications.

The surface charge can be analyzed by electrophoretic light scattering technology to measure the zeta potential based on the electrophoretic mobility under an electric field. It is a very important parameter specially when considering cellular toxicity and the uptake by the target cells.

It was already demonstrated the importance of preparing our novel formulation in a high (100/1) N/P ratio, in order to obtain a better transfection activity in the presence of serum, and potentially a greater biological activity *in vivo* applications. On the other hand, when the polyplexes surface charge is extremely positive the interactions established between the polyplexes and the target cells may result in cytotoxicity by disassembling of cell membranes.[89]

In Figure 15b, it is displayed the zeta potential of each of the formulations studied. All of them are predictably positively charged, taking into account the excess of cationic charges, oscillating between +38 mV and +59 mV. The polyplexes prepared at the 100/1 N/P ratio have, in all cases, a more positive surface charge than the corresponding ones prepared at the 50/1 N/P ratio, being this justifiable by the presence of more amino groups.

Similarly to what was observed in size measurements, P_1- and P_2-based polyplexes have a very close zeta potential, around +40 mV, leading to believe that their difference in terms of biological activity and cytotoxicity is most probably due to a different polymer composition and structure, and consequently to a distinct interaction with the DNA.

Even it is widely recognized that the size and charge of the polyplexes are greatly related to their performance, it is clear by the obtained results that they are not the only determinant factors and that the transfection activity of a polyplex formulation is hard to predict based only on its physicochemical properties.

3.2.2 DNA Condensation

As previously discussed, a good gene delivery system has to be able to protect the load from the moment it is administrated up to reaching the target. The cationic polymers do so by condensing the DNA and consequently shielding it from the potential damages it could suffer.

Ethidium bromide (EtBr) is a monovalent DNA-intercalating agent with fluorescent properties used to detect the accessibility to DNA, since its fluorescence increases strikingly when it forms a complex with DNA. As illustrated in Figure X, bPEI-based polyplexes were prepared in five different N/P ratios (from 10/1 to 100/1) and used as a control, and P_1-, P_2-, and their combination (C_A to C_E)-based polyplexes were tested in four different N/P ratios.

All of the formulations show a dependence on their N/P ratios (Figure 16), where the higher N/P ratios allow a lower EtBr access to the DNA, since most probably an increasing amount of polymer will cause a higher condensation of DNA in the polyplexes, as it was possible to observe by a decrease in the particle size for higher N/P ratios, when comparing 50/1 with 100/1 N/P ratios (Figure 15a).

As observed in the biological activity and cell viability experimental assays, P_1 and P_2-based polyplexes behave very differently. Whereas P_1-based polyplexes show very low levels of EtBr access to DNA, P_2-based polyplexes present the highest levels of all studied polyplexes (Figure 10). This very low condensing capacity observed for all the N/P ratios of P_2-based polyplexes may be related to their lack of success as

