UNIVERSIDADE DA BEIRA INTERIOR
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Gene Therapy
Development of a new nanocarrier system for mitochondrial gene therapy

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Tese para obtenção do Grau de Mestre em
Ciências Biomédicas
(2º ciclo de estudos)

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The content of this work is the sole responsibility of the author:

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(João Miguel Almeida Santos)

"The fundamental cause of trouble in the world is that the stupid are cocksure while the intelligent are full of doubt."

- Bertrand Russell (1872-1970)

Acknowledgments

Over a year has passed, one year of dedication to an MsC thesis that is finally coming to an end. What I feel proud about is not the work done but the experience and knowledge that I have gained during the past year. The truth is, not everything went according to planned and there were times when constant failures lead me to think that the best I could do was just give up. Yet I was able to reach the end thanks to the support I have been receiving from friends, family and colleagues and for those people I dedicate this page of my thesis.

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To finish, I would like to dedicate this thesis to the person which I believe to be the most important one in my life and where I got most of my strength to never give up, my girlfriend Carina Tomás.

To all those people who may have contributed to my success and the success of this thesis I want you to know that I feel really appreciated and that none of this would ever had been possible without your help.

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João Santos

Resumo

As mitocôndrias são organelos únicos pois possuem o seu próprio genoma, o ADN mitocondrial (ADNmt). Apesar de bastante pequeno quando comparado com o ADN nuclear (ADNn), mutações ao nível do ADNmt são bastante frequentes devido à falta de mecanismos de protecção e de reparação. Como consequência, citopatias e doenças associadas à mitocôndria são bastante frequentes afectando essencialmente órgãos e tecidos onde existe muito dispêndio de energia como é o caso dos músculos e do cérbero. Logo, o desenvolvimento de um novo e eficiente protocolo para terapia génica mitocondrial (MGT) é visto como uma proposta aliciante.

Durante esta tese de Mestrado, tentamos criar um novo nanosistema que consiga entregar eficazmente ADN plasmídico (pDNA) à mitocôndria para que no futuro possa ser usado em terapia génica mitocondrial (MGT).

Assim, este projecto de investigação pode ser dividido em três etapas principais:

1. O isolamento e purificação de três plasmídeos (pUC19, pVAX1-*LacZ* e pcDNA3-myc-*FLNa* S2152A);
2. A síntese e caracterização de nanopartículas com afinidade para a mitocôndria;
3. O estudo da capacidade das nanopartículas efectuarem transfecção celular e dirigirem-se à mitocôndria;

As nanopartículas desenvolvidas, através do método de co-precipitação oferecem-nos qualidades únicas como a sua biocompatibilidade, alta eficiência de encapsulamento de ADN e baixo custo de produção.

A transfecção celular foi alcançada com sucesso sendo que, tais resultados, podem contribuir em grandes avanços na correcção de defeitos mitocondriais, oferecendo-nos uma nova estratégia terapêutica no combate a diversas patologias desde o cancro, às doenças de Parkinson e Alzheimer.

Palavras Chaves

ADNmt, pDNA, terapia génica mitocondrial, nanopartículas, mitocôndria.

Resumo Alargado

As mitocôndrias são organelos únicos que possuem o seu próprio genoma, o ADN mitocondrial, uma molécula com dupla cadeia circular com aproximadamente 16.5 mil pares de base (kbp) e que codifica 13 polipéptidos, 2 ácidos ribonucleicos ribossomais (rARN) e 22 ácidos ribonucleicos de transferência (tARN).

Apesar de conhecidas como as principais geradoras de energia dos seres vivos, sobre a forma de ATP, as mitocôndrias são também importantes por desempenharem outras funções relevantes para o bem-estar do organismo como a produção e regulação de espécies reactivas de oxigénio, regulação da entrada de cálcio na célula e apoptose.

Ainda que o ADNmt pareça insignificante quando comparado com o DNA nuclear (ADNn) a verdade é que mutações ao nível do ADNmt são bastante frequentes e estima-se que este seja, pelo menos, 10 vezes mais mutável que o nuclear. Tal discrepância é essencialmente devido à falta de mecanismos de protecção e reparação, onde o único conhecido até ao momento é o reparo por excisão de bases, "Base Excision Repair" (BER).

Assim, torna-se preponderante a investigação de novas formas terapêuticas tendo como alvo as doenças mitocondriais e, em especial, a manutenção da integridade do genoma mitocondrial. Pondera-se cada vez mais a incorporação da terapia génica como uma abordagem adequada a deficiências mitocondriais, surgindo assim a terapia génica mitocondrial (MGT). Para o sucesso desta terapia, é necessário o desenho de um vector adequado que seja capaz de incorporar um tamanho ilimitado de ADN inserido, seja facilmente produzido e em larga escala, pudesse ser direccionado para organelos celulares específicos, não permitisse a replicação autónoma do ADN e pudesse garantir uma expressão génica a longo prazo. Adicionalmente, o vector seria biocompatível e não-imunogénico.

Apesar do sucesso desta terapia ao nível nuclear, os avanços na área mitocondrial têm sido escassos. Alguns progressos importantes na formulação de um vector baseado em ADN mitocondrial foram reportados, mas contudo, apresentando limitações ao nível da transfecção celular.

Tendo em conta esta lacuna e a pouca investigação centrada na terapia génica mitocondrial, o objectivo desta tese passa por desenvolver um novo sistema à escala nano para a entrega de ADN plasmídico (ADNp) à mitocôndria numa abordagem terapêutica inovadora para tratamento de doenças mitocondriais.

Este projecto de investigação inclui 3 etapas fundamentais:

1. Isolar e purificar três plasmídeos com diferentes tamanhos;

2. Desenvolver e caracterizar novas nanopartículas com características adequadas para uso em terapia génica;

3. Transfecção *in vitro* direccionada à mitocôndria.

Foram amplificados, numa cultura de *Escherichia coli*, e isolados com sucesso três plasmídeos (pUC19, pVAX1-*LacZ* e pcDNA3-myc-*FLNa* S2152A) com recurso ao kit Qiagen.

Procedemos em seguida à criação de nanopartículas contendo rodamina$_{123}$ (rho$_{123}$). Estas nanopartículas têm como ideia a encapsulação de ADN plasmídico (ADNp) e rho$_{123}$ através de um simples método de co-precipitação de carbonato de cálcio ($CaCO_3$). Criaram-se dois protocolos, destinados à entrega de 1µg e 10µg de pDNA. Procedemos, de seguida, à sua caracterização de modo a averiguar qual o mais eficiente para futuros estudos.

A caracterização envolveu a identificação da morfologia através da microscopia electrónica de varrimento (SEM), análise da capacidade de encapsulamento de ADNp por parte das nanopartículas, determinação do tamanho médio das nanopartículas, do potencial ζ e do poder protector das nanopartículas perante a presença de nucleases.

Por último, procedemos ao crescimento de uma cultura celular de fibroblastos humanos, avaliámos a viabilidade celular através do ensaio MTT e procedemos à avaliação *in vitro* da eficiência de transfecção por microscopia confocal.

As nanopartículas apresentam uma forma praticamente circular e um potencial ζ entre os valores de -8mv e -10mv para ambos os sistemas. No caso do sistema de 1µg, a percentagem de encapsulamento varia entre os 50% e os 60%, o tamanho médio entre os 300nm e os 400nm e as nanopartículas encontram-se bastante degradadas na presença de 5µg/ml de DNAse. Nos sistemas de 10µg, a percentagem de encapsulamento varia entre os 55% e os 70%, o tamanho médio entre os 350nm e os 550nm e as nanopartículas só começam a degradar-se para valores superiores a 10µg/ml de DNAse. Quando comparados, os resultados obtidos durante o teste da protecção sugerem que o sistema de 10µg seja o mais indicado para fins terapêuticos.

A transfecção e transporte para a mitocôndria foi observada com sucesso através de estudos de microscopia confocal. Apresentamos deste modo um novo método para a entrega de ADNp à mitocôndria que apresenta vantagens únicas em aspectos como: encapsulamento, biocompatibilidade e custo de produção.

O trabalho desenvolvido ao longo desta tese pode contribuir fortemente para o sucesso da terapia génica mitocondrial (MGT) e talvez num futuro próximo, as nanopartículas desenvolvidas possam oferecer uma nova estratégia terapêutica no combate a diversas patologias, das quais, se destacam as doenças de Parkinson e Alzheimer consideradas grandes flagelos da vida moderna.

Abstract

Mitochondria are unique organelles that have their own genome, the mitochondrial DNA (mtDNA). Although quite small compared to nuclear DNA (nDNA), mutations in mtDNA are quite frequent due to the lack of protection and repair mechanisms. Per consequence, cytopathies and diseases are quite common and mostly associated with high energy demanding tissues such as muscles and the brain. Therefore, the development of a new and efficient mitochondrial gene therapy protocol is seen as a promising approach.

During this MSc thesis we try to bring together a new nanocarrier system with the ability to deliver plasmid DNA into the mitochondria, for future application in mitochondrial gene therapy (MGT).

Hence, the development of this research project can be divided itself into three main stages:

1. Isolation and purification of three plasmid DNAs (pUC19, pVAX1-*LacZ* and pcDNA3-myc-*FLNa* S2152A);
2. Synthesis and characterization of nanoparticles with mitochondria affinity;
3. *In vitro* study of mitochondrial transfection ability.

The newly developed nanoparticles, created through a co precipitation method, offer us unique features such as: biocompatibility, plasmid DNA (pDNA) encapsulation efficiency and low manufacturing cost.

We were able to successfully achieve transfection into the mitochondria which may result in a huge step in the correction of mitochondrial defects, offering new therapeutic strategies for a variety of pathologies ranging from cancer to Parkinson and Alzheimer´s diseases.

Keywords

mtDNA, pDNA, mitochondrial gene therapy, nanoparticles, mitochondria.

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Lista de Acrónimos

AP	Apurinic/apyrimidinic
APE	Apurinic/apyrimidinic endonucleases
ATP	Adenosine triphosphate
BER	Base excision repair
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
$CaCO_3$	Calcium Carbonate
CARD	Caspase Recruitment Domain
Caspase	Cysteinyl aspartate-specific proteases
Cl^-	Chloride
Co_3^{2-}	Carbonate
DA	Dalton
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DQAsomes	DeQuAlinium-based liposome-like vesicles
EE	Encapsulation Efficiency
H_2O_2	Hydrogen Peroxide
IMM	Inner mitochondrial membrane
IMS	Inner mitochondrial space
kbp	Kilobase Pair
kDA	KiloDalton
KSS	Kearns-Sayre syndrome
LHON	Leber hereditary optic neuropathy
LP-BER	Long-pathway BER
LS	Leigh syndrome
MELAS	Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes
MERRF	Myoclonic Epilepsy with Ragged Red Fibers
MIDD	Maternally inherited diabetes and deafness
MMR	Mis-match Repair
MOMP	Mitochondria Outer Membrane Permeabilization
mtBER	Mitochondrial base excision repair
mtDNA	Mitochondrial DNA
Na^+	Sodium
Na_2CO_3	Sodium Carbonate
NaCl	Sodium Chloride

NARP	Neuropathy, ataxia, retinitis pigmentosa
nDNA	Nuclear DNA
NER	Nucleotide excision repair
$O_2^{\bullet-}$	Superoxide anion
OC	Open Circular
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative Phosphorylation
pDNA	Plasmid DNA
polγ	Polymerase Gamma
PS	Pearson syndrome
PFA	Paraformaldehyde
PTP	Permeability Transition Pore
Rho_{123}	$Rhodamine_{123}$
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SC	Supercoiled
SEM	Single Electron Microscopy
SOD	Superoxide Dismutase
SP-BER	Short-pathway BER
TEM	Transmission Electron Microscopy
tRNA	Transfer RNA

Índex

Introduction

The human genome is the complete set of human genetic information stored within the 23 chromosome pairs of the cell's nucleus but also the small closed double-stranded circular molecule situated inside the mitochondria, the mitochondrial DNA (mtDNA). Responsible for encoding 13 proteins as well as 2 ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) (1), damage to mtDNA ends up affecting high energy-demanding tissues like brain, heart and muscles.

In recent reports, it was shown that 1 in 4 healthy individuals inherit a mixture of wild-type and variant mtDNA, process known as heteroplasmy (2). Even less frequently, less than 1 in 200 inherit a potentially pathogenic variant of mtDNA (3) whereas 1 in 10.000 manifest clinically a mtDNA disease (4). It is presumed that the mutation rate of mtDNA is at least 10 times bigger than nuclear DNA (nDNA), leading to a wide range of metabolic and neuromuscular syndromes. In addition, mutations and/or polymorphism variance in mtDNA genes are related with Parkinson and Alzheimer's diseases, diabetes and a greater susceptibility to develop cancer.

Gene therapy consists of the introduction of genetic material into cells for a therapeutic purpose with the objective of correcting or treating a deficient gene responsible for a disease (5). Over the last decades, gene therapy has had remarkable advances becoming a feasible treatment option (6). With such achievements, new strategies and shifts should be made to further improve the conventional therapy systems.

The first reference to gene therapy was presented in 1963, when Joshua Lederberg suggested the control of nucleotide sequences in human chromosomes, coupled with selection and integration of the desired genes (7). This idea ended up being accomplished only in 1980 with the introduction of two functional genes into mammalian cells (8). Virus-based vectors were once the most commonly used gene delivery systems in gene therapy because of their highly efficient infection rate and ability to integrate therapeutic genes into the host chromosome to ensure stable and long term gene expression. However, the initial enthusiasm for the use of viruses in gene therapy has diminished due to its lack of safety associated with fatal adverse effects reported in previous studies (9). Meanwhile clinical trials, concerning the usage of non-viral vectors, has been constantly growing day-by-day due to its improved safety features, high cell viability and rapid manufacturing timeline (10).

The interest of incorporating gene therapy into mitochondria started with the observation of patients suffering from myopathy (11) and optic neuropathy (12), both studies observed for the first time mutations in mtDNA. The incorporation of a therapeutic vector, to target the mitochondrial DNA errors, brings potential advantage in the reestablishment of oxidative phosphorylation energetic metabolism in mutated cells and represents an innovative approach in the therapeutics of mitochondrial pathologies.

A successful mitochondrial gene therapy (MGT) would lead to the cellular uptake and intracellular targeting of a therapeutic nucleic acid or protein specifically to the mitochondrial compartment (13). The design and development of an adequate mitochondrial transfection vector is quite relevant and deserves more attention from scientific community once this first step can compromise the entire gene therapy operation.

Nanomedicine plays an important role in biotechnology with the creation of new gene delivery systems. Recent nanocarriers ensure protection against enzymatic degradation, are able to by-pass the innate immune system, have good biodistribution, reduced side-effects, safety, no toxicity, reduced inflammation and ensure the therapeutic payload to be released in the desired intracellular compartment (14).

1. Understanding the mitochondria

Mitochondria are membrane-enclosed organelles found in most eukaryotic cells that range from 0.5 to 1 micrometer (μm) in diameter. Responsible for producing most part of cell's energy in the form of adenosine triphosphate (ATP), mitochondria play an essential role in the life cycle of the cell (15).

Human genetic information can be found in the form of nDNA but we should not neglect the existence of a second form of genome found inside the mitochondria, the mtDNA. Of nearly 17kb or 10.000 KDa, circular double-stranded mtDNA is present in hundreds to thousands of copies in each cell (16) (Figure 1). MtDNA consists predominantly of coding DNA, with the exception of the control region that has mainly regulatory functions.

A unique feature of mitochondria is the possession of proteins encoded from two separate genomes. While all mtDNA encoded proteins are mitochondria exclusive, most of mitochondrial proteins are nuclear encoded, synthesized in the cytosol and finally directed to the mitochondria by specific targeting sequences (17).

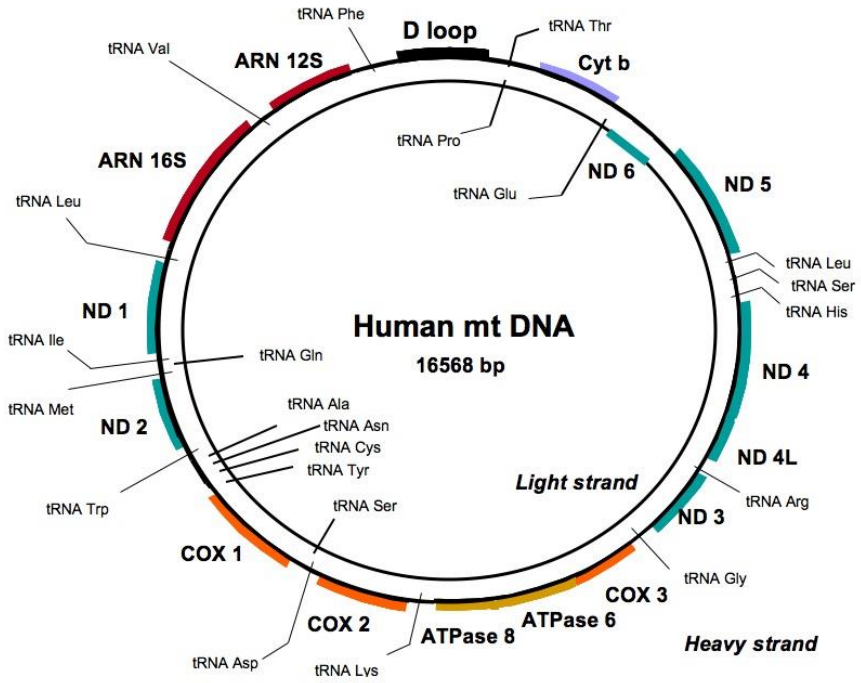

Figure 1. Human Mitochondrial DNA. Circular double-stranded molecule of 16,568 (bp) in length consisting predominantly of coding DNA that codes for 13 polypeptides, 2 ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) (Adapted from (18)).

The control region is a three stranded D-loop of nearly 600bp that promotes the origin of mtDNA replication. The leading strand has historically been termed Heavy (H) while, logically, the other strand was labeled as Light (L). The D loop is thus defined as a three-stranded structure with the nascent leading H strand defining the origin of leading-strand replication (O_H) at its 5 ' end (19). DNA synthesis occurs unidirectionally, after the growing of H strand has elongated to two-thirds or more of its total length, the origin of lagging L-strand replication is exposed on the displaced parental H strand and initiation of daughter L-strand synthesis begins leading into two distinct progeny circles being segregated. This process is finalized by the synthesis of a new D loop (20).

