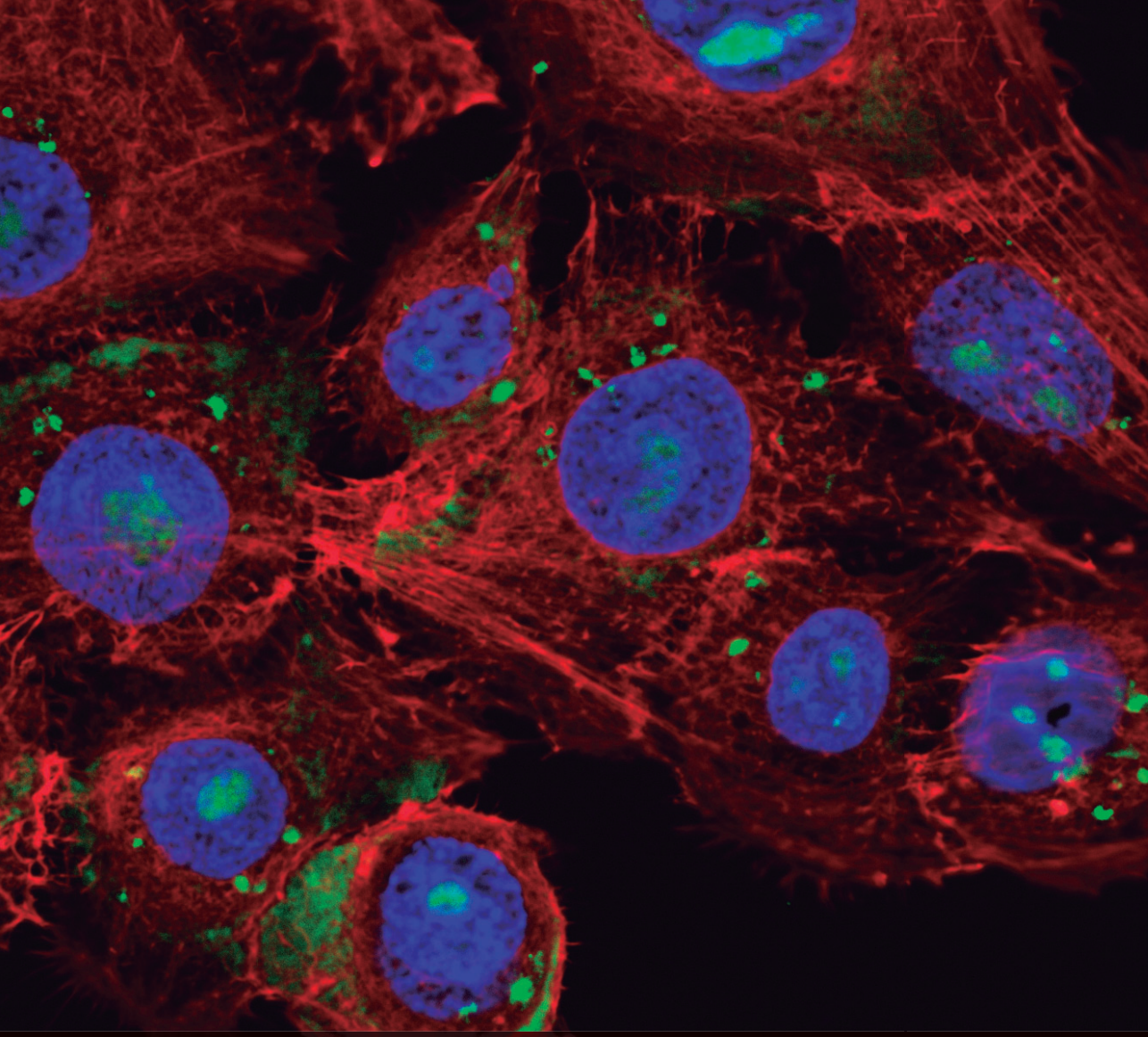

Development and characterization of non-viral vectors based on cationic niosomes to address cystic fibrosis disease by gene therapy approach

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Vitoria-Gasteiz, 2022

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Volverán las mañanas felices,
la época en la que dejemos de ahogarnos en conversaciones de ascensor
intentando sostener una imagen que nos protege de nosotros mismos
a la vez que ayuda a mantener el equilibrio
de esta obra de teatro a la que llamamos existencia.
Nadie avisó de los baches y las caídas.
Aun así, un pie delante del otro, desafiantes.
¿Qué importa lo que diga el mundo y cuán fiera sea la tormenta?
No dejes que te arrastre y te hunda bajo la arena,
porque si logras mirar más allá del tiempo y del espacio,
de los números, los colores y las formas,
hay un segundo en el que todo cobra sentido
y hasta el miedo desaparece.
Al fondo, entre las sombras, la luz ha dibujado una frase.
Beti libre.