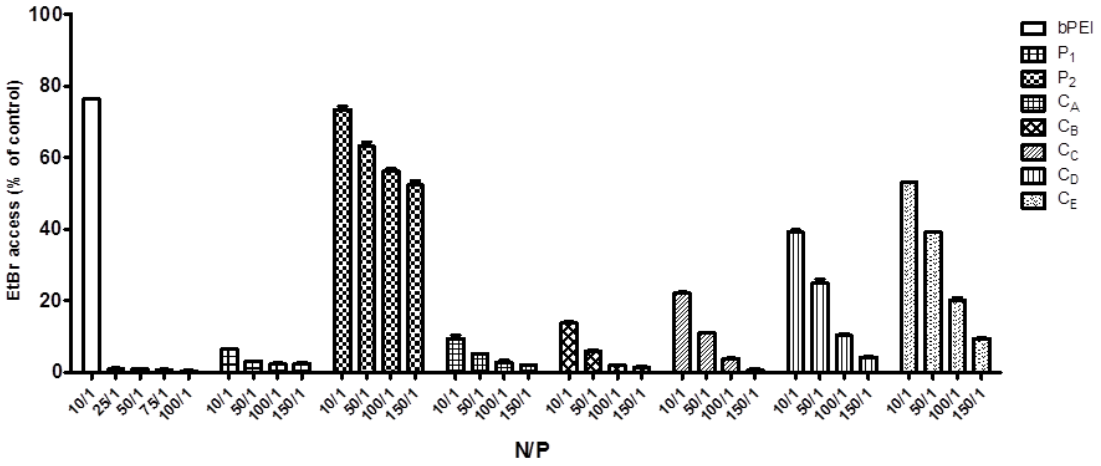

Figure 16. Accessibility of ethidium bromide to DNA of the different polyplexes prepared at different N/P ratios. Polyplexes prepared with bPEI, P_1, P_2, C_A, C_B, C_C, C_D and C_E and containing 1µg of DNA, were incubated with EtBr as described in 'Materials and methods'. The amount of DNA available to interact with the probe was calculated by subtracting the values of residual fluorescence from those obtained for the samples and expressed as the percentage of the control. Control corresponds to free DNA in the same amount as that associated with the polyplexes (100% of EtBr accessibility). The data are expressed as EtBr access (% of control) (mean ± SEM, obtained from triplicates). The results are representative of at least two independent experiments.

transfection mediators, as they can release the DNA too soon, causing it to be degraded before reaching the nucleus.

The accessibility of EtBr to the DNA of the polyplexes prepared with both polymers is dependent on the present proportion of polymer P_2. The higher the proportion of P_2 in the combination, the higher the percentage of EtBr accessing to DNA, which means that unlike other characteristics, such as the transfection efficiency, the condensation of DNA is inversely proportional to the amount of the polymer P_2 in the polyplexes. Our best formulation, C_E-based polyplexes prepared at the 100/1 N/P ratio, presents a relatively low percentage of EtBr access (20%). Even though the control formulation, bPEI-based polyplexes, did not allow EtBr access at high N/P ratios, a very low access of this probe can also mean a worse transfection efficiency. In fact, when polymers condense so strongly the DNA it is not properly released when reaching the proximity of the nucleus, resulting in reduced biological activity.

It is also noteworthy that this assay gives information on the protection of DNA, since EtBr is a smaller molecule than nucleases, and consequently if polyplexes have the ability to restrain the access of EtBr to DNA, they most probably have the ability to protect it from the nucleases attack.

In turn, agarose gel electrophoresis assay offers information on the degree of DNA complexation of the polyplex. This technique is based on the fact that free DNA will be able to move towards a positive electrode, as it is negatively charged. On the other hand, DNA that is still complexed in the polyplexes will not move.

The observed results (Figure 17) are concordant with the data obtained in the EtBr intercalation assay. Of all the polyplexes tested (bPEI-, P_1-, P_2- and C_E-based polyplexes), P_2-based formulation was the only one that demonstrated a reduced capacity of complexing the DNA. C_E-based polyplexes, which did allow a slight accessibility of EtBr to DNA (figure 16), have proven to efficiently complex the DNA.

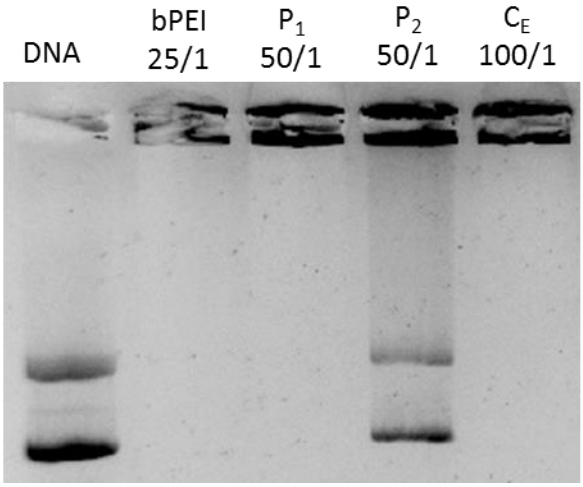

Figure 17. Agarose gel electrophoresis of different polyplexes. The polyplexes with bPEI, P_1, P_2 and C_E were prepared with 1µg of pCMV.Luc at the indicated polymer/DNA N/P ratios.