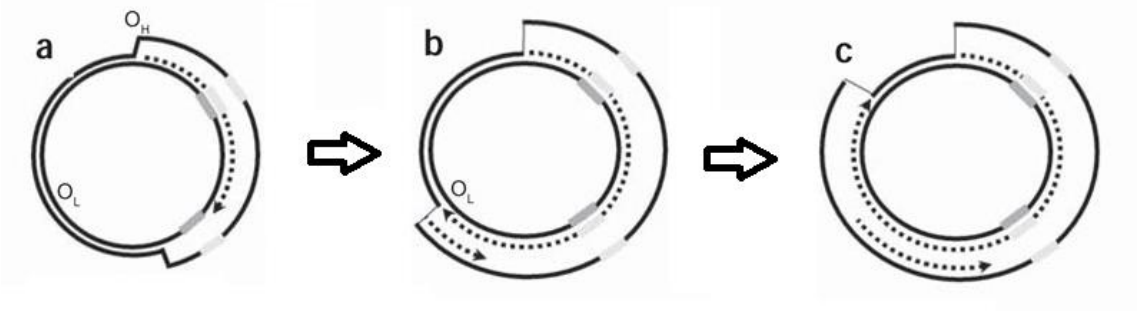

Figure 2. mtDNA replication. a) Replication of mtDNA begins in the D loop at the O_H, displacing the light strand from the heavy strand. b) when the new synthetized heavy strand reaches at O_H replication of the light strand begins in the opposite direction. c) both strands replicate in different directions until both strands have been fully replicated. (Adapted from (21))

1.1. Functions of mitochondria

Mitochondria are cytosolic double-membrane organelles that have been considered the powerhouse of the cell. Mitochondria, however, participate in a high number of other cellular processes such as ion homeostasis, redox signaling, apoptotic and necrotic cell death, as well as the control of cell cycle and cell growth (22, 23)

Even though the mitochondria contribute to a huge number of cellular processes, four crucial functions to the cell homeostasis are enlightened.

1.1.1. Generation of energy

All biological and molecular events require energy to function properly. Energy is available in the form of ATP (adenosine triphosphate) which is mainly produced through aerobic cellular respiration of carbohydrate and glucose, which represent most of the source of biological energy of the human body. On the other side, reduced energy levels threaten cellular homeostasis and integrity (24). The principal source of ATP production is the oxidative phosphorylation (OXPHOS) which takes place in the mitochondria (25).

OXPHOS begins with the entry of electrons into the respiratory chain through complex I (NADH ubiquinone oxidoreductase) (26) or complex II (Succinate ubiquinone oxidoreductase) (27). Electrons from complex I or complex II are subsequently transferred to complex III (Ubiquinol cytochrome c oxidoreductase) then to cytochrome c, the second mobile electron carrier, and finally to complex IV (Cytochrome c oxidase). Complex IV is the terminal enzyme in the electron transfer chain reducing O_2 to H_2O by using the delivered electrons (28). This whole process ends up creating a membrane potential that promotes the conformational change of complex V (ATP synthase) resulting in the generation of ATP (29) (Figure 3).

All 13 polypeptides encoded by the mtDNA are subunits of the OXPHOS system: complex I (7 polypeptides), complex III (1 polypeptide), complex IV (3 polypeptides) and complex V (2 polypeptides) (30).

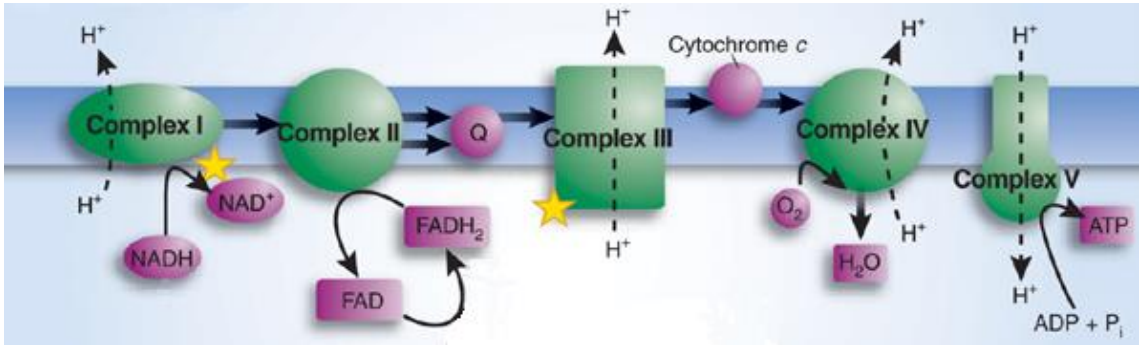

Figure 3. Electron transport chain of mitochondria. The function of the electron transport chain is to produce a transmembrane proton electrochemical gradient as a result of the redox reactions ending with the production of ATP. (Adapted (31))

1.1.2. Generation and regulation of ROS

Reactive oxygen species (ROS), are oxygen derivatives, that are oxidized and easily converted into radicals (32). A wide range of mitochondrial ROS-induced damages have been described, which can lead, either individually or collectively, to a cellular energetic catastrophe

All the mitochondrial enzyme complexes can generate ROS or at least contribute to their appearance (33). ROS are produced by mitochondria during oxidative metabolism through the one-electron reduction of molecular oxygen (O_2), forming superoxide anion ($O_2^{\bullet -}$). Superoxide is the proximal ROS produced by mitochondria and is converted to hydrogen peroxide (H_2O_2) through the action of superoxide dismutases (SODs) both within the mitochondria and in the cytosol (34).

H_2O_2 generated in mitochondria may act as a signaling molecule in the cytosol (35) however another possibility is to infuse within the cell and be eliminated by cytosolic or mitochondrial antioxidant systems such as catalase, glutathione peroxidase, and thioredoxin peroxidase (36).

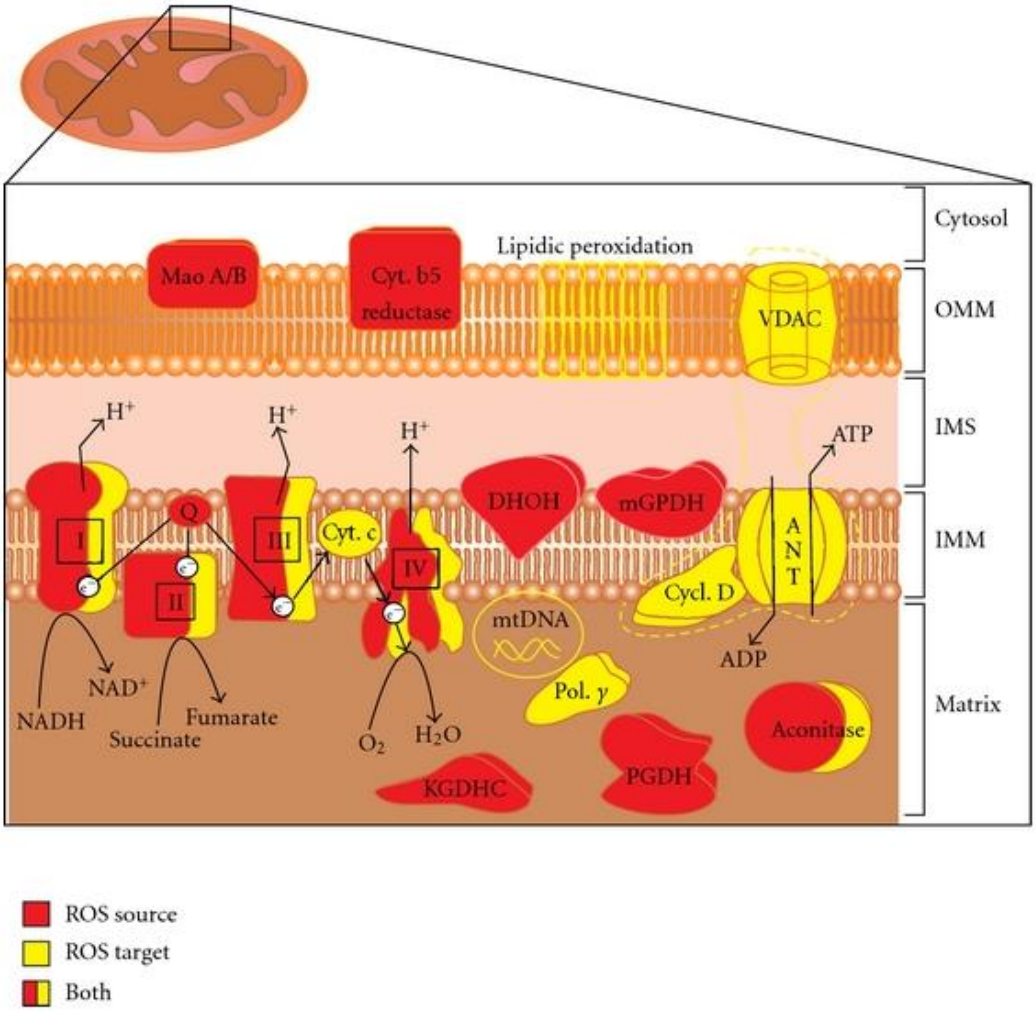

Figure 4. Mitochondrial sources of ROS and mitochondrial ROS targets. ROS generators (red) and ROS targets (yellow) are present all over mitochondria. Aconitase and complex I through IV are both sources and targets of ROS. (Adapted from (37).

1.1.3. Calcium regulation

Calcium (Ca^{2+}) is a highly versatile second messenger that controls critical cellular responses in all eukaryotic organisms (38). Mitochondria has the ability to act a Ca^{2+} buffer therefore having an important part in regulating cytosolic Ca^{2+} signals (39).

Ca^{2+} uptake by mitochondria alters the activity of mitochondria in multiple ways. An increase in the free Ca^{2+} concentration within the mitochondrial matrix results in the increase of $[Ca^{2+}]_{mit}$ which leads to a larger respiratory rate, H^+ extrusion and ATP production. However, prolonged increases in $[Ca^{2+}]_{mit}$ can induce the opening of the mitochondrial permeability transition pore (PTP) leading to mitochondrial swelling, cytochrome *C* release, and cell death by apoptosis (40).

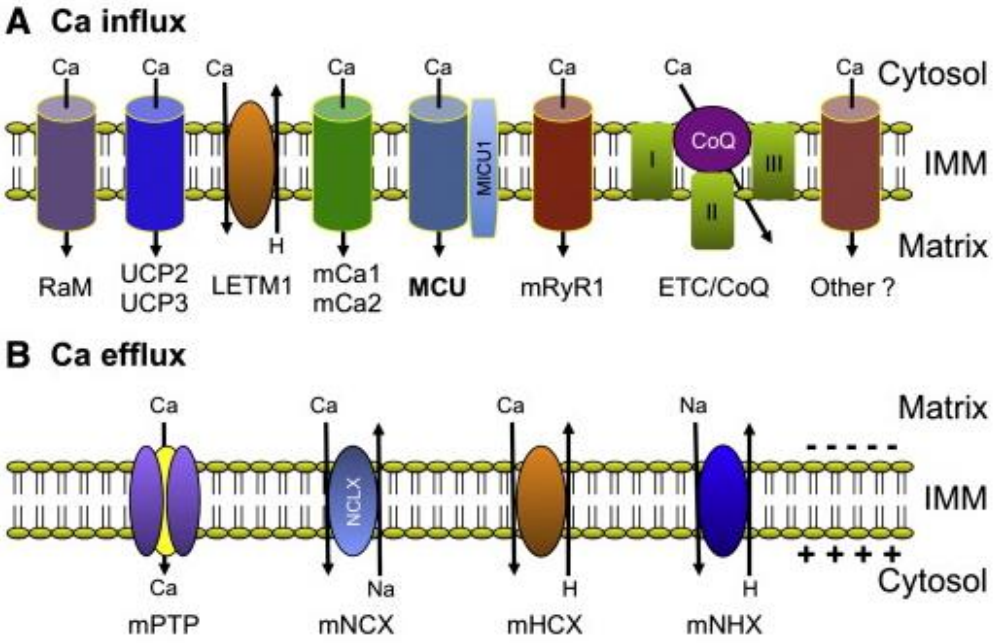

Figure 5. Mitochondrial Ca transport pathways. A) Mitochondrial Ca uptake and mechanisms and pathways located at the IMM. B) Mitochondrial Ca extrusion mechanisms and pathways located at the IMM. (Adapted from (41))

1.1.4. Regulate Apoptosis

Apoptosis is a programmed form of cell death, characterized by the activation of cysteinyl aspartate-specific proteases (caspases) and the systematic breakdown of dying cells into easily phagocytized apoptotic bodies. (42, 43)

There are two alternative pathways that initiate apoptosis: one is mediated by death receptors on the cell surface (extrinsic pathway) while the other is mediated by the mitochondria (intrinsic pathway). The intrinsic pathway is activated in response to intracellular stressors, induced by several stimuli including DNA damage (44).

These stress signals trigger the mitochondria outer membrane permebilization (MOMP) resulting of the activation of certain proapoptotic BCL-2 family members (45). MOMP then facilitates the release of cyt c which interacts with Apaf-1 stimulating it into a caspase-activating complex known as the "Apaf-1 apoptosome". The apoptosome subsequently recruits the initiator procaspase-9 through caspase recruitment domains (CARDs) present in the N-termini of both Apaf-1 and procaspase-9. Once bound, active caspase-9 then processes the effector caspase-3 and induces death (46).

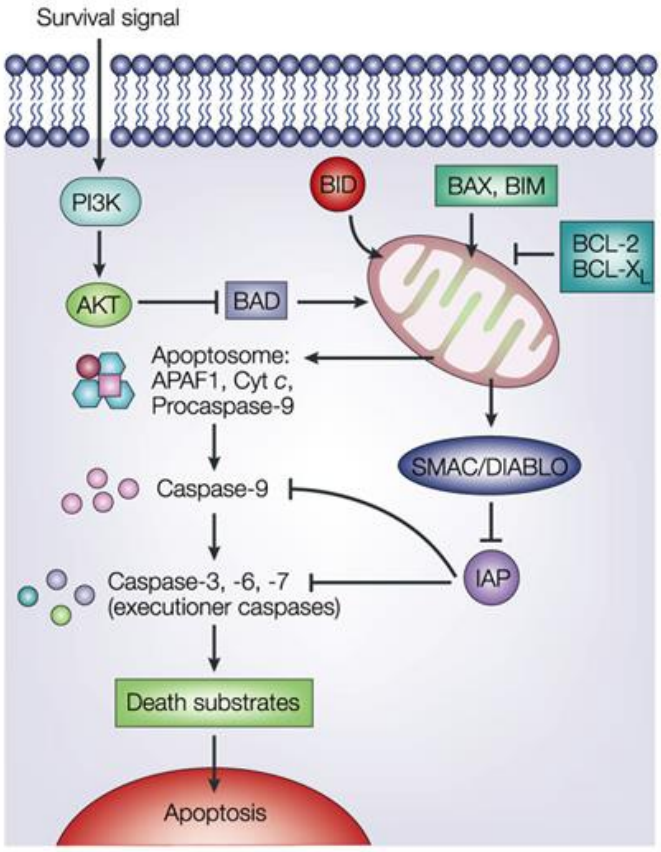

Figure 6. Apoptosis intrinsic pathway. Activation of apoptosis starts with MOMP, generally as a result of the activation of pro-apoptotic members. MOMP then facilitates the release of several pro-apoptotic factors from the mitochondria into the cytoplasm where they promote cell death (47).

2. Maintaining mtDNA integrity

Mammals normally inherit their mtDNA just prior to fertilization (48) and immediately after mtDNA replication (49). Such method of inheritance supposedly tend to make copies of identical mtDNA, (homoplasmy) (50). However, recent studies reveal that is not unusual for two different copies of mtDNA, wild-type and mutated, to be present inside of the mitochondria, process known as heteroplasmy (2, 51).

MtDNA is highly mutable due to the limited mtDNA protection and repair mechanisms but also because of the proximity to the electron transport chain, ROS formation site and even nuclear genome (52) which may contribute to the high diversity in the levels of mutated mtDNA. However, that does not explain the high rate of mutation and the high number of mutated mtDNA copies present in some populations. Furthermore, it is quite hard to explain why a woman with two children, one is healthy, while the other infant may have a devastating and fatal disorder.

2.1. Inheritance and clonal expansion

It is widely accepted that in the cells of most animals, mtDNA is inherited solely from the mitochondria of the oocyte, making the inheritance, with few exceptions, maternal. The maternal mode of mtDNA transmission gives rise to a genetic asymmetry between females and males, whereas the nuclear genome is inherited in equal measure through males and females, enabling selection to act on male and female traits in both sexes (53).

Although the maternal inheritance of mtDNA is conserved in many species, it is not understood why paternal mitochondria and mtDNA should be eliminated from zygotes. Three possible explanations are suggested to why there is no paternal inheritance: 1) paternal mitochondria and/or mtDNA could be heavily damaged by reactive oxygen species produced during spermatogenesis and the long swim of the sperm. 2) Uniparental inheritance may prevent further heteroplasmy. 3) Uniparental inheritance may be the mechanism to prevent further potentially deleterious mtDNA (54).

Yet, uniparental inheritance makes it even stranger why a healthy mother gives rise to a child with high percentage of mutated mtDNA. One hypothesis, the Mitochondrial Genetic Bottleneck, possibly the most well accepted explanation, does however explain why this strange phenomenon may happen (55-57). The Bottleneck suggests that a healthy mother can give birth to an unhealthy child as long as she has some mutated mtDNA. The idea is that in the primary oocyte, a small number of mother's mitochondria are randomly selected. Once the oocyte becomes mature, an expansion of the few randomly chosen mtDNA will have occurred. Since the sperm does not contribute with mitochondria, if those randomly selected mtDNA correspond to a high percentage of mutated mtDNA, it means that the offspring will definitively harbor a high percentage of mutated mtDNA and will probably manifest a severe disease even though the mother did not have any symptoms (Figure 7).

The degree of heteroplasmy can vary between tissue to tissue and individual to individual. When the percentage of mutant copies reaches above a critical point, the normal cell phenotype fluctuates and a new diseased phenotype may appear. This process is known as the threshold effect (58). While the necessary value to reach the threshold varies for different tissues and the type of mutation, it does explain why some offspring's have some diseases while the mother does not.