La M.O.D.A.

Glossary

2D: two-dimensional

3D: three-dimensional

AAV: adeno-associated virus

ADU: arbitrary densitometric units

AdV: adenovirus

AFM: atomic force microscopy

AON: antisense oligonucleotide

AP: aqueous phase

AU: arbitrary units

bac-ORI: bacterial origin of replication

BBB: blood brain barrier

BEGM: bronchial epithelial growth medium

BRB: blood retinal barrier

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

CCF: cross-correlation function

CCK-8: Cell Counting Kit-8

CF: cystic fibrosis

CFTR: cystic fibrosis transmembrane conductance regulator

CME: clathrin-mediated endocytosis

CNS: central nervous system

COVID-19: coronavirus disease 2019

CpG: cytosine-phosphate-guanine

CPP: cell penetrating peptide

CRISPR: clustered regularly interspaced short palindromic repeats

CuFi-1: human cystic fibrosis airway epithelial cells

CvME: caveolae-mediated endocytosis

DAPI: 4′,6-diamidino-2-phenylindole

DCM: dichloromethane

DLS: dynamic light scattering

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DOPE: phosphatidylethanolamine

DOSPA: 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate

DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane

DOTMA: 1,2-di-O-octadecenyl-3-trimethylammonium propane

DPBS: Dulbecco's Phosphate Buffered Saline

DPI: dry powder inhalers

DSB: double-strand break

DSC: differential scanning calorimetry

dsDNA: double stranded DNA

dsRNA: double stranded RNA

DTPA: 2,3-di(tetradecyloxy)propan-1-amine(hydrochloride salt)

ECM: extracellular matrix

EGFP: enhanced green fluorescent protein

EMA: European Medicines Agency

F508del: deletion of a phenylalanine at position 508

FD: freeze-drying

FDA: Food and Drug Administration

FEV1: forced expiratory volume in first second

FITC: fluorescence isothiocyanate

FITC-pEGFP: fluorescence isothiocyanate labeled pCMS-EGFP

FSC: forward scatter

FSK: forskolin

GMP: good manufacturing practice

GOI: gene of interest

GP: general polarization

gRNA: guide RNA

GTMP: gene therapy medicinal product

HBSS: Hanks' Balanced Salt Solution

HDR: homology-directed repair

HEK-293: human embryonic kidney

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLB: hydrophilic-lipophilic balance

$\mathbf{I_{440}}$: intensity value at 440 nm

$\mathbf{I_{490}}$: intensity value at 490 nm

IBMX: 3-isobutyl-1-methylxanthine

iPS / iPSCs: induced pluripotent stem cells

ITC: isothermal titration calorimetry

Lac-PEI: lactosylated polyethylenimine

Laurdan: 6-Dodecanoyl-2-Dimethylaminonaphthalene

LDH: lactate dehydrogenase

LDV: Lasser Doppler Velocimetry

LNP: lipid nanoparticle

mcDNA: minicircle DNA

MC-LG: McCMV-fLuc-2A-EGFP

MFI: mean fluorescence intensity

MFSD8: major facilitator superfamily domain-containing 8

miRNA: micro RNA

mMDI: mechanical metered dose inhaler

mRNA: messenger RNA

MSCs: mesenchymal stem cells

NGS: next-generation sequencing

NHEJ: non-homologous end joining

NLS: nuclear localization signal

o/w: oil in water

OC: open circular DNA

ODN: oligodeoxynucleotides

OP: organic phase

ORI: origin of replication

PAM: protospacer adjacent motif

PBS: phosphate buffered saline

pc-LG: pcDNA3.1-fLuc-2A-EGFP

pDNA: plasmid DNA

PEG: polyethylene glycol

pEGFP: plasmid encoding enhanced green fluorescent protein

PEI: polyethylenimine

PLL: poly-L-lysine

pMDI: pressurized metered dose inhaler

PS: phosphatidylserine

PVDF: polyvinylidene fluoride

PVP: poly(N-vinyl-2-pyrrolidone)