4. Conclusions and Future Perspectives

CONCLUSIONS AND FUTURE PERSPECTIVES

Gene therapy was envisioned as a treatment to several genetic diseases, many years ago. Today, it has not yet reached its full potential, as its implementation has been delayed by the lack of suitable vectors to transport and deliver genetic material into the target and it is many times regarded as the future of therapeutics rather than its present.

Non-viral vectors, of which polymers and lipids are the most relevant, have been demonstrated as the best alternative to viral vectors as genetic material carriers, mostly as a result of their safeness and versatility. However, this class of vectors still lacks some characteristics that are crucial for their definitive affirmation as the used systems in gene therapy, namely levels of transfection efficiency similar to those obtained with virus-based vectors. A great number of studies have been performed in the past decades in the attempt to find better vectors, whether by altering molecules already used in vectors, combining them, or creating/testing new molecules.

In this context, in the present work a set of new vectors was designed by combining two polymers in different proportions and their potential as DNA delivery systems was evaluated. In order to accomplish this, the polyplexes formulations were submitted to different studies having been evaluated several parameters, namely transfection activity (through luminescence, flow cytometry and fluorescence microscopy), cytotoxicity, particle size, surface charge and protection of DNA.

The obtained results were conclusive: all tested polymer combinations have the ability to mediate gene transfer. However, the detected transfection activity is dependent on the polyplexes N/P ratios, being the polyplexes prepared at the 50/1 and 100/1 N/P ratios the ones with the best transfection efficiency in all combinations. The cell viability, measured after incubation with the polyplexes, is also dependent on the

relative amount of polymer present in the nanosystem: higher N/P ratios present higher cytotoxicity.

In the presence of serum, the luciferase transgene expression mediated by polyplexes prepared at the 50/1 N/P ratio decreases drastically. However, in these experimental conditions, this parameter is not affected (for HeLa cells) or is even increased (for COS-7 cells) for polyplexes prepared at the 100/1 N/P ratio. The higher luciferase activity observed in the presence of serum for polyplexes prepared at the 100/1 N/P ratio is indicative that they perform well even in conditions that are not usually favorable for *in vitro* assays. Taking into consideration the results obtained in the transfection activity and cytotoxicity assays, the best developed formulation was the C_E-based polyplexes prepared at the N/P ratio of 100/1. Our work revealed that this novel formulation presents a transfection activity that is approximately 320, 187 and 19 times higher than that obtained with the best formulation of bPEI-based polyplexes, in COS-7, HeLa and MDA-MB-231 cells, respectively, proving its high effectiveness in different cell lines and positioning our nanosystem as a much better delivery system than one of the most successful polymers for genetic material delivery. These results were confirmed by other experimental assays, namely flow cytometry and fluorescence microscopy.

Regarding the physicochemical characteristics, the best C_E-based polyplexes revealed suitable properties for *in vivo* applications, namely a good DNA condensation, which could be a determining factor on the protection of genetic material from damages before reaching the target, an adequate small particle size, and a surface charge that even being positive do not impair their transfection activity in the presence of serum.

Even though the relation between the observed results *in vitro* and the registered performance *in vivo* is usually not linear, the preliminary feedback about the best C_E-based polyplexes formulation is that it could be a very potent vector for therapeutic applications. However, there is still a long way to go before clinical applications and more studies need to be done to confirm this assumption.

In a first phase, more studies will be necessary to characterize the polyplexes physicochemical characteristics and their biological activity *in vitro*. To better understand the systems potential behavior *in vivo,* it would be of interest to study their interactions with serum components, and their mean diameter and surface charge in the presence of serum. The long-term stability of the polyplexes is also an important characteristic that must be studied, in order to evaluate their potential to be stored and used in subsequent *in vivo* applications. The pathway by which polyplexes are internalized by target cells is also very important to know in order to understand the mechanisms associated to their biological activity and to be able to improve it.

In a second phase, it will be very interesting to have the possibility to improve our formulation characteristics, namely conferring them specificity to target cells, by introducing a ligand into the polyplexes surface, and reducing their levels of cytotoxicity (specially at high concentrations) and increasing their potential blood circulation time, by introducing a biocompatible polymer, such as PEG, at their surface.

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