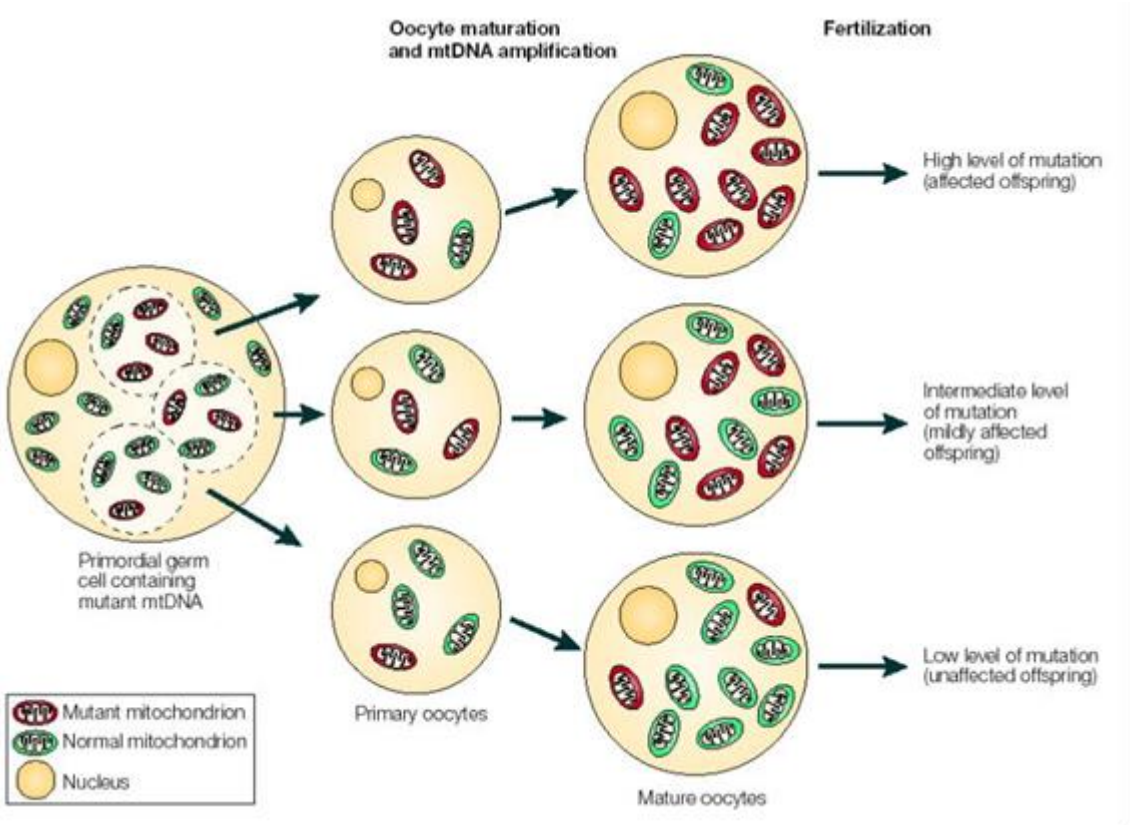

Fig.7. Mitochondrial genetic bottleneck. During production of primary oocytes, a selected number of mtDNA molecules are transferred into each oocyte. Oocyte maturation is associated with the rapid replication of this mtDNA population. This may lead to a random shift of mtDNA mutational load between generations and is responsible for the variable levels of mutated mtDNA.(Adapted from (59))

2.2. Repair mechanisms of the mitochondria

When comparing mitochondrial genome to nuclear genome, we may think that since smaller, mutations at mtDNA should be less likely to occur. Actually, mtDNA is presumed to be ten times more mutable than nDNA. In comparison to nDNA, mtDNA has no real protection mechanism like the chromatine to pack DNA. Moreover, the close proximity to ROS formation sites makes mtDNA errors quite frequent.

In order to maintain genomic integrity, different DNA repair pathways have evolved. Without efficient cellular DNA repair mechanisms, DNA stability and cellular survival are seriously compromised. DNA repair mechanisms have been extensively investigated in the nucleus, where different repair pathways occur: nucleotide excision repair (NER); base excision repair (BER) and mismatch repair pathway (MMR).

Although these mechanisms have mostly been investigated in the nucleus, our knowledge regarding mitochondrial DNA repair pathways has significantly increased during the last decade (60).

Over the last two decades it was confirmed that mitochondria do possess effective DNA repair mechanisms (61), and the understanding of how these mechanisms function has significantly increased in the last few years. The first DNA repair pathway that was described to actively take place in mammalian mitochondria was the BER pathway. Today, MMR, thought to occur exclusively in the nucleus has been described to take place in mammalian mitochondria (62, 63). However, there has not been space to conclude the existence of MMR in mtDNA and how does it works.

BER is the primary and best known pathway described for repair of small DNA modifications in the mitochondrial genome caused by alkylation, deamination, or oxidation (64, 65). It starts with recognition of the damage followed by enzymatic processing steps that aim to remove the lesion and restore genomic integrity (66). Although nDNA also possess a BER mechanism, mitochondria base excision repair (mtBER) has rather unique features (67). BER facilitates the repair of damaged DNA via two general pathways: the short-pathway BER (spBER) leads to the repair of a single nucleotide while the long-pathway BER (lpBER) produces a repair of at least two nucleotides (68).

The first step of BER is catalyzed by DNA glycosylases, which are responsible for initial recognition of the lesion. Some DNA glycosylases may be bifunctional and possess AP lyase activity (69). Mitochondrial and nuclear DNA glycosylases are both encoded by the same nDNA gene however generated by alternative splicing and transcription (70). Repair of these lesions promote the migration of various enzymatic processes to induce DNA single-strand breaks and spontaneously generation of purinic/apyrimidinic (AP) sites. Among those processes, AP endonuclease 1 (APE1) is the most important and indispensable enzyme to the cleavage of AP sites and continuation of mtBER (71, 72).

Once the AP site has been processed by APE1, the following step in the BER pathway is catalyzed by a DNA polymerase gamma (polγ), which inserts the correct nucleotide(s) in the generated gap. Polγ is the only known DNA polymerase in mammalian mitochondria and it is responsible for all aspects of mtDNA synthesis, including all replication and recombination of the mitochondrial genome (73). During the SP-BER, one single nucleotide is incorporated into the gap by polγ, while the LP-BER involves the incorporation of several nucleotides and additional enzymes (74).

The final step of the mitochondrial BER pathway is the nick sealing catalyzed by a DNA ligase. While two DNA ligases are described in the nucleus (I and III), in mammalian mitochondria only DNA ligase III has been detected, acting both in replication and repair (75) (Figure 8).

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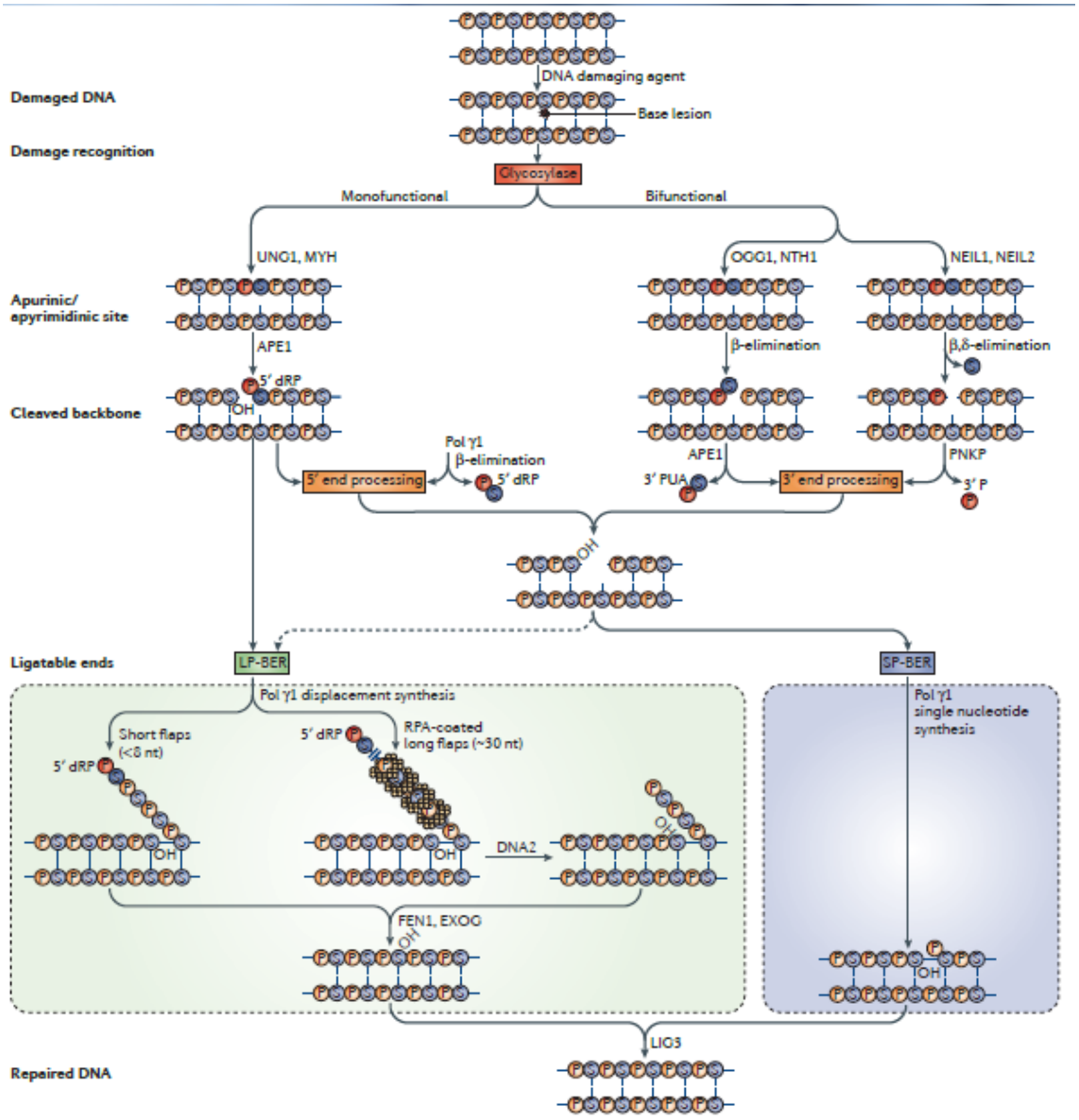

Figure. 8. Base Excision Repair in mitochondria. Major steps: 1) recognition and removal of the modified base; 2) processing of the generated AP site; 3) incorporation of the correct nucleotide(s); 4) strand ligation (60)

2.3. Mutations and Diseases

The first association of mtDNA with human disease was in 1988, with the observation of pacients suffering from myopathy (11) and optic neuropathy (12). Originally considered rare, recent epidemiological studies indicate that pathogenic mtDNA mutations are a significant cause of human disease, affecting millions of individuals all around the world. Currently more than 250 different provisional or confirmed pathogenic changes have been reported associated with a diverse array of clinical phenotypes (76).

Mitochondrial diseases represent a genetically and clinically heterogeneous group of inherited metabolic disorders characterized by impaired energy production. Their heterogeneity is due in part to the biochemical complexity of mitochondrial respiration and the fact that 2 genomes, 1 mitochondrial and 1 nuclear, encode the protein subunits of the respiratory chain complexes, as well as, their import and assembly proteins (77). One interesting observation is that mitochondrial genome only code proteins involved with organelle gene expression, electron transport and oxidative phosphorylation. As a consequence, mutations in mtDNA will mostly present a deficit in ATP production.

The clinical presentations of mitochondrial diseases are highly variable and the symptoms are often initially vague and non-specific. A mitochondrial disease should be considered in patients of any age with apparently unexplained combinations of symptoms and signs, rapid progressive course and multi-organ involvement, generally affecting brain and the muscles due to their high energy demand (78).

2.3.1. Cytopathies

Mitochondrial cytopathy is a term used to describe a number of diseases which have their appearance due to disturbance in mitochondrial metabolic pathways (79). Mitochondrial diseases are quite complex and correspond to a group of heterogeneous multisystem disorders that mostly affect the function and sometimes the structure of an organ, usually the brain muscle and the heart (80). According to epidemiological studies, at least one in 8000 people under the age of 65 suffers or is at risk of having a mitochondrial disorder in the future (81).

Inherited and sporadic mitochondrial cytopathies may have quite varied effects since the mutation rate of mtDNA varies from organ to organ within the body. Commonly, minor exercise intolerance is often observed in pacients with no serious illness or disability. Severe cases often involve into more complex disorders (Figure 9)

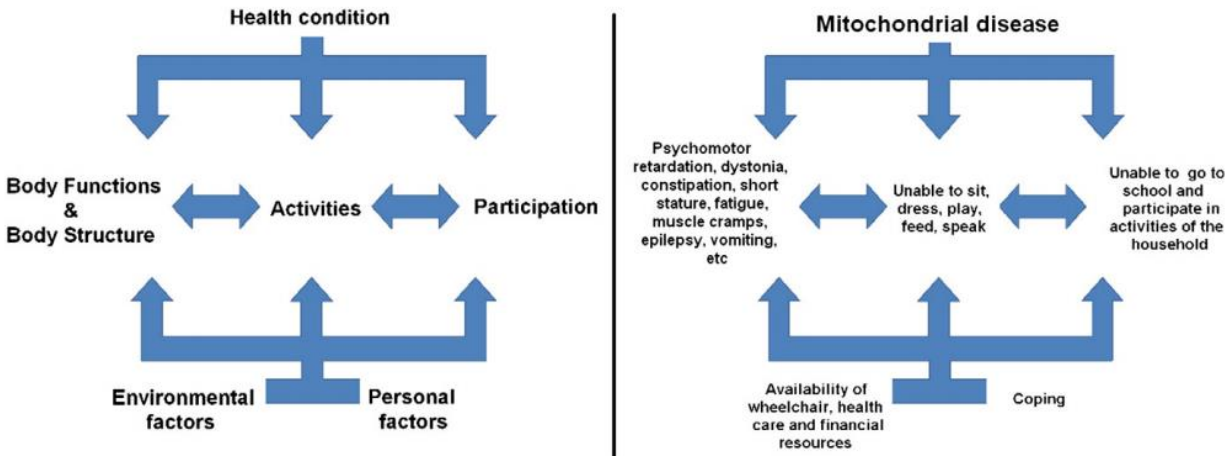

Figure 9. Severe mitochondrial disease condition. Symptoms, limitations and disabilities of a typical patient suffering from mitochondrial disease (right side). (Adapted (82))

Tissues known to be affected by mitochondrial diseases are (79, 82-84):

1. The muscles: Usually manifest lack of energy and exercise intolerance due to the low muscle tone (Hypotonia). Cramps and muscle pains are also observed.
2. The brain: A wide variety of mental problems such as: dementia, mental disorders, development delay and neuro-psychiatric disturbances. Stroke and stroke-like episodes are quite common.
3. The heart: myopathy, heart blocks and cardiac dysrhythmia are the most likely causes associated to the heart
4. The kidneys: Proximal renal tubular dysfunction (Fanconi syndrome) resulting in the loss of electrolytes.
5. The eyes: Optic neuropathy and retinitis pigmentosa are the common conditions usually leading to some vision loss and in some cases permanent loss.
6. The ears: Sensory-neural hearing loss leading to deafness
7. Endocrine System: A wide variety of conditions such as weight loss, diabetes, hypoparathyroidism and exocrine pancreatic dysfunction.

Although, the amount of research behind mitochondrial cytopathies has been growing during the past years, there is currently no established treatment for mitochondrial disorders since the association of mitochondria and disease is quite recent and because it usually refers to multi-systemic symptoms. At this moment, the best approach for treatment are: pharmacological and nutritional agents, diets supplemented with vitamins and co-factors and exercise based therapy (85).

2.3.2. Mutations and mitochondrial diseases

Mutations all over mitochondrial genome have been observed and some specific diseases are often associated to mutations in specific mitochondrial genes and mitochondrial tRNA, while rRNA mutations appear to be less frequent compared to the other two.

Somatic mutations, mutations that were not present in the germ cells but occurred through time, may occur in the mitochondria. Point mutations and deletions are the most common. Point mutations occur essentially due to three factors: base substitution caused by errors in polymerase y (86); proximity to ROS formation sites and lack of histones. No nucleotide excision repair (NER) mechanisms are present, as BER is the only repair mechanism known to work at the site of mitochondria. On the other side, no elucidative studies are able to explain the mechanism behind the origin of mtDNA delections (21, 87).

Mutations in mtDNA genes are a quite frequent cause of mitochondrial cytopathy resulting in a huge variety of clinical phenotypes associated with severe metabolic dysfunctions, including progressive cardiomyopathy, encephalopathy, leukodystrophy, Leigh´s syndrome or ragged red fibbers syndrome and premature age-related symptoms (11, 88-96). In addition, mutations and/or polymorphism variance in mitochondrial genes are related with Parkinson, Huntington´s and Alzheimer´s diseases, diabetes and the greater susceptibility to develop cancer (97-111).

Although, approximately 200 different mitochondrial diseases have been reported (112), the most important diseases that are caused due to defects on the mtDNA are summarized in Table 1.

2.3.3. Aging

Aging is a degenerative process that is characterized by a decline in physiological function and an increased chance of developing a disease and death. These changes, that occur in all organisms, are believed to be associated with the metabolic activity and therefore with the mitochondria.

The initial idea originated in 1956, when Harman proposed the theory of “Free Radical Theory of Aging” (113). The idea assumes that free radicals, produced from normal metabolism, could be the cause of aging and aging-related degenerative diseases. Later on, the same Harman, suggested that mitochondria could be the main producer and the major target of free radicals and therefore, an organelle responsible for aging (114).

As mentioned before, mitochondria are the main cellular energy sources that generate ATP through the process of OXPHOS located at the IMM (25). While the OXPHOS system has the main goal of producing ATP, it also becomes the primary intracellular source of ROS species (33).