RISC: RNA-induced silencing complex

RNA: ribonucleic acid

RNP: ribonucleoprotein

S/MAR: scaffold/matrix attachment region

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

SC: supercoiled DNA

SD: standard deviation

SDS: sodium dodecyl sulfate

SEM: scan electronic microscopy

SFD: spray freeze-drying

shRNA: short hairpin RNA

siRNA: small interference RNA

SPQ: 6-Methoxy-N-(3-Sulfopropyl) Quinolinium

SSC: side scatter

ssDNA: single stranded DNA

ssRNA: single stranded RNA

TALEN: transcription activator-like effector nuclease

TEM: transmission electron microscopy

Tm: thermal melting temperature

WWTPs: wastewater treatment plants

ZFN: zinc finger nuclease

Index

Chapter 1

INTRODUCTION

1. State of the art

The field of gene therapies involves the techniques that allow "to modify or manipulate the expression of a gene or to alter the biological properties of living cells for therapeutic use" [1]. The objective of gene therapy medicinal products (GTMPs) is to treat or cure diseases by the addition, deletion, replacement, repair or regulation of a genetic sequence within the cell nucleus. These products are made of two main components: a genetic construct and a vector or a delivery system [1,2]. In addition, they can be classified into three main groups according to the composition and the mechanism of action.

Gene therapy approaches can be targeted to somatic or germline cells. Somatic cell therapy involves the correction of a genetic mutation in the patient that will not be passed on to the next generations. On the contrary, when the target cells are germ cells (sperm or ovum), these changes will be transferred also to the offspring of the treated patient [3,4].

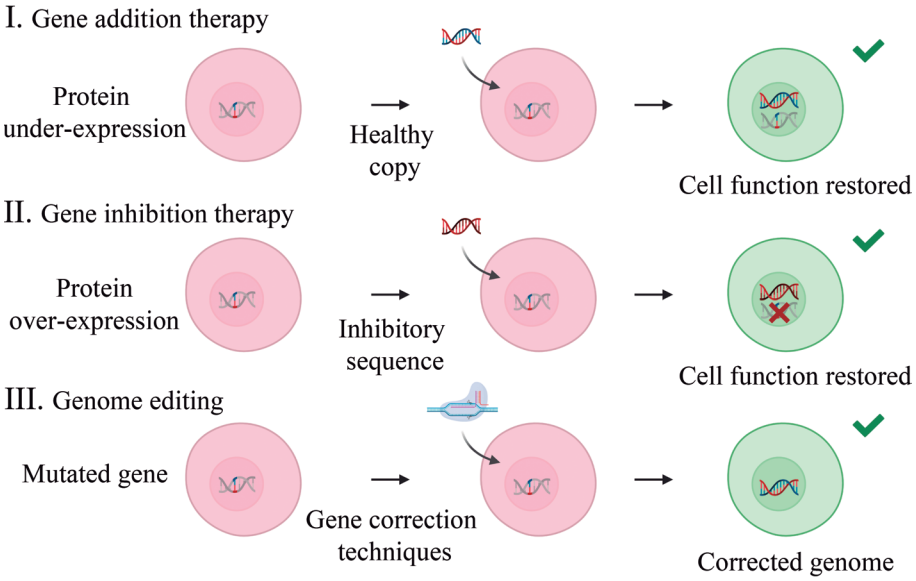


Figure 1. Schematic representation of the different gene therapies according to their mechanism of action.

Regarding the mechanism of action, we can identify three different strategies (Figure 1): gene addition therapy, gene inhibition therapy and genome

editing [5]. Gene addition therapies are usually a suitable option in the case of genetic disorders with protein under-expression, as they involve the incorporation of a healthy copy of the affected gene into the target cells. On the other hand, genetic disorders with protein over-expression can be treated with gene inhibition therapies, which consist of the incorporation of inhibitory sequences into the target cells [1,4]. Lastly, genome editing strategies are based on the incorporation of different molecules with the ability to modify ("edit") mutations in the genome [6].

In order to accomplish the aim of gene therapy, it is necessary to bind the genetic material to a vector or delivery system. These vectors can carry three main groups of genetic material: DNA, RNA and genome editing tools [7].