Table 1. Pathology associated to mitochondrial genome. (Adapted from (115))

PATHOLOGY	MITOCHONDRIA
Leigh syndrome (LS) Neonatal subacute encephalopathy with bilateral symmetric midbrain and basal ganglia necrosis on MRI	**Gene** ND3 - (116-118) ND5 - (119, 120) ND6 - (121, 122) ATPase6 - (123, 124)
Leber hereditary optic neuropathy (LHON) Profound, painless loss of central vision	**Gene** ND1 - (125-127) ND4 - (126, 128) ND6 - (129, 130)
Neuropathy, ataxia, retinitis pigmentosa (NARP) Neuropathy (Damage of the nerves),Ataxia (Lack of Muscle Coordination),Retinitis pigmentosa (Vision inpairness and/or blindness)	**Gene** ATPase 6 - (131, 132)
Mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) Encephalopathy (Brain disorder),Lactic acidosis (Low pH in blood caused by excessive lactate), Stroke-like episodes (Acute pain that resembles a stroke)	**Gene** ND1 - (133-135) ND5 - (120, 136, 137)
Pearson syndrome (PS) Sideroblastic anemia, pancytopenia, exocrine pancreatic insufficiency and renal tubulopathy	**Deletion** (138, 139)
Myoclonic Epilepsy with Ragged Red Fibers (MERRF) Myoclonus, epilepsy, progressive ataxia, muscle weakness and degeneration, deafness, and dementia.	**tRNA** Leu (140, 141) Lys (142, 143)
Kearns-Sayre syndrome (KSS) External ophthalmoplegia, pigmentary retinopathy, elevated CSF protein, cerebellar ataxia, and cardiac conduction defects	**Delection** (144, 145)
Maternally inherited diabetes and deafness (MIDD) Form of diabetes that is often accompanied by hearing loss	**tRNA** Leu (146, 147)

The Mitochondrial theory of aging proposes that during the course of the years, mtDNA suffers somatic mutations causing a decline in mitochondrial bioenergetics function contributing to aging. During normal conditions, mitochondria produce low levels of ROS and that low amount of ROS can be easily removed by antioxidants and free radical scavenging enzymes. However, mitochondria lack protection mechanisms and oxidative damage cause damage to the mtDNA leading to the appearance of mutant mtDNA. The accumulation of mutant type mtDNA usually results in the dysfunction of the respiratory chain, leading to an increased production of ROS and even more oxidative damage to the mtDNA. This becomes a vicious cycle, resulting in the decline of cellular and tissue functions resulting in the low amount of energy leading to apoptosis (148, 149) (Figure 10).

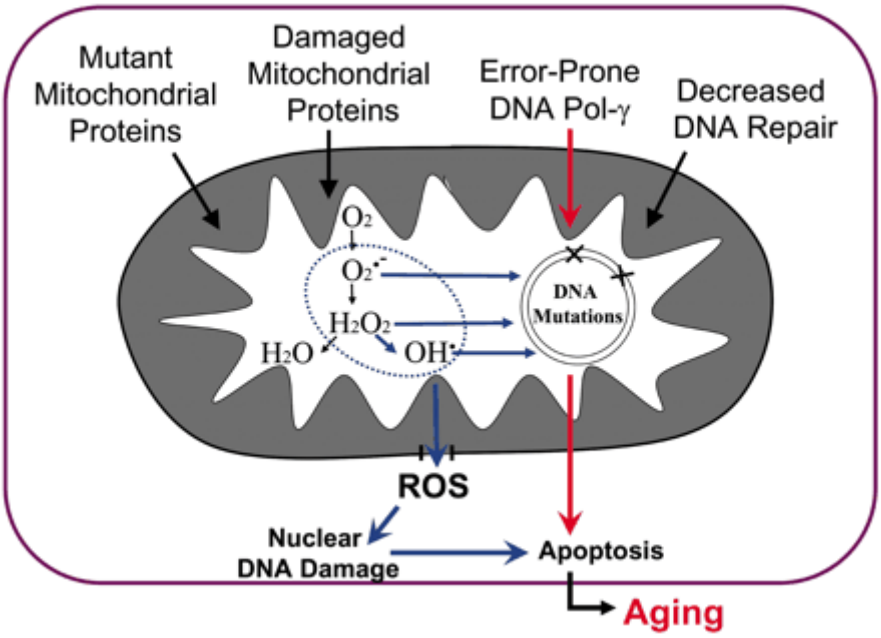

Figure 10. The mitochondrial theory of aging. Multiple factors may cause mtDNA mutations which then leads to an increased production of ROS species. ROS species then contribute to even more mutations leading to a never ending process of aging (149).

3. Mitochondrial Gene Therapy

Over the last decades we have seen the harmful effects mitochondrial diseases may have in a patient's life. Health care professionals have a great dilemma at hands since classical interventions have revealed unsuccessful in the fight against mitochondrial disorders.

From a medical perspective, treatment of mitochondrial disorders is a must but at the same time an incredible challenge. Defective mitochondrial proteins may be due to either nDNA or mtDNA mutations or delections. While conventional gene therapy approach may work in the correction of nDNA troubles, solutions to mtDNA are yet fictional.

Nowadays, it is well recognized that the future approach of mitochondrial medicine may involve the targeting of mitochondria. Focusing on the development of new therapies to correct mitochondrial disorders has become an active and expanding research field (150).

Introducing a mitochondrial gene into mitochondrial matrix might turn out to be the only approach to achieve permanent cure against mtDNA diseases. Up until today, two possible strategies have been suggested to fight against such disorders.

One good strategy is to avoid the threshold effect. As stated before, mitochondrial diseases generally manifest when mutant mtDNA reaches above a critical point in cells. If wild-type mtDNA, isolated from the pacient's healthy tissues, were to be administered into the damaged tissue, swapping with mutated mtDNA may occur and the threshold level may not be reached and the disease not manifested (151).

The other approach is the introduction of a therapeutic mitochondrial gene into the matrix which is seen as a major hurdle. Although electroporation (152) and biolistic transformation (153) have been suitable methods utilized to introduce DNA into the mitochondria, no evidence supports their success for human MGT. Endocytosis is nowadays seen as the most promising solution due to the ability of mitochondria to receive exogenous DNA.

An adequate mitochondrial gene therapy (MGT) system should compromise a minimum number of requisites:

1) The carrier system should initially be taken up by the host cell through an internalization mechanism such as endocytosis. Once inside the cell, our carrier should target the mitochondria instead of other intracellular organelle such as the nucleus, acting as a mitochondriotropic agent.
2) The genetic material inside must be able to traverse both OMM and IMM and reach the matrix where it may meet its target. The target may vary from a vast list which comprises all forms of nucleic acids (mtDNA, rRNA, tRNA) depending on specific aim.
3) The carrier system should bring beneficial effect to the mitochondria. The introduction of genetic material may be beneficial by correcting either a mtDNA mutation or modulation of gene expression. However, compromising the integrity or survival of the cell even if coupled with good results may not be a viable MGT.
4) The mitochondrial transfection vector should ensure long and sustained gene expression.

3.1. Non-Viral Gene Therapy

Gene therapy has evolved during the last decade becoming a trend as a therapeutic approach against a big number of incurable diseases. The possibility of trying to treat these patients, by providing replacement copies of the defective gene, leads to a huge impact in the traditional clinical management (5).

The idea was initially originated in 1963, when Joshua Lederberg suggested that it would be possible to control a nucleotide sequence in human chromosomes, coupled with selection and integration of the desired genes (7). This idea ended up being accomplished only in 1980 with the introduction of two functional genes into mammalian cells (8).

The two main types of gene therapy are somatic cell gene therapy and germ-line gene therapy. Germ-line gene therapy is an interesting concept which consists in the introduction of a gene into reproductive cells (sperm or eggs) or later on the zygote, which results in the transmission of a beneficial gene into the offspring. Even more interesting is that this therapy can be extended into the mitochondria. As shown by Tachibana and colleagues, mtDNA can effectively be replaced in oocytes reproducing embryonic stem cells similar to controls (154). As fascinating it may sound, ethical aspects puts a halt, resulting in less and less studies around this concern and therefore no imminent application in humans, seems to be, even possible (155). Therefore, most of the research, nowadays, is centered on somatic cells.

The delivery of genetic material into the cell can be accomplished by using viral and non-viral vectors. Viral vectors exploit the intrinsic ability of the viruses to target the nucleus. These vectors were once the most commonly used gene delivery systems in gene therapy, because of their highly efficient infection rate and ability to integrate therapeutic genes into the host chromosome ensuring sustained gene expression (156). However, several disadvantages presented by virus vectors such as, the given antigenicity, possibility of virus recombination (157), potential oncogenic effects (158, 159), difficulty in large scale production and instability of storage along with fatal cases associated with a severe inflammatory response have instigated the search for new vectors. When targeting mitochondria is concerned, the choice becomes easier. Since no virus is known to transfect the mitochondria, the intrinsic ability of the viruses becomes obsolete. As a result non-viral vectors, such as the plasmid DNA (pDNA), become the only viable option as transformation is considered (Figure 11).

The pDNA, discovered by Joshua Ledberg in 1952 (160) is a small circlular double-stranded DNA molecule that can replicate independently within the cell. Usually ranging from 1kbp to 1.000kbp, plasmids are widely used as non-viral vectors since they are able to integrate exogenous DNA and then replicate this exogenous within the cell target.

Once transfection is achieved by the therapeutic gene, its purpose may not always be the same. Successfully delivered genes could have different therapeutic approaches such as: modify the defective host gene, replace the deficient host gene, to insert into the host

genome or just to stay in the nucleus with no integration occurring within the host genome (161).

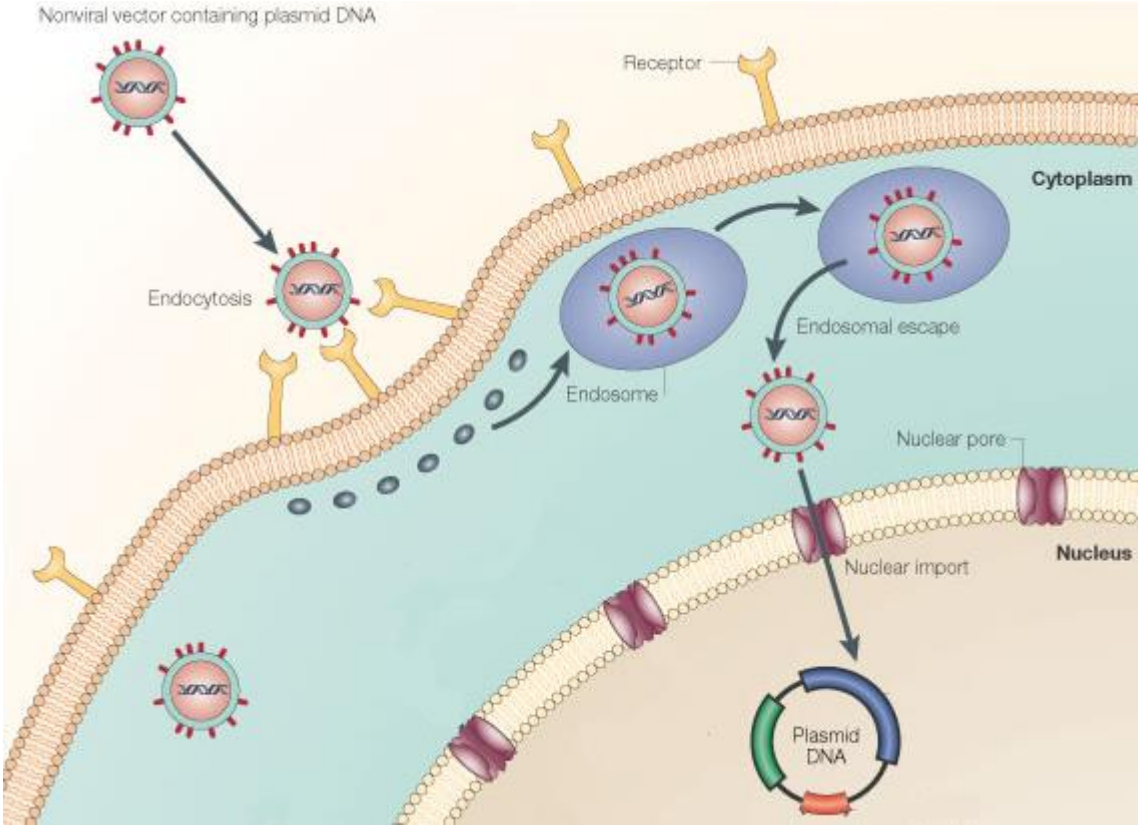

Figure 11. Transfection Schematics. Non-viral vectors are capable of receptor-mediated endocytosis. In the cytoplasm, endosomal escape and nuclear import, ending with the delivery of pDNA carrying a therapeutic gene into the nucleus. Adapted from (162)

Non-viral gene therapy has evolved to the point that there are huge varieties of methods to deliver the pDNA, ranging from conventional methods such as injection of naked DNA and electroporation to the usage of nanoparticles (163) and gels (164).

3.2. Traversing mitochondrial membrane

As is well known, to the proper function of the mitochondria both mtDNA and nDNA play important roles. In order to correct mutated mitochondrial genes, gene therapy emerges as a viable possibility; but are we able to deliberately introduce nucleic acids at the site of mitochondria in a transfection process?

Targeting DNA into mitochondria should present itself as a hard challenge involving several obstacles. Mitochondrial membranes are of lipophilic nature preventing the entrance of big molecules such as peptides and DNA unless there is an active transport mechanism (165). Moreover, if it ends up entering the cell via the endocytic pathway it may become entrapped in the endosome and eventually end in the lysosome where degradation takes place (165).

Milana Koulintchenko and colleagues first demonstrated that plant have a transmembrane potential-dependent mechanism of plamid-like DNA uptake into mitochondria (166) and later, through five different assays that mammalian mitochondria possess a natural competence for DNA uptake (167). Further observation of both works proposed that DNA intake in both plants and mammals is achieved through voltage-dependent anion channel and that mammalian can intake both ss (single-stranded) and ds (double-stranded) DNA while plants can only internalize ds DNA (166, 167). As previously described, mitochondria are able to successfully integrate exogenous DNA and although the idea behind how DNA can transverse OMM has started to become understood and accepted until today the mechanisms behind the IMM are still unknown.

Transfection of plamid DNA into the mitochondria has been successfully achieved (168, 169) but transformation, incorporation and expression of transfected DNA has only been reported in *Saccharomyces cerevisiae*, *Chlamydomonas reinhardtii* (170) and *Candida glabrata* (171) while in mammals it is still a pipe dream.

3.3. Nanotechnology

A major research thrust in the biochemical/pharmaceutical technology is still the development of efficient and safe controlled release systems for the sustained delivery of drugs and bioactive agents. To be used therapeutically, these systems should be able to deliver the drug and/or gene at a specified rate and time period. Furthermore, they can be targeted to a particular organelle or cell type.

Nanotechnology provides appropriate knowledge and tools for the design and creation of new suitable biocompatible formulations for gene delivery purposes. This technology can be applied to mitochondrial gene therapy providing nanosystems to carry genetic information to mitochondria, since mitochondria size range from 0,5 to 1 (μm) in diameter (15). Furthermore, the idea that nanosystems have unique physical and biological properties that might be used to overcome the problems of gene delivery, has gained interest in recent years because they can ensure protection against enzymatic degradation, are able to by-pass the innate immune system, have good biodistribution, reduced side-effects, safety, no toxicity, reduced inflammation and the therapeutic payload can be released in the desired target (14). Until today, different types of carrier systems have been developed with the purpose of gene therapy application: nanoparticles, dendrimers, nanocages, micelles, molecular conjugates

and liposomes. Unfortunately, mitochondria targeted therapy represents a significant challenge and only a few nanocarriers are, at the moment, seen as possible candidates (Figure 12). For instance, considerable improvements have been made with dequalinium cationic vesicles (DQAsomes) with mitochondria affinity as vehicles for drugs and DNA to this organelle. These mitochondriotropic vesicles bind and condense plasmid DNA and release it when in contact with cardiolipin-rich liposomes mimicking mitochondrial membranes (172, 173).

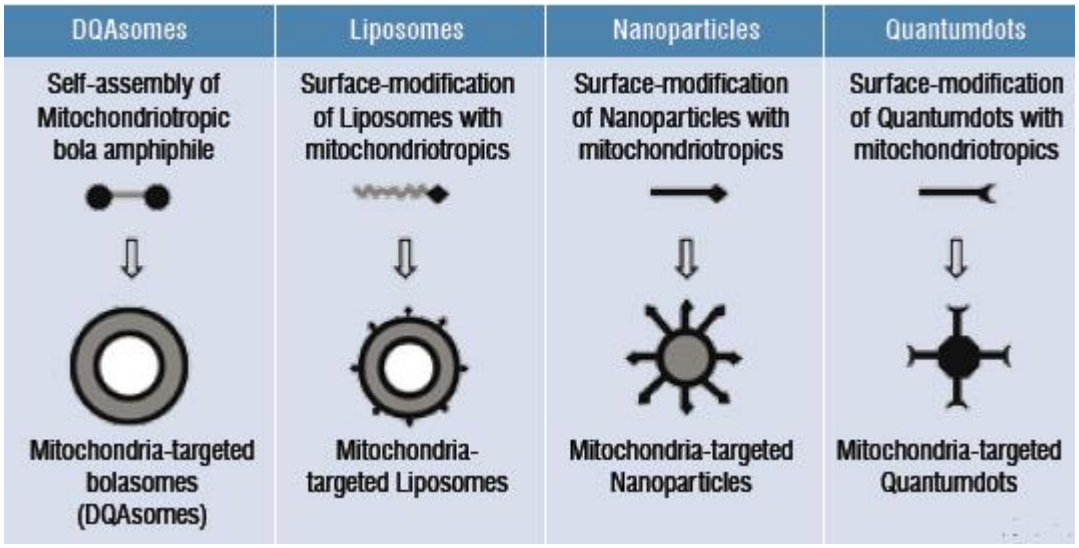

Figure 12. Mitochondria-specific Nanotechnology. : DQAsomes (DeQuAlinium-based liposome-like vesicles), nanolipid vesicles, solid nanoparticles and quantum dots are the typical nanocarrier systems used when approaching the mitochondria (Adapted from (174))

3.4. Salt as an efficient nanocarrier

Although the development of efficient methods to produce gene delivery vehicles for gene therapy purposes started decades ago, it has not still reached a point where we can say that we have found the perfect nanocarrier system. Several promising gene delivery systems including polymeric (175), silica based (176) and liposomal (177) have been developed. However, the biocompatibility displayed by these systems is not satisfactory.

One of our main concerns, when designed the mitochondrial nanocarrier, was its biocompatibility and biodegradability. The nanocarrier system, proposed in this thesis, is based on calcium carbonate ($CaCo_3$) and was prepared through the technique of co-precipitation of calcium (Ca^{2+}), an inorganic cation, with carbonate (CO_3^{2-}), an inorganic anion.

Calcium is essential for living organisms since it functions as an important signal for many cellular processes. It is the major material used in mineralization of bone and teeth and it is

required for the normal function of all muscles and nerves. On the other side, carbonate works as a buffer in the blood.

Calcium carbonate is widely used medicinally as an inexpensive dietary calcium supplement. Calcium carbonate is composed of three important elements that are present in almost all organic and inorganic materials: carbon, oxygen and calcium. $CaCO_3$ is a white, non-toxic and odorless salt (178) that allure many scientists to its usage as a nanocarrier system due to their good biocompatibility, biodegradability, wide range of resources, size and low cost (179).

Although the usage of $CaCO_3$ nanocarriers has been presented as a plausible choice for gene therapy (180-183), its application as a MGT has not, until now, been demonstrated.

Aims of the Project

In addition to the cell nucleus, mitochondrion is the unique organelle with its own genome-the mitochondrial DNA. Mitochondrial genome is considered a hotspot for mutations due to the lack of protection and repair mechanisms. Mutations in mtDNA genes are a quite frequent cause of mitochondrial cytopathy and conventional treatments are, in most of the cases, inefficient. Mitochondrial gene therapy, thus, emerges as a new perspective to correct such anomalies.

Although MGT is seen as a promising approach, transformation is not yet possible in mammals and, even transfection, has hardly ever been achieved into the mitochondria. The application of virus in MGT seems impossible, since no virus is known to target the mitochondria. Therefore we shift our attention to the plasmid, a non-viral vector which is widely used as a therapeutic agent in gene therapy.

Taking all the previous information into account, during this MsC thesis, it was tried to bring together a new nanocarrier system that can effectively deliver pDNA into the mitochondria, for future application in mitochondrial gene therapy.

The first aim of this work consisted in the isolation and purification of three plasmids with different sizes. Then, we proceeded to the development of $CaCO_3$-pDNA-rho_{123} nanoparticles through a co-precipitation method. We then designed two different protocols, in order to encapsulate 1μg and 10μg of pDNA and characterized each one and compared them. Lastly, we proceeded to the nanocarrier *in vitro* evaluation.

The success of this work is based on the design and preparation of a suitable vector that can represent a promising tool for progresses in mitochondrial gene delivery purposes, contributing for new therapies centered in mitochondria.

Materials and Methods

1. Materials

1.1. Reagents

Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM: Ham's F12) was obtained from Biochrom AG (Germany). Rhodamine 123, Tween 20, Triton X-100, paraformaldehyde (PFA) and Deoxyribonuclease I from bovine pancreas (DNAse I) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Agarose and Green Safe were obtained from NZYTech (Lisboa, Portugal). Mitotracker® Orange CMTMROS was obtained from Invitrogen™ (Eugene, Oregon, USA). Sodium Carbonate anhydrous was obtained from Panreac Quimica SA (Barcelona, Spain). Calcium chloride was obtained from BDH Prolabo (Leuven, Belgium). Cellulose powder was obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Tween 20 was obtained from Applichem (Darmstadt, Germany). Normal Human Dermal Fibroblast (NHDF) adult donor cells, Ref. C-12302 (cryopreserved cells) were purchased from PromoCell.

1.2. Plasmid

Both pUC19 and pVAX1-*LacZ* were obtained from Invitrogen (Carlsband, CA, USA) while pcDNA3-myc-*FLNa* S2152A was obtained from Addgene plasmid 8983 (pcDNA3-based plasmid) (Cambridge, MA, USA).

The pDNAs (Figure 13,14,15) were designed with the usage of SnapGene Viewer 2.1.

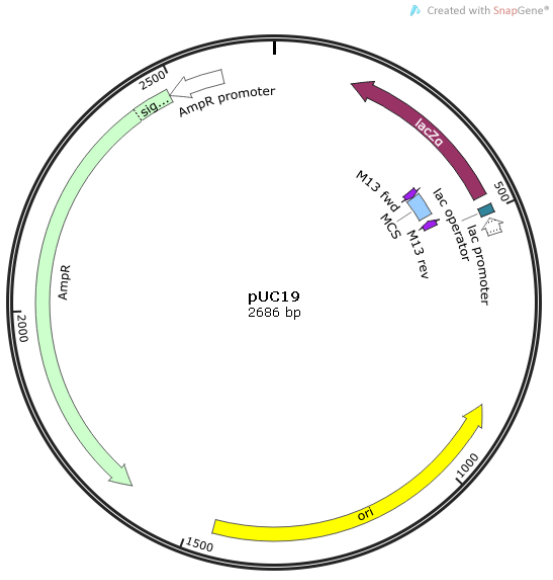

Figure 13. pUC19 mapping. Representation of the main features of the 2686bp pUC19.

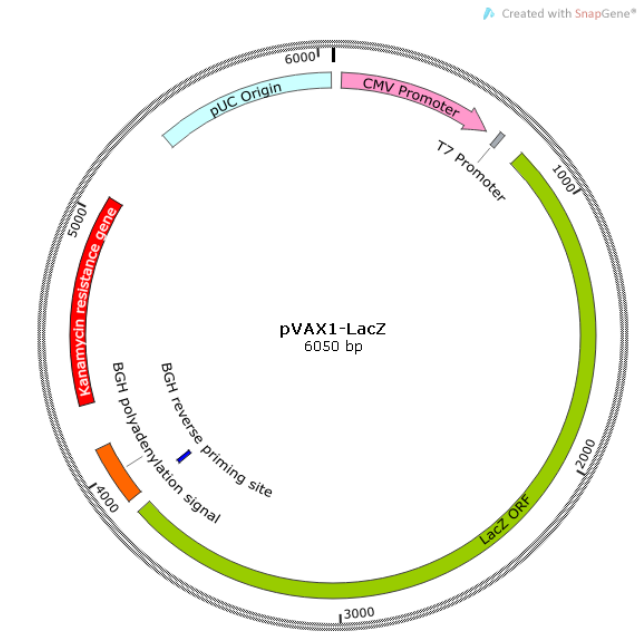

Figure 14. pVAX1-*LacZ* mapping . 6050 bp pVAX1-*LacZ* has the exact same features as pVAX1 (2999bp) but containing the additional gene, ß-galactosidase.

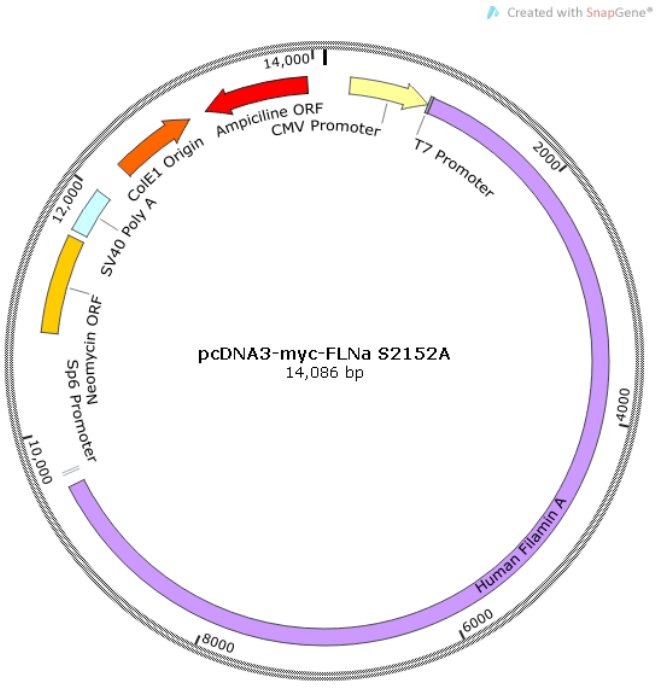

Figure 15. pcDNA3-myc-*FLNa* S2152A mapping. The 14,086bp pcDNA3-myc-*FLNa* S2152A has the exact same features as the pcDNA3 (5446bp) but containing the additional gene, Huma Filamin A.

2. Methods

2.1. Bacterial Growth and Plasmid Purification

The 2.6kbps plasmid pUC19, the 6.1kbps pVAX1-*LacZ* and the 14kbps pcDNA3-myc-*FLNa* S2152A were amplified by fermentation carried out in a 500 mL Erlenmeyer using a Terrific Broth medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4) supplemented with the appropriate antibiotic; 100 µg of ampicillin/mL for the cells transformed with pUC19, 30 µg of kanamycin/mL for the cells transformed with pVAX1-*LacZ* and a combination of ampicillin/mL with neomycin/ml for the cells transformed with pcDNA3-myc-*FLNa* S2152A , adapted from (184).

The bacterial growth was carried out overnight, at 37ºC under 250 rpm shaking, and the cells were harvested at the late log phase (OD600 nm ≈ 9) by centrifugation.

Plasmid purification was achieved using QIAGEN® Plasmid Purification kit. Shortly, cells were suspended, lysed and precipitated followed by a double centrifugation at 20,000 x g for 30min at 4 ◦C with the recovery of supernatant containing plasmid DNA. Supernatant was added to a QIAGEN-tip to remove most contaminants followed by DNA elution, precipitation and centrifugation at 15,000 x g for 30 min, 4 ◦C. The pellet was recovered and the pDNA concentration estimated through UV-VIS analysis and finally suspended in the suitable buffer and stored at -80ºC.

2.2. Agarose gel electrophoresis

Electrophoresis is a technique that consists in the usage of an electric field applied to a gel matrix that permits the separation and identification of nucleic acids based on their size and charge. The electrophoresis experiments were carried out by running a 1% agarose gel (Hoefer San Fransisco, Ca, USA) stained with Green Safe (1 µg/mL). Electrophoresis was carried out at 110V for 20 minutes with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA pH 8.0). Gel visualization occurred under UV light in a Vilber Loumat system (ILC Lda, Lisbon, Portugal).

2.3. Synthesis of $CaCO_3$-pDNA-Rho_{123} Nanoparticles

Plasmid DNA solution containing 1 µg of plasmid DNA, 40 uL of $CaCl_2$ solution (0.07 g/mL) and 0.1 µg of Rhodamine 123 (Rho_{123}) were mixed and then diluted with deionized water to make a solution A with a total volume of 75 µL. 40 µL of Na_2CO_3 solution (0.66 µg/mL) was mixed together with 1 µg of cellulose and then diluted with deionized water to make solution B with a total volume of 75 µL. Solution A was then gently added to Solution B with the help

of a Pasteur pipette to form a final Solution C with a total volume of 150 µL containing $CaCO_3$-pDNA-Rho_{123} nanoparticles (181).

Similarly, plasmid DNA solution containing 10 µg of plasmid DNA, 120 µL of $CaCl_2$ solution (0.03 g/mL) and 0.5 µg of Rho_{123} were mixed and then diluted with deionized water to make a solution A with a total volume of 290 µL. 255 µL of Na_2CO_3 solution (0.0425 µg/mL) was mixed together with 5 µg of cellulose and then diluted with deionized water to make solution B with a total volume of 260 µL. Solution A was then gently added to Solution B with the help of a Pasteur pipette to form a final Solution C with a total volume of 550 µL containing $CaCO_3$-pDNA-Rho_{123} nanoparticles (183).

2.4. Particles Morphology

Recently formed nanoparticles were centrifuged (10.000 g, 20 min, 25 ºC) and the pellet recovered. The pellet was suspended in a solution containing 20 µL deionized water with 20 µL tungsten. 10 µL of the recently formed solution was set in roundly shaped cover-slip and left at room temperature overnight to dry.

In the following day, the samples were sputter coated with gold using an Emitech K550 sputter coater (London, England) and then analyzed by scanning electron microscope (SEM) (Hitachi S-2700, Tokyo, Japan), operated at an accelerating voltage of 20 kV with variable magnifications.

2.5. pDNA Encapsulation Efficiency

The encapsulation efficiency (EE) of the pDNA was determined after centrifugation (15.000 g, 20 min, 25 ºC) and recovery of the supernatant. The supernatant corresponded to the unbound pDNA or, in other words, the pDNA that was not encapsulated into $CaCO_3$ nanoparticles. The concentration of unbound pDNA was determined by Uv-vis analysis at 260 nm in a NanophotometerTM (Implen, Germany). Desionized water was used to perform the blank experiment. At least three independent measurements were performed.

To determine EE values we resorted to the following formula:

$$Encapsulation\ Efficiecny\ (\%EE) = \frac{Total\ amount\ of\ pDNA\ -\ unbound\ pDNA}{Total\ amount\ of\ pDNA} x\ 100$$

2.6. Nanoparticles Size

Samples were injected into a Quartz flow cell, with the help of a Pasteur Pipette. Size information was obtained using Dynamic Light Scattering option from Zetasizer Nano ZS

(Malvern Industries, United Kingdom) which measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes-Einstein relationship. Incorporation of Non-Invasive Back Scatter technology (NIBS) gives the highest sensitivity along with the largest size and concentration range. To perform the blank, pDNA free sample were used and to validate the results, at least three measurements were performed. All results were recorded and analyzed with the help of Malvern Zetasizer software v6.34.

2.7. Zeta (ζ) Potential

Samples were introduced into a Dip Cell with the help of a Pasteur Pipette. ζ Potential information was obtained using Laser Doppler Micro-electrophoresis option from Zetasizer Nano ZS (Malvern Industries, United Kingdom) where an electric field is applied to a solution of molecules or a dispersion of particles which then move with a velocity related to their ζ potential. This velocity is measured through an interferometric laser technique named Phase Analysis Light Scattering enabling the calculation of the ζ potential and ζ potential distribution. To perform the blank, pDNA free sample were used and to validate the results at least three independent measurements were performed. All results were recorded and analyzed with the help of Malvern Zetasizer software v 6.34.

2.8. Protection Test

Sample of pDNA nanoparticles of 20 µL were incubated with 5 µL of DNAse I solution with a concentration of either 5 µg/mL or 10 µg/mL at 37 ºC for a period of 1 hour. The control group was also incubated at 37 ºC for 1 hour but with DNAse I free. Followed the incubation period, samples were run in an agarose electrophoresis gel with the same settings as stated in point 2.2.2.

2.9. Cell Culture Growth and Maintenance

Previously frozen Human Fibroblast cell line were taken from cryogenic storage and thawed in a 37 ºC water bath. Immediately after the cells were thawed, cells were seeded into a sterile 75 cm^2 T-Flask appropriate for cell culture with the help of a 1 mL sterile serological pipette. Next, 10 mL of DMEM/F12 media (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) was added and the flask was sealed and placed in an incubator at 37ºC, 5% CO_2 and 90% humidity for an appropriate cell growth. After 24 hours, the cell culture was checked through microscopy in order to determine if the cell were actually adherent to the bottom. When the media started changing from a pink color to a slight orange/yellow color it

was replaced by 10 mL of new fresh media to replenish the nutrients and remove any unattached, dead cells. The cell culture was then monitored daily and typically, media was replaced every 2 to 3 days. Growth was carried until nearly 80% of confluence was attained. At this point, three different procedures could occur: 1) cells were used to perform experiments; 2) cells were subcultured in two to three new 75 cm^2 T-Flasks supplemented with 10 mL of media; 3) cells were transferred to a 150 cm^2 T-Flask supplemented with 25 mL of media to even further promote growth.

To harvest the cells, firstly the media was exchanged with a PBS solution (NaCl 8 g/L, KCl 0.2g/L, Na_2HPO_4 • 2 H_2O 1.8g/L, KH_2PO_4 0.3g/L with a 7.4 pH) to remove any dead cells and detritus present. Thereafter, the PBS solution was removed and 5 mL of recently thawed Trypsin solution has been added to promote the detachment of the living cells that were aggregated to the bottom of the flask. Past 3 minutes, 5 mL of media was added to stop the trypsin effect followed by a small centrifugation of 150 g for 5 min at 25 ºC and recovery of the pellet, containing the live cells.

To determine the number of living cells, the recently recovered pellet was gently suspended in 5 mL of new media. Then, a small sample of 20 µL was taken and mixed together with 20 µL of trypan blue. Live cells were then counted with the help of a Neubauer chamber, where every white dot represented a living cell.

Using the following formula we were, then, able to estimate the number of living cells.

$$Total\ nº\ of\ Cells\ = nº\ of\ counted\ cells\ x\ 10^5$$

2.10. Cell Cytotoxicity

Human fibroblast cells were plated at a density of 2 $x10^4$ cells per well in a 96 well plate, previously, ultraviolet irradiated for 35 minutes to decontaminate. $CaCO_3$-pDNA-Rho_{123} nanoparticles were then added to each well and incubated at 37 ºC with 5% CO_2 humidified atmosphere, for 24 and 48 hours. After incubation, the redox activity was assessed through the reduction of the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). 100 µL of MTT dye solution (0.05 mg/mL in Krebs) was added to each well, followed by incubation for a period of 2 hours at 37 ºC, in a 5% CO_2 atmosphere.

Followed the incubation period, the medium was aspirated and cells were treated with 50 µL of HCl (0.04 N) in isopropanol for 40 minutes. Absorbance at 570 nm was measured using a Biorad Microplate Reader Benchmark where the spectrophotometer was calibrated to zero absorbance using culture medium without cells. Experiments were repeated three times in triplicate to validate the results.

To determine the cell viability we resorted to the help of the following formula:

$$Cell\ Viability\ (\%)\ = \frac{Test\ (Abs)}{Control\ (Abs)}\ x\ 100$$

2.11. *In Vitro* Transfection

Fibrobast cells (30,000 cells) were seeded into a 24 well plate containing round shaped lamella for a period of, at least, 24 hours to assure that the cells are able of adhesion. Then, the cells were incubated with $CaCO_3$-pDNA-Rho_{123} nanoparticles for a period of 3 hours at 37ºC to promote the transfection.

Past the first incubation period, cells were stained with 200 nM of Mitotracker Orange CMTMROS for a period of 60 minutes at 37ºC. MitoTracker probes, which passively diffuse across the plasma membrane, can only accumulate in active mitochondria. Once the mitochondria are labeled, the cells can then be treated with fixative step.

To fix the cells we start by incubating them with paraformaldehyde (PFA) 4% for a period of 15 minutes. To finish the staining procedure we incubate the cells with 1 μM Hoescht 33342 for a period of 10 minutes.

Cells were then mounted by placing the round shaped lamellas in a lamina with the help of Entellan solution and visualized through confocal microscopy (ZEISS LSM 710, Oberkochen, Germany). Probes excitation and emission wavelength are stated in the next table (Table 3).

Table 3. Confocal Microscopy Probes

Probe	Excitation (nM)	Emission (nM)
Rhodamine 123	505	560
Hoescht 33342	343	483
Mitotracker Orange CMTMROS	554	576

Between incubation periods, cells were washed twice with a PBS solution. Furthermore, all procedures after incubation with nanoparticles were done in the dark to ensure the probes efficacy.