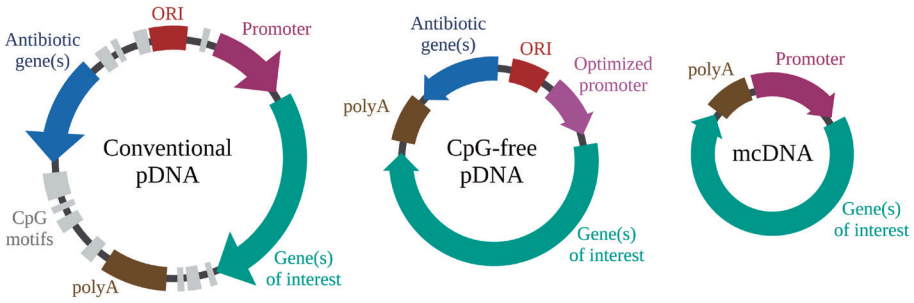


Figure 2. Different DNA constructs: conventional pDNAs, CpG-free pDNAs and mcDNAs. CpG: cytosine-phosphate-guanine. mcDNA: minicircle DNA. ORI: origin of replication.

Plasmid (pDNA) is a circular extrachromosomal double-stranded DNA molecule that has two main sections: a transcription unit, which consists of an expression cassette with the gene(s) of interest (GOI) and regulatory sequences required for the expression; and a bacterial backbone that contains an origin of replication (ORI) and the antibiotic resistance gene(s) used for amplification in bacteria -with subsequent extraction and purification processes [8,9]. As pDNA is not integrated into the cell genome, its expression will be gradually lost after successive cell divisions (except for slowly dividing cells) [8]. The bacteriological origin of the plasmids can give rise to biosafety concerns. Therefore, minicircle DNA (mcDNA) technology emerged as an attempt to reduce the possible immunogenic responses. mcDNA contains the

same expression cassettes as conventional plasmids but lacks both the backbone sequences and the unmethylated cytosine-phosphate-guanine (CpG) dinucleotides (unnecessary sequences), which makes the final product smaller [8,10,11]. Another approach used to decrease the immunogenicity and to increase the transfection efficiency of pDNA is the elaboration of CpG-free plasmids. These plasmids are free of unmethylated CpG motifs and have optimized promoters [12,13]. Figure 2 represents the different DNA constructs. In this regard, it is necessary to use the appropriate genetic material since the size and composition of the DNA released from the vectors or delivery systems influence both the transfection efficiency and the sustained transgene expression [10].

Other gene therapy approaches are based on the release of RNA. While DNA needs to reach the nucleus to fulfil its function, RNA should be released in the cytoplasm, so this strategy is not always considered as gene therapy but as an oligo-based gene therapeutic approach [14]. This approach uses therapeutic RNA that can mimic or antagonize the functions of the endogenous RNA. Nevertheless, the poor stability of the RNA molecule in presence of RNases often hampers its release in the target site [15]. RNA-based therapies include the administration of molecules such as messenger RNA (mRNA), small interference RNA (siRNA), CpG oligodeoxynucleotides (ODN), aptamers, microRNA (miRNA), antisense oligonucleotides (AONs) or short hairpin RNA (shRNA) [12,13,15,16].

In recent years, new genome-editing tools have been incorporated into the list of available gene therapies. These tools include meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPR) that are able to modify genes in their natural chromosomal location and cause permanent changes in the cell genome. The edition mechanism consists of a double-strand break (DSB) at a specific and desired location and its subsequent repair via two different mechanisms: homology-directed repair (HDR) if DNA template is present, or non-homologous end joining (NHEJ), which involves random extraction and introduction of nucleotides [6,17,18].

In order to achieve the desired action in each strategy, the genetic material has to reach the target cells. Administration of naked genetic material is the simplest and safest method. However, transfection efficiencies are usually very low, mainly due to degradation caused by enzymes present in the extracellular medium, and the cellular internalization capacity that is hampered by the electrostatic repulsive interactions between the negative charges present both in the genetic material and the cell membranes [4,19]. In order to address the disadvantages of naked genetic material administration, delivery systems emerged to improve protection, targeting and cell internalization. The development of efficient and safe vectors involves the design, characterization and evaluation of the systems, ensuring the capacity to carry, protect and deliver the genetic material, promote its internalization, and control the duration of gene expression [20,21]. Other factors such as dosage, route of administration, biosafety issues, storage stability or reproducibility must also be taken into account [19,22]. Vectors can be classified into two main groups according to their nature: viral and non-viral vectors (Figure 3).

Viral vectors are genetically modified viruses that possess natural ability to penetrate cells and integrate the released genetic material into the host genome, obtaining high transduction efficiencies [4]. However, they have a low genetic cargo packing capacity and an expensive and complex production process. In addition, they are often associated with biosafety issues due to their high immunogenicity and cytotoxicity, especially in multi-dose approaches [19,23,24]. Integrating viral vectors can insert their genes directly into the host genome which increases the probability of mutagenesis. In contrast, the genetic material of non-integrating viral vectors remains in an episomal form and results in transient expression [23,25]. The most commonly used viral vectors are retroviral, lentiviral, adeno-associated viral (AAV) and adenoviral (AdV) vectors [21-23,26].