2.12. Statistical Analysis

Graph design and statistical analysis were carried out using GraphPad Prism 6 software. Statistical analysis was carried out for Encapsulation Efficiency Test, Nanoparticle Size, ζ Potential Test and MTT Assay. In all these tests we analyzed parameters such as the media and standard deviation followed by a two-way Anova to access the comparison between pDNA and system.

Results

1. Plasmid Amplification and Purification

To yield pDNA successfully, mainly three steps are involved: growth of the bacterial culture, harvesting and lysis of the bacteria and purification of pDNA.

E. Coli growth is normally carried out in liquid medium due to the high pDNA production rate (185). Generally, pDNA encode at least one antibiotic resistance gene so that bacteria that have been successfully transformed may multiply. The plasmids pUC19 and pVAX1-*LacZ* have an antibiotic resistance gene, penicillin and kanamycin respectively, while pcDNA3-myc-*FLNa* S2152A has two, penicillin and neomycin.

The last two steps are performed through Qiagen Kit where gDNA, RNA and proteins are denatured. In the end, we should obtain two forms of pDNA: supercoiled (sc) and open circular (oc).

2. $CaCO_3$ Nanoparticles synthesis

Both nanoformulations presented were synthesized through a co-precipitation method, in two steps that involve the preparation of solution A and B that merge together as demonstrated in the Figure 16. Co-precipitation is a phenomenon where a solute that would normally remain dissolved in a solution precipitates out on a carrier that forces it to bind together instead of remaining dispersed. In this case, the $CaCl_2$ molecule contributes by releasing both calcium (Ca^{2+}) and two chloride (Cl^-) ions while, the Na_2Co_3 molecule also contributes with the release of two sodium (Na^+) and one carbonate (CO_3^{2-}) ions. Then, the anion CO_3^{2-} reacts with the cation Ca^{2+} resulting in the $CaCO_3$ salt which is insoluble in water. At the same time, anion Cl- is reacting with the cation to promote the formation of two molecules of sodium chloride (NaCl) that opposed to $CaCO_3$ is soluble in water. The reaction scheme is summarized in Figure 17.

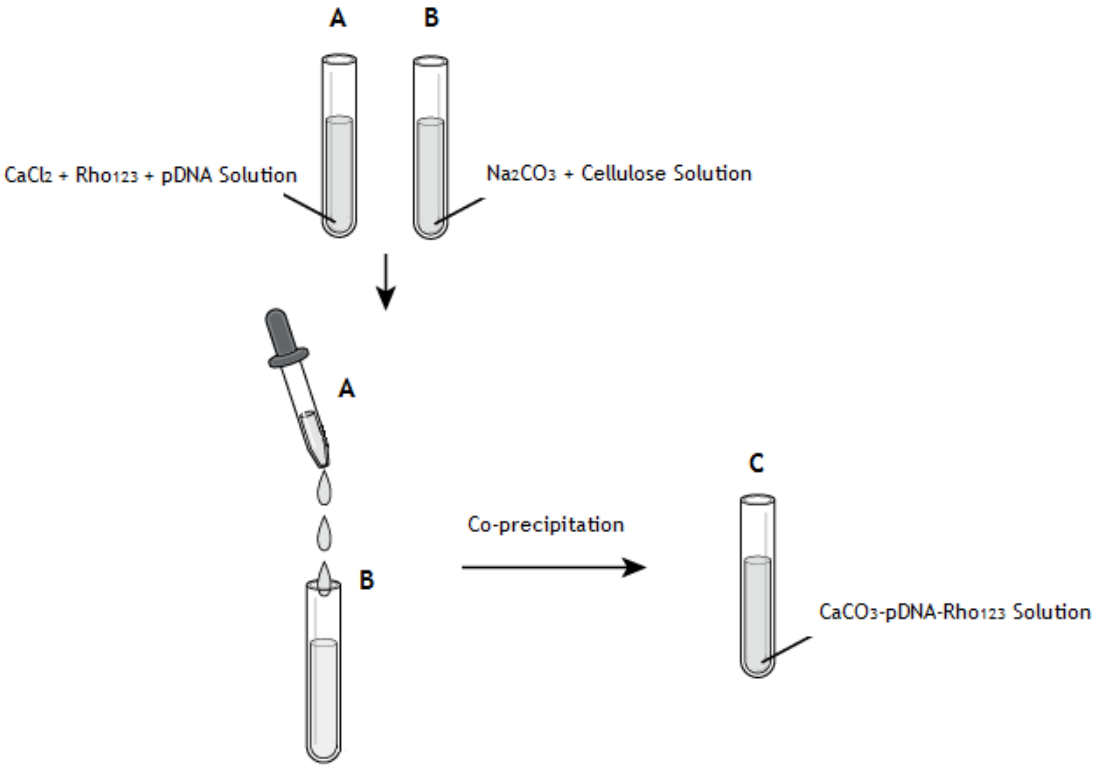

Figure 16. $CaCO_3$ Nanoparticles synthesis. Solution A containing $CaCl_2$, Rho_{123} and pDNA is gently added to a solution B containing Na_2CO_3 and Cellulose creating a final solution C containing $CaCO_3$-pDNA-Rho_{123} Nanoparticles.

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Rhodamine 123, 2-(6-Amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester, is a cationic fluorescent dye often used as a fluorometer to label respiring mitochondria. The dye distributes according to the negative membrane potential across the mitochondrial inner membrane emitting a green fluorescent light. Loss of potential will result in loss of the dye and, therefore, the fluorescence intensity (186). Furthermore, rho_{123} has been proved to act as a mitochondriotropic agent (187). The term mitochondriotropics refers to any molecules displaying a high degree of mitochondrial affinity (13).

$$CaCl_2(aq) + Na_2CO_3(aq) \longrightarrow CaCO_3(s) + 2NaCl(aq)$$

Figure 17. $CaCO_3$ Co-precipitation. Both aqueous solutions, $CaCl_2$ and Na_2CO_3 precipitate together to form a solid compound $CaCO_3$ followed with two molecules of the NaCl solution.

Cellulose is a linear polysaccharide polymer and its structure consists of long polymer chains of glucose units connected by a beta acetal linkage. Cellulose is seen as a promising approach in drug delivery systems since they offer antimicrobial activity by inhibiting the formation of bacterial biofilms while being biocompatible and nontoxic (188). Even more, the lack of stability by the $CaCO_3$ nanoparticles may be attenuated by the effect of cellulose.

Initially known for offering stability and protecting plants, cellulose is thought to reinforce nanoparticles turning them tougher in the presence of agents (189).

The presence of Rho_{123} in both solution A and C makes the colorless solution become a bit orange. For characterization purpose, where the effect of rho_{123} is of no interest, $CaCO_3$-pDNA-rho_{123} nanoparticles formation was carried normally. However, when formed for *in vitro* experiments, $CaCO_3$ nanoparticles were formed covered from light. Nanoparticles formation were carried prior to any test and left in ice to ensure preservation.

3. Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is a technique that uses a beam of highly energetic electrons to yield information about the topography and morphology of objects at a very fine scale. In this case, SEM permits us to understand some important aspects of $CaCO_3$ nanoparticles such as the shape, size and surface properties, features that conventional light microscopes cannot convey due to the lack of resolution. Images provided by SEM are presented for the different pDNA systems and initial loading amounts in Figures 18, 19 and 20.

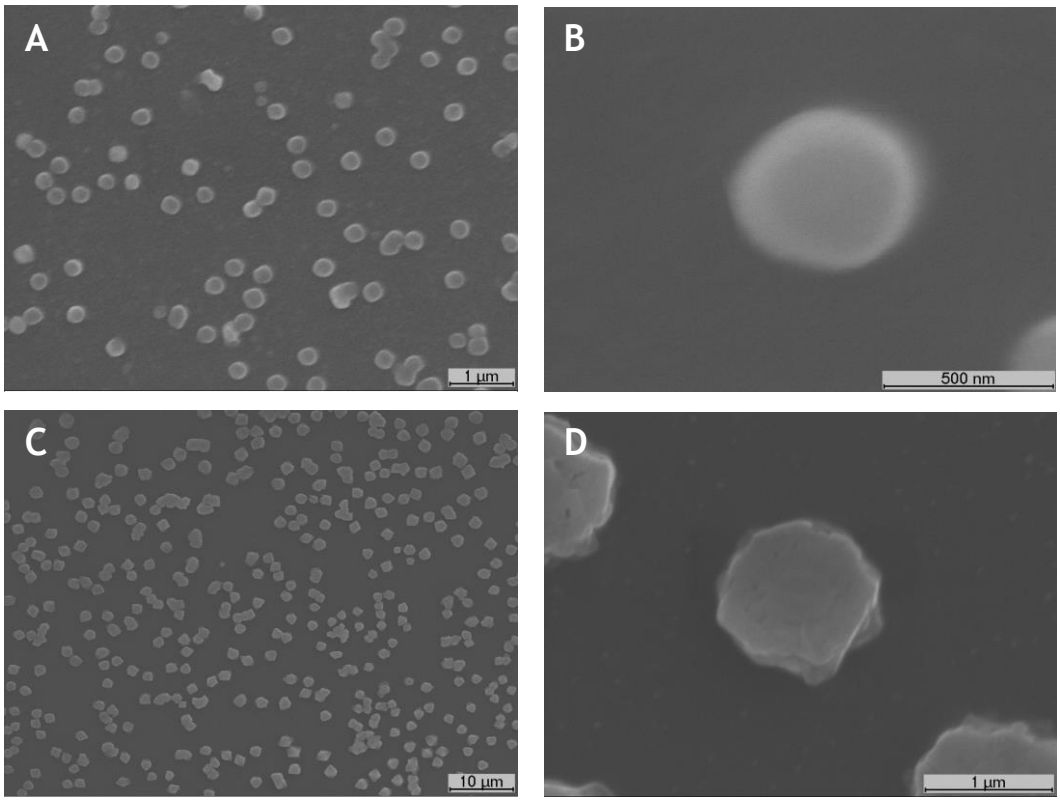


Figure 18. **pUC19 Nanoparticles Morphology**. Images A and B correspond to 1µg system, while images C and D correspond to 10µg system.

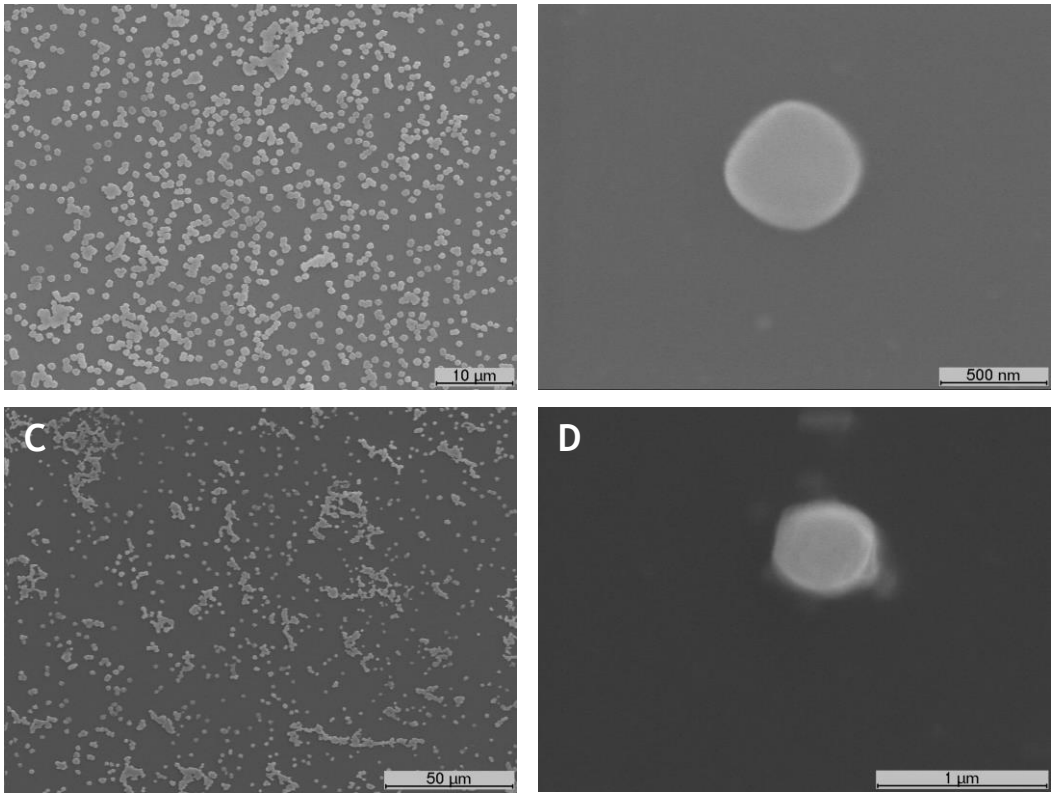


Figure 19. pVAX1-*LacZ* Nanoparticles Morphology. Images A and B correspond to 1µg system while images C and D correspond to 10µg System

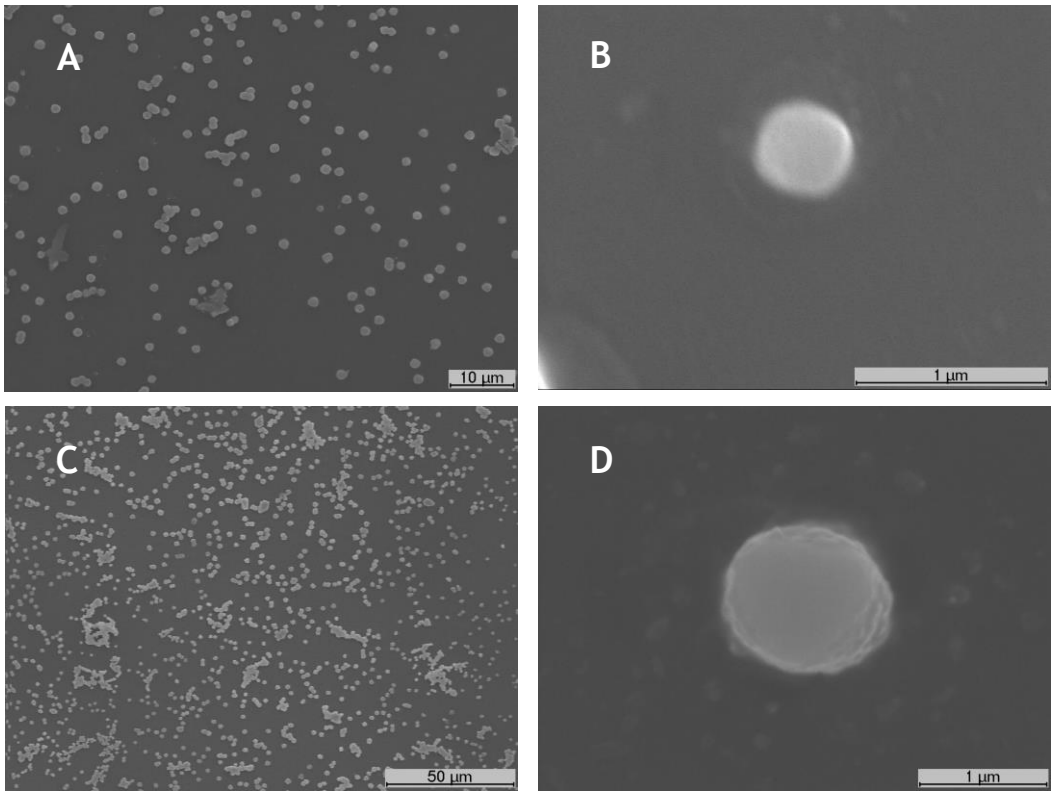
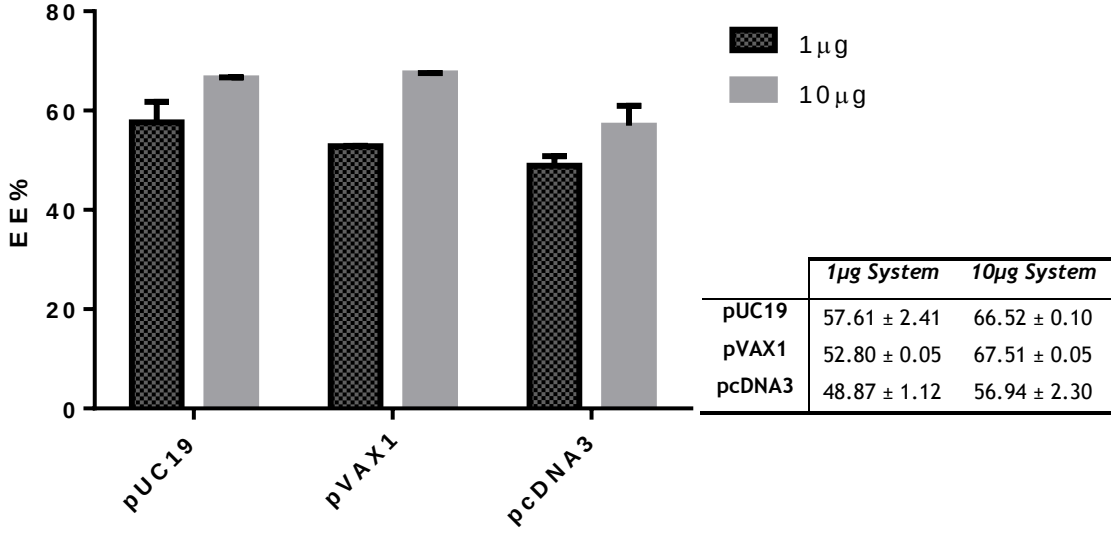


Figure 20. pcDNA3-myc-*FLNa* S2152A Nanoparticles Morphology. Images A and B correspond to 1µg system while images C and D correspond to 10µg System

The scanning electron micrographs exhibit nanoparticles with a round shaped size that seems to range from, approximately, 500nm to 1µm. Another conclusive aspect is that 1µg system nanoparticles appear to have a more defined spherical morphology and they have slightly lower sizes compared to the 10µg system, where aggregation is notorious and the round shaped form is somehow irregular.

4. Encapsulation Efficiency

To design a suitable pDNA system for gene therapy purposes, the pDNA encapsulation efficiency is a relevant issues to consider. Figure 21 presents the pDNA encapsulation efficiency for the three pDNA nanosystems where larger pDNA encapsulation efficiencies were achieved for all pDNA vectors with 10µg. The encapsulation efficiency led to significant results for both pDNA (31.26% variation) and system (56.01% variation). When comparing pDNA, it is notorious a decay of EE% by increasing the pDNA size in 1µg systems. On the other side, for 10µg systems it was only observed decay for the larger plasmid. This data indicates that size of pDNA affects %EE where pDNA with smaller size are encapsulated more effectively compared to pDNA of larger size.



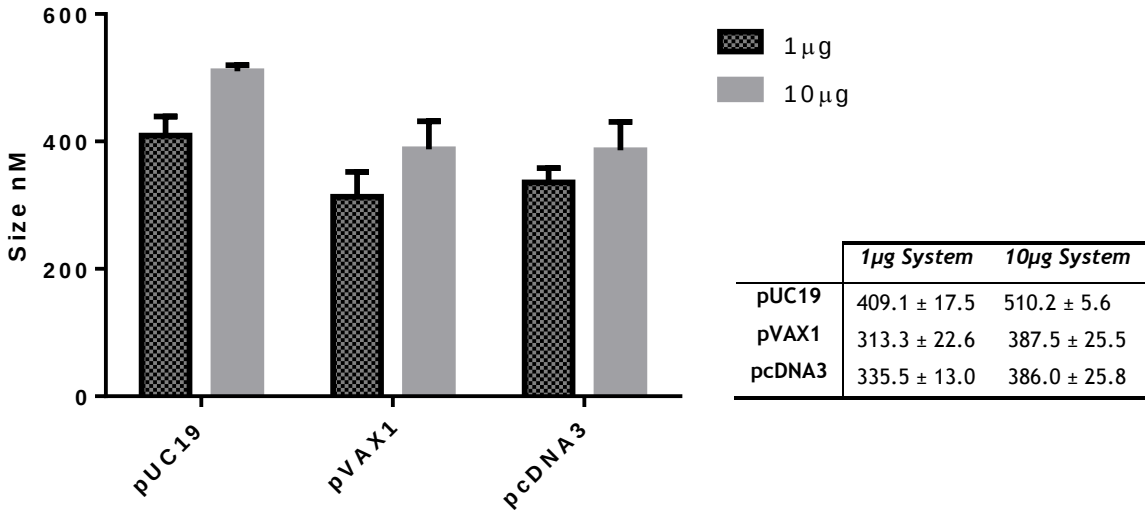
	1µg System	*10µg System*
pUC19	57.61 ± 2.41	66.52 ± 0.10
pVAX1	52.80 ± 0.05	67.51 ± 0.05
pcDNA3	48.87 ± 1.12	56.94 ± 2.30

Figure 21. Encapsulation Efficiency of $CaCO_3$-pDNA-Rho_{123} Nanoparticles. All values are represented as Mean ± SEM with N=3. Two-way Anova treatment reveal a plasmid $p<0.0001$, system $p<0.0001$ and an interaction of $p=0.0802$.

When comparing the 1µg and 10µg systems, %EE was higher in the 10µg systems. This suggests that the protocol designed for 10µg system can encapsulate more pDNA when compared to the 1µg system protocol.

5. Nanoparticle Size

A quite relevant property of a nano vehicle is its size. The mean size of the nanoparticles is presented in Figure 22. The results were rather interesting and significant for both pDNA (51.33% variation) and system (29.97% variation). When comparing 1μg and 10μg systems, it was found that 10μg had larger size across all three plasmids when compared to the 1μg systems. On the other hand, pDNA results had no real conclusive idea since pUC19 had values away higher when compared to pVAX1-*LacZ* and pcDNA3-myc-*FLNa* S2152A .



	1μg System	*10μg System*
pUC19	409.1 ± 17.5	510.2 ± 5.6
pVAX1	313.3 ± 22.6	387.5 ± 25.5
pcDNA3	335.5 ± 13.0	386.0 ± 25.8

Figure 22. $CaCO_3$-pDNA-Rho_{123} Nanoparticles Size. All values are represented as Mean ± SEM with N=3. Two-way Anova treatment reveal a plasmid $p<0.0005$, system $p<0.001$ and an interaction of $p=0.4615$.

Interesting, is that the pDNA become non-significant, when pUC19 is taken out of the equation. As interesting as it may seem, the systems prepared with pVAX1-*LacZ* and pcDNA3-myc-*FLNa* S2152A having almost the same size may be pure coincidence and no relationship between nanoparticles and pDNA size may be taken. In summary, all carriers present diameter sizes in the nano scale, what make them appropriate for cell uptake and internalization in practical applications concerning gene delivery.

6. ζ Potential

ζ potential describes the electrostatic interactions of cells and particles in a fluid environment. The liquid layer surrounding the particle exists as two parts; an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to gravity), ions

within the boundary move with it. Those ions beyond the boundary stay with the bulk dispersant. The potential at this boundary (surface of hydrodynamic shear) is the ζ potential.(190). A representation of this subject is shown in Figure 23.

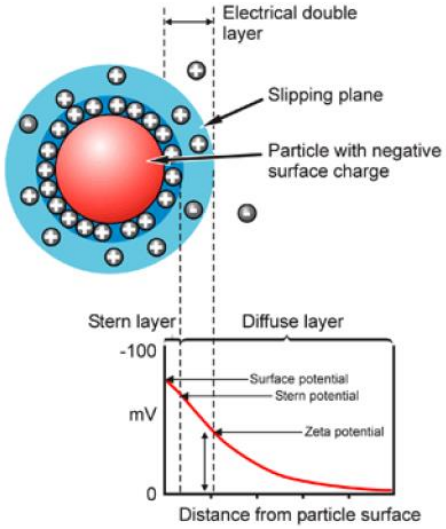
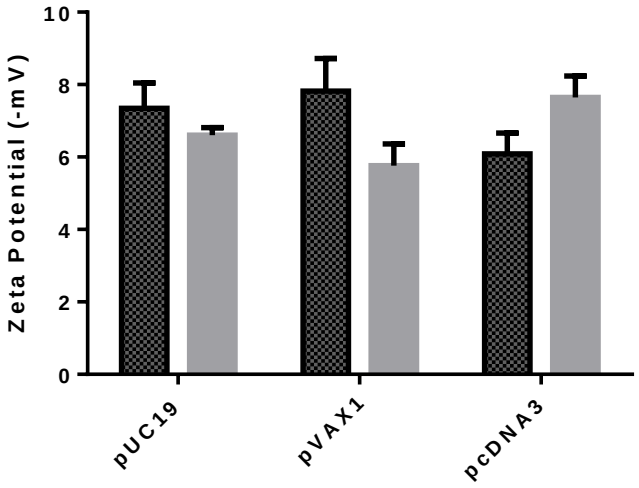


Figure 23. Understanding nanoparticles ζ potential. ζ potential is the potential that is situated between the stern layer and the slipping plane. (Adapted from (190))

An important notion to retain is that nanoparticles and the cells interact according to the magnitude of their ζ potential and not the surface charge of each. ζ potential values, higher than 30 mV, either positive or negative, can lead to monodispersity while values lower than 5mV can lead to agglomeration. Typically, pH can affect ζ potential values, for such it is important to understand that a ζ potential test should be accompanied with a pH measurement. In the case of this work, pH values were around 7.



	1µg System	*10µg System*
pUC19	7.34 ± 0.41	6.60 ± 0.12
pVAX1	7.82 ± 0.52	5.76 ± 0.35
pcDNA3	6.08 ± 0.33	7.65 ± 0.34

Figure 24. ζ Potential of $CaCO_3$-pDNA-Rho_{123} Nanoparticles. All values are represented as Mean ± SEM with N=3. Two-way Anova treatment reveal a plasmid $p>0.05$, system $p>0.05$ and an interaction of $p<0.05$.

The prepared nanoparticles display ζ potential values ranging from -8mV to the -6mV, as presented in Figure 24. This characteristic is particularly important when considering cell transfection mechanism and the ability of the produced nanoparticles to transpose the cellular barriers and reach the cellular organelle of interest. For therapeutic purposes, in general it is adequate to have a ζ potential of approximately -5 to -15 mV since most biological cells have ζ potentials in this range.

Statistically, the results were found non-significant between plasmids and system. This data suggests that neither pDNA nor initial loading amount does really influence the ζ potential charge.

7. Protection Test

To reach the therapeutic target, the vector will suffer from enzymes activity. Therefore, it was crucial to demonstrate that $CaCO_3$ nanoparticles actually protect the encapsulated pDNA against serum nucleases, since this is an important issue affecting both pDNA stability and transfection efficiency. The nanoparticles were incubated with DNase I (5 μg ml−1 and 10 μg ml−1) and agarose gel electrophoresis was employed to verify the protection effect of the nanoparticles to maintain plasmids stability (Figures 25-27).

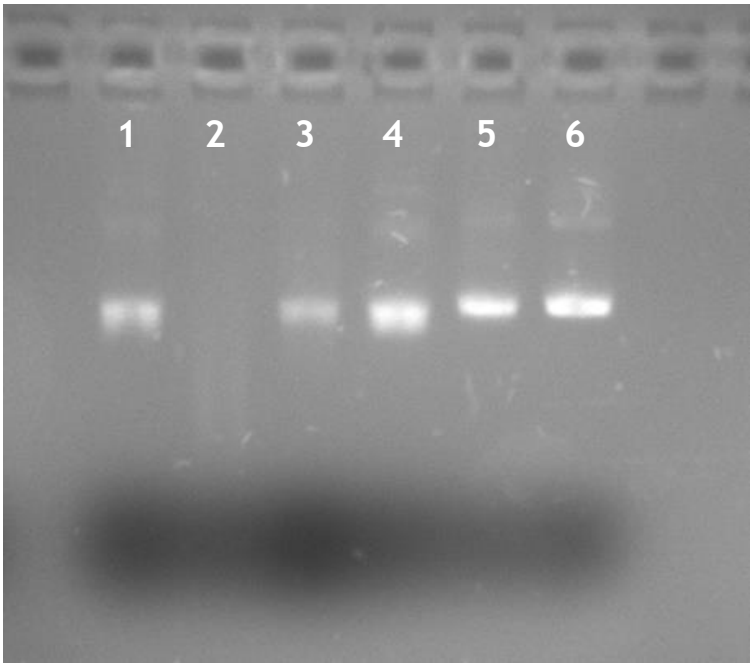


Figure 25. Agarose gel electrophoresis of pUC19 nanocarriers followed by incubation with DNAse I for 1h at 37ºC. Lane 1 and 4 – pDNA, Lane 2 and 5 – pDNA+ DNase I (10μg/mL) and Lane 3 and 6 – pDNA +DNase I (5μg/mL). Lane 1-3 1μg system and lane 4-6 10μg system.

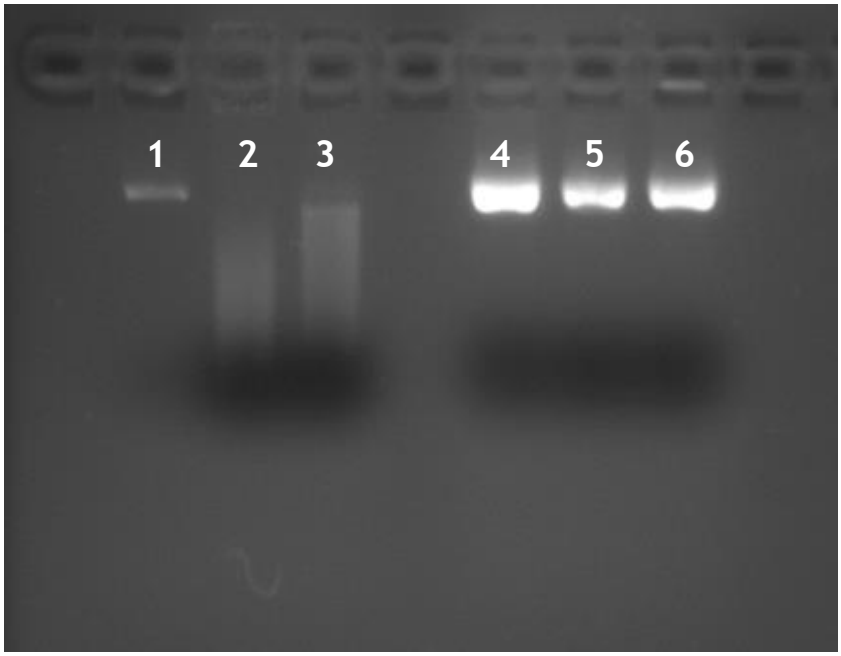


Figure 26. Agarose gel electrophoresis of pVAX1-*LacZ* nanocarriers followed by incubation with DNAse I for 1h at 37ºC. Lane 1 and 4 – pDNA, Lane 2 and 5 – pDNA+ DNase I (10µg/mL) and Lane 3 and 6 – pDNA +DNase I (µg/mL). Lane 1-3 1µg system and lane 4-6 10µg system.

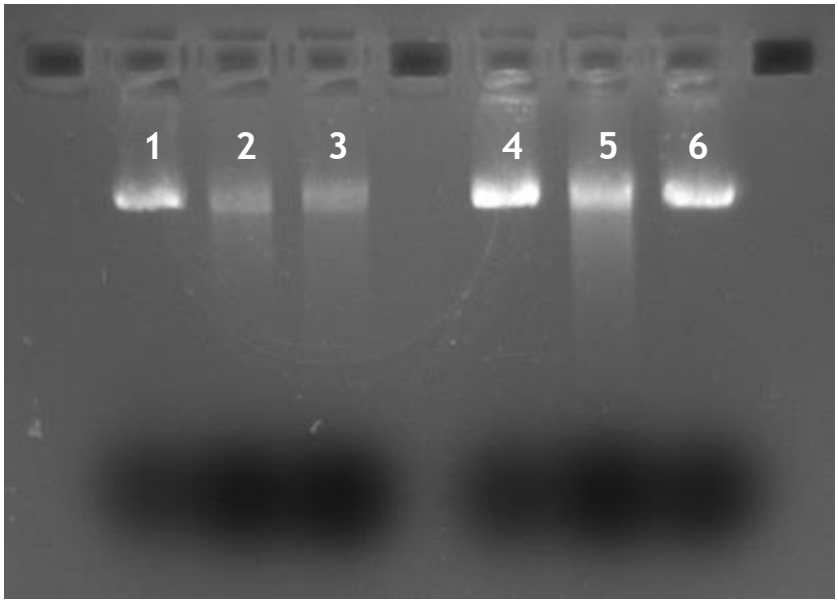


Figure 27. Agarose gel electrophoresis of pcDNA3-myc-*FLNa* S2152A nanocarriers followed by incubation with DNAse I for 1h at 37ºC. Lane 1 and 4 – pDNA, Lane 2 and 5 – pDNA+ DNase I (10µg/mL) and Lane 3 and 7 – pDNA +DNase I (5µg/mL). Lane 1-3 1µg system and lane 4-6 10µg system.

Observing the results it is obvious that 10µg system offer a better protection of the pDNA when compared to the 1µg system, for all the three considered plasmids. When prone to 5µg/mL of DNAse I, 10µg system did not suffer any significant difference compared to the

control. When the DNAse I concentration was raised to 10μg/mL we still do not observe any changes in pUC19 and pVAX1-*LacZ*. However, pcDNA3-myc-*FLNa* S2152A started to form a small haul corresponding to the small DNA fragments that were affected by DNAse I.

On the other side, 1μg systems were incubated with exactly the same concentration but the results are totally different. When incubated with 5μg/mL of DNAse I, hauls are quite common affecting more pVAX1-*LacZ* compared to the other two. When the concentration was the double, the pDNA was even more prone to DNAse I activity. Both pUC19 and pVAX1-*LacZ* were totally destroyed while pcDNA3-myc-*FLNa* S2152A did not suffer that much compared to the 5μg/mL DNAse I assay.

8. MTT Assay

MTT is a yellow, water-soluble, tetrazolium salt. The MTT assay is a simple non-radioactive colorimetric assay to measure cell cytotoxicity or viability. Metabolically active cells are able to convert this dye into a water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. The formed crystals can be dissolved and quantified by measuring the absorbance of the solution at 570 nm. To determine the pDNA nanoparticles cytotoxicity, the MTT assays were performed at 1 and 2 days after fibroblast cells having been seeded on top of the different pDNA nanoparticles. The results, described in Figure 28, suggest that both nanoparticles are non-toxic to cells since every formulation promoted dehydrogenase activity. These formulations do not have an acute cytotoxic effect, and thus, this system should not elicit an inflammatory response that can ultimately result in failure to achieve normal cell growth and function. Cell viability ranged between the 79% to the 86% between all plasmids and system. All plasmids had a small decay past 48 hours but this decay never exceeded the 2%. In general, the smaller the pDNA, larger cell viability. The same occurred in both systems, with the exception, of pVAX1-*LacZ* of 10μg systems, where cell viability surpassed pUC19.

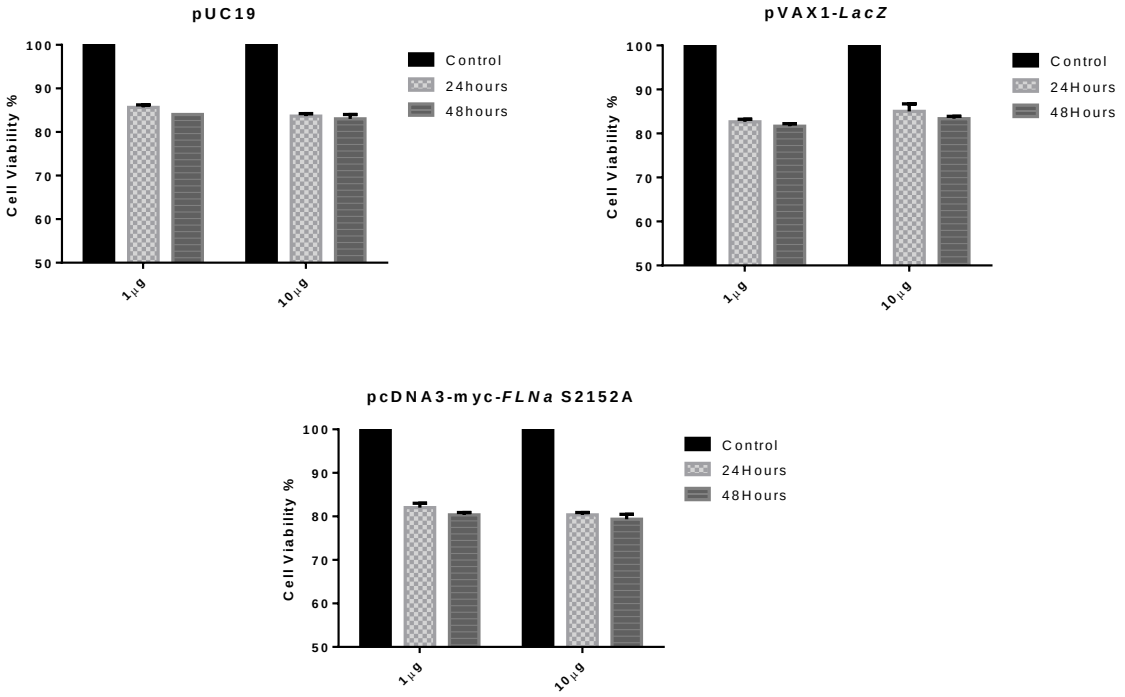


Figure 28. Cell Viability after incubation of Fibroblast cells with $CaCO_3$-pDNA-Rho_{123} nanoparticles for all three pDNA, for 24 and 48 hours. All values are represented as Mean ± SEM with N=3 followed by a Two-way Anova treatment, * $p<0.05$ when compared to control . Control was automatically defined as 100% Cell Viability.

9. *In vitro* study

Transfection is the process of deliberately introducing nucleic acids into the cell. Typically, a successful gene therapy procedure should be able to reach the nucleus and then perform its therapeutic purpose. In this work, the goal was slightly different as we intend to target the pDNA delivery to the mitochondria, instead of the nucleus. Following this approach, as first we were interested to know if cellular uptake and internalization mediated by $CaCO_3$-pDNA-Rho_{123} nanoparticles was possible and, secondly, to assess more closely the effectiveness of intracellular target. Fluorescence confocal microscopy has been applied, through a co-localization study with stained mitochondria and nucleus. Images can be visualized in Figure 29 for pVAX1-*LacZ* 10μg system.

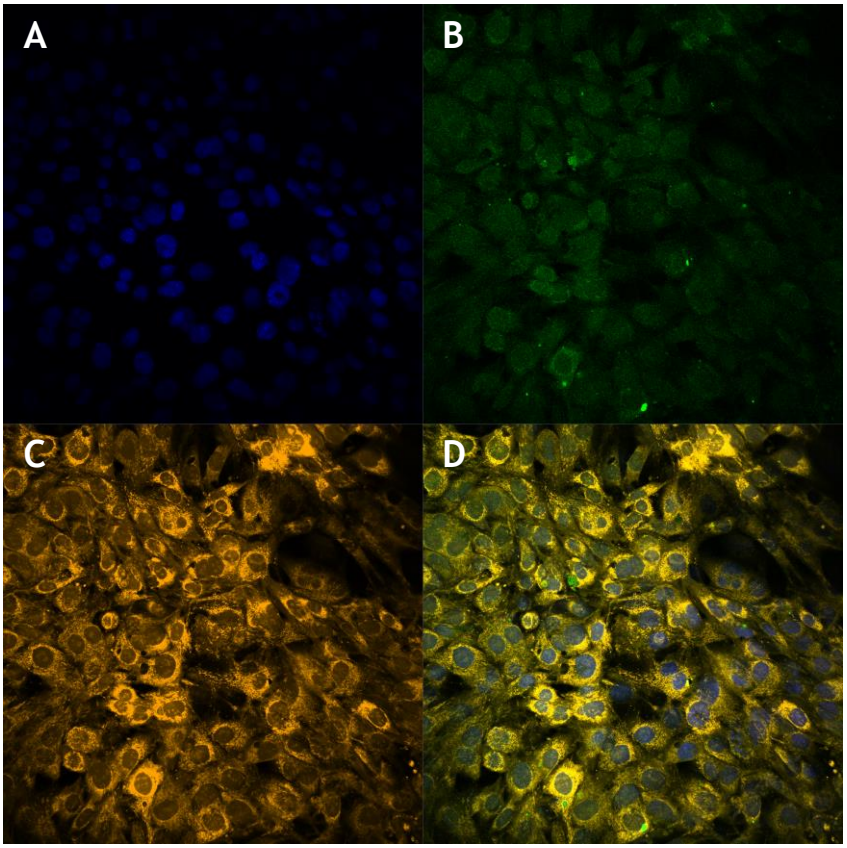


Figure 29. $CaCO_3$-pDNA-Rho_{123} naoparticles transfection ability and co-localization study. A) Nucleus stained blue by Hoescht 33342 B) Nanoparticles stained green due to the presence of rho123 in its composition. C) Mitochondria stained orange by Mitotracker Orange CMTMROS D) Merged image.

As observed, nanoparticles were able to enter the cell and cause a green stain, as illustrated in image B. At the same time, comparing image B and C they look like they correspond to mitochondria staining with different dyes, rhodamine 123 in (B) and Mitotracker Orange in (C). To finish, image D appears to have been stained yellow, and thus, supporting the hypothesis that the developed nanoparticles target mitochondria.

The next aim was to compare the plasmids transfection and target ability for both 1µg and 10µg systems (Figure 30).

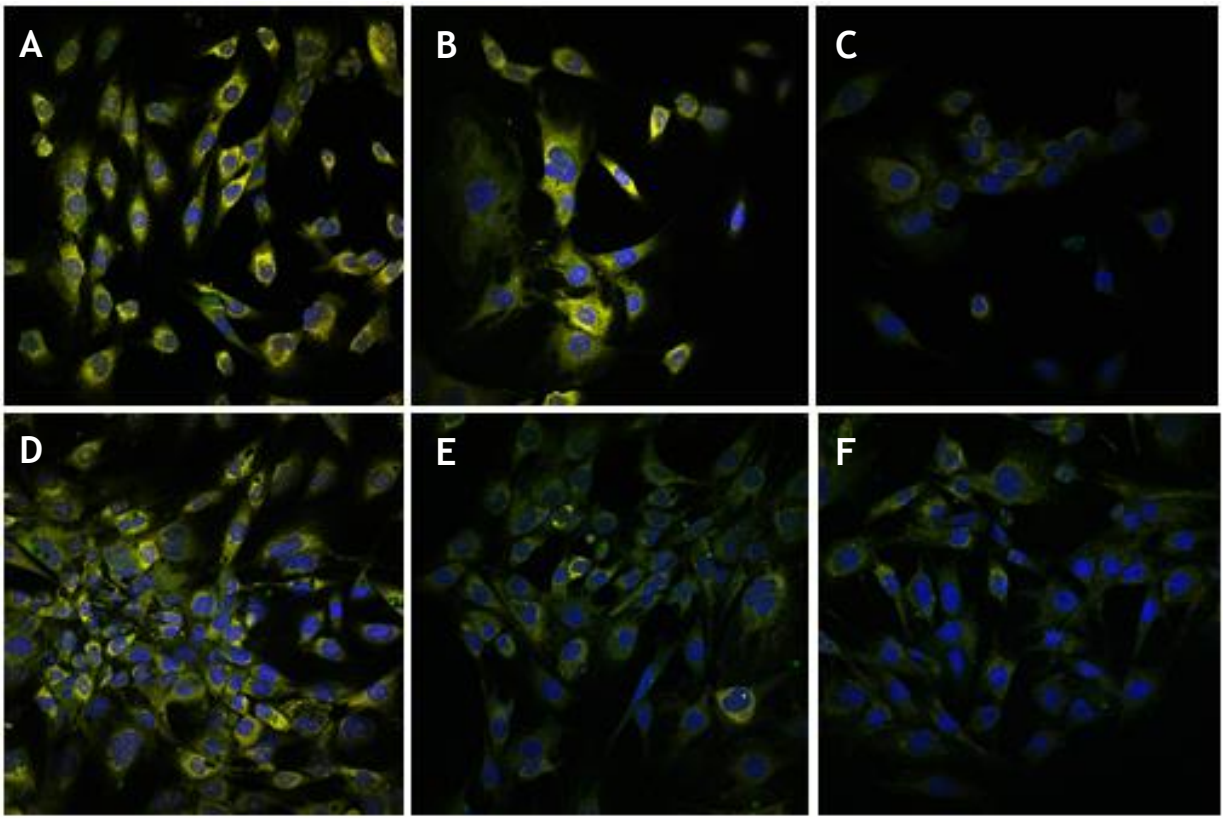


Figure 30. $CaCO_3$-pDNA-Rho_{123} nanoparticles transfection ability for the three plasmid systems. A) pUC19 1µg system; B) pVAX1-*LacZ* 1µg system; C) pcDNA3-myc-*FLNa* S2152A 1µg system; D) pUC19 10µg system; E) pVAX1- *LacZ* 10µg system; F) pcDNA3-myc-*FLNa* S2152A 10µg system. All images result from the cell staining treatment with Hoescht 33342 (blue) and Mitotracker Orange CMTMROS (Orange).

As observed, all pDNA vectors were able of cell transfection and, it seems that the delivery can be directed to the site of mitochondria.

In summary, we have strong evidences that the nanoparticles were cell internalized and can be guided to mitochondria. Although, this study is quite fascinating and innovative, it is, however, hard to make certain that pDNA was indeed delivered to this organelle.

Discussion

The search for the treatment of incurable diseases has been growing exponentially during the last decades. Disorders at mitochondrial level are responsible for a wide range of cytopathies and diseases generally related to neuromuscular pathologies. Although the knowledge behind mitochondria and mtDNA has grown, treatment options are limited and quite unsuccessful. To correct and solve such lacuna in the health care system, we tried to bring together a new nanocarrier system with affinity towards the mitochondria that can offer us unique and interesting features as a mitochondrial therapeutic method.

The Use of pDNA: As mitochondrial gene therapy is concerned, the usage of a plasmid is a must. Therefore, the initial step of this work consisted in the isolation of three pDNA which differ in size: the 2.7kbp plasmid pUC19, the 6.1kbp pVAX1-*LacZ* and the 14kbp pcDNA3-myc-*FLNa* S2152A. Plasmids have been isolated in a culture from bacteria, *E. coli*, and purified using an appropriate kit. Both isoforms, open circular and supercoiled are present. We are, however, aware that for transfection purposes, requirements such as purity and structural stability are relevant. Moreover, according to the guidelines provided by regulatory agencies pDNA should be enriched in the sc isoform (191). This conformation renders DNA to be more compact while ensuring enhanced transfection and expression rate in eukaryotic cells (192).

In line with this, future experiments must be focused in the purification of the sc pDNA. Colleagues have already succeeded on this issue by arginine affinity chromatography, where sc pDNA is recovered with high yield, structural stability and in a single purification step (163, 193).

These plasmids were essentially used as model vectors, allowing a variety of experiments and enriching our knowledge in areas concerning mitochondrial gene therapy. Although their use contributed for relevant advance, future research on this topic must devote attention to the design of reporter plasmids recoded for exclusive expression in mammalian mitochondria, since the codon usage of mitochondria differs from that of the nucleus.

Nanoparticles formation and characterization: To this point, only a few works refer to the encapsulation of pDNA in $CaCO_3$ nanoparticles and their use in the delivery of genetic material into mitochondrion has never been addressed (182, 194, 195). In this work, we initially test the encapsulation of different pDNA. Until now no work has ever contributed to such a vast knowledge of how $CaCO_3$ encapsulates the pDNA, this idea deserves even more attention when considering the unusual theme of mitochondrial transfection (169).

Plasmid DNA nanoparticles were prepared using a co-precipitation method. To target delivery to the site of mitochondria, rhodamine 123, a fluorescent dye, has been incorporated

in pDNA nanoparticles. Additionally, and in an attempt to enhance gene transfection and retard the particles size, a polysaccharide (cellulose) has also been incorporated in the nanosystem. Two different formulations, 1μg and 10μg of initial pDNA loading amount, have been considered. Both carriers are spherical, biocompatible, able of promote pDNA protection and stability and exhibit negative zeta potential values. In summary, $CaCO_3$-pDNA-rho_{123} nanoparticles offer us the following characteristics:

- *Morphology and size:* A round shaped form ranging between the 300nm and the 500nm
- *Encapsulation Efficiency:* Encapsulation that almost reaches the 70% in some cases. A value definitely high when compared to systems whose objective is the encapsulation of pDNA.
- *ζ Potential:* Values quite similar to the ζ Potential of the human cells therefore promoting a better interaction between the nanoparticles and the cell
- *pDNA protection:* $CaCO_3$ is able to protect the pDNA until the presence of 10μg/mL of DNAse I.
- *MTT Assay:* Cell viability was higher than 80% even after 48 hours in the presence of the nanoparticles.

Each system presents unique advantages. However, the encapsulation efficiency and the protection test turn the 10μg system a more viable option for future studies.

pDNA nanoparticles show a rod-like shape and have diameter sizes in the nano scale, that make them appropriate for cell uptake and internalization in practical applications concerning gene delivery. Particles exhibiting lower sizes possess the ability of fast diffusion and can more easily reach the target cell, where they can exert its therapeutic function. Complementary with the size, there is also a need to correlate this parameter with the surface potential of the nanosystem. This property is particularly important when considering transfection mechanism that involves the interaction with cell membrane. The negative zeta potential values displayed by our systems are a consequence of the presence of cellulose, which has a negative charge for pH values below 9. The negatively charged cellulose chains on the particle surface can contribute for improved colloidal stability.

Despite these parameters, the formulations can incorporate large pDNA amounts and are biocompatible ensuring its potential use in clinical applications. Moreover, structural stability after encapsulation is preserved and this topic deserves attention when considering intracellular trafficking; the enzymatic degradation in the serum can, along with all other particle properties described, compromise the entire transfection mechanism. Based on this, we found that, as a whole, 10 μg pDNA nanoparticles present better characteristics to be further used in the development of a sustained system for gene delivery.

In vitro study: Nanocarrier mediated delivery has been investigated by fluorescence confocal microscopy in a study where nucleus and mitochondrion have been stained with adequate fluorescent dyes. The obtained results, from a co-localization experiment, are consistent with significant accumulation of nanoparticles in the mitochondria. Nonetheless, some may argue that confocal images do not prove a successful transfection and that the observed green fluorescence is derived from free rhodamine that stains mitochondria. To prove that the staining was caused by the nanoparticles containing rho123, we present in Figure 31 a comparison between a cell staining treatment with rhodamine 123 (A) and a cell staining treatment with Hoescht, for visualization of the nucleus, when intracellular internalization has been mediated by $CaCO_3$-pDNA-Rho nanoparticles (B). Image A seems like the staining is caused by small green dots and it is not uniform. On the other side, image B has a uniform stain. This data undoubtedly supports our idea that these nano vehicles can target mitochondria.

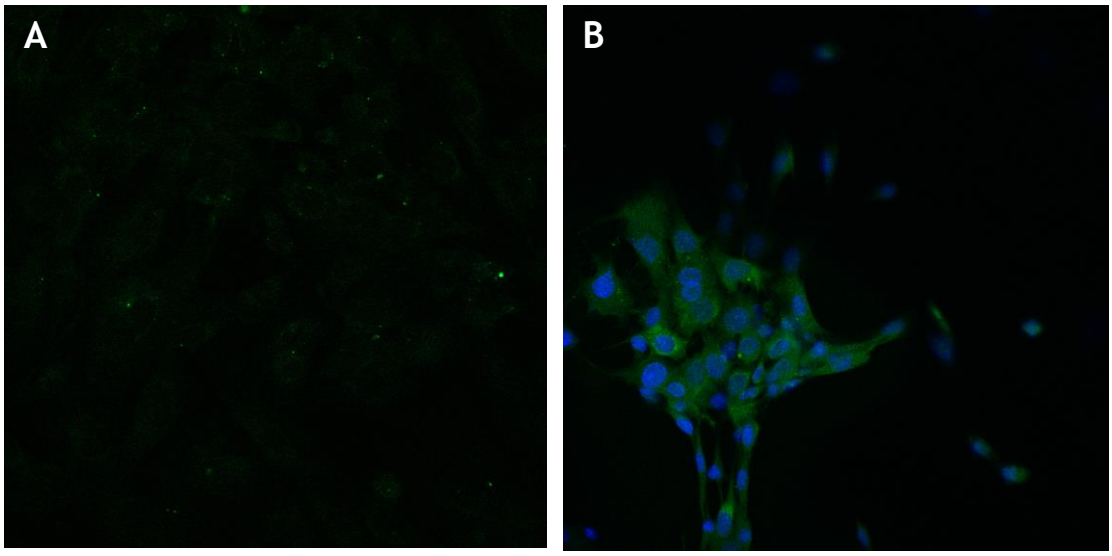


Figure 31. Comparing Rhodamine 123 Staining. A) Nanoparticles Rho123 green stain. B) Rho123 Stain

It is, however, not clear if pDNA can be successfully delivered to mitochondria. To estimate this issue, rhodamine fluorescence intensity displayed by cells can be quantified using spectroscopy. Once more, the creation of vectors that can encapsulate mitochondrial reporter genes will greatly help to clarify this point.

Conclusions and Future Perspectives

Mitochondrial gene therapy appears to be quite promising as a valuable approach to reestablish normal metabolic function. Scientific community seeks, however, a suitable mitochondrial vector able to carry a therapeutic gene into this organelle with the ultimate goal of functional protein expression. We developed novel mitochondrial-targeted plasmid DNA nanoparticles by incorporation of rhodamine 123, a fluorescent amphiphile with mitochondria affinity. These biocompatible nanocarriers have suitable sizes for gene therapy purposes, negative zeta potential values and, depending on pDNA loading amount, are able to protect the encapsulated pDNA from nucleases digestion. Furthermore, the pDNA vectors can be easily internalized into cultured fibroblasts and fluorescence confocal microscopy confirmed targeted delivery to mitochondria. Therefore, we succeeded in the creation of a new system to further be applied in, the poorly studied, area of mitochondrial gene therapy. The acquired knowledge on these pDNA based vectors is crucial as a model, for progresses in the design of systems that would be capable of mitochondrial genes encapsulation, cell transfection, targeted mitochondrial delivery by using mitochondriotropic agents, and, as a final goal, protein expression.

This work marks an important advance in the mitochondrial gene therapy field and we hope it can highlight the relevance of mitochondrion as a crucial intracellular organelle to reach.

Unfortunately, it is not today that we can prove that transformation of mammal mtDNA is possible. However, it is expected that in the near future mitochondrial gene therapy will establish a new hallmark in medicine. As a result patients suffering from mitochondrial diseases, such as Leigh's Syndrome or neuromuscular cytopathies, namely, Alzheimer's and Parkinson's may finally gain a new perspective of treatment improving their quality of life.

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