Non-viral vectors present lower biosafety issues, immunogenicity and mutagenicity than their viral counterparts. Their elaboration process is cheaper, easier, scalable, and obtains better reproducibility. Moreover, a multi-dose administration regimen is possible and, their genetic cargo packing capacity is nearly unlimited in size. However, their low transfection efficiency remains the

main challenge to be overcome, but continuous advances in the field bring us closer to this goal [19,25,27]. The main non-viral transfection approaches include physical and chemical methods. On the one hand, physical transfection methods include naked genetic material, needle injection, gene gun (or ballistic injection), electroporation, sonoporation, photoporation magnetofection, hydroporation, nucleofection or microbubbles [20,25,28]. On the other hand, chemical transfection vectors are classified according to the chemical nature of their compounds, such as polymers, lipids, peptides, inorganic materials, dendrimers or hybrid systems [20,27].

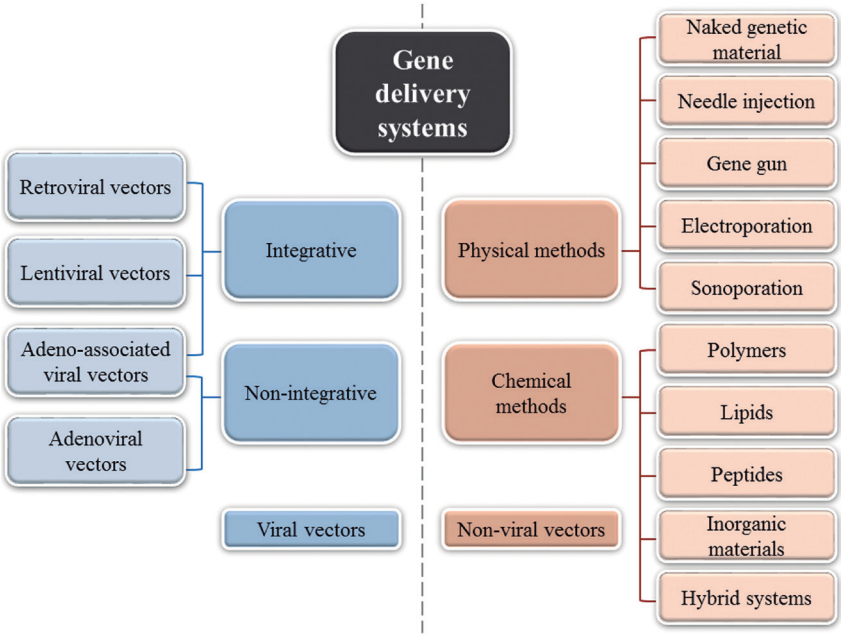


Figure 3. Overview of the main gene delivery systems: viral and non-viral vectors.

So far, the most widely used non-viral vectors are lipid vectors, more specifically cationic lipid-based vectors for gene delivery purposes, among which liposomes stand out. Liposomes are phospholipidic spherical vesicular systems formed by a lipophilic bilayer, whose main application is drug and gene delivery in nanomedicine [29,30]. In fact, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved the Onpattro® formulation for nucleic acid delivery purposes [31,32]. In addition, research in this field has recently led to the development and approval of

lipid nanoparticle-formulated mRNA vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for human use [33]. Remarkably, niosomes emerged as an alternative to liposomes that have gained attention because of their higher chemical and storage stability, lower toxicity, with cheaper and easier elaboration process [34-36]. Niosomes are non-ionic surfactant vesicles with a bilayer structure that, in the case of gene delivery, are made of three components (Figure 4): non-ionic surfactants, cationic lipids and, optionally, "helper" components [34,35,37].

The main component of niosomes is the non-ionic surfactant. These surfactants are essential for stabilizing emulsions, preventing particle aggregations and properly integrating the genetic material. In addition, they can be considered non-toxic, because they are free of ionic charges [38,39]. We can differentiate four groups of non-ionic surfactants according to their chemical structure: alkyl ethers, alkyl esters, alkyl amides and esters of fatty acids [40]. Among them, polysorbates (Tween®) [38,41], sorbitan fatty acid esters (Span®) [42], polyoxyethylene alkyl ethers (Brij®) [43] and poloxamers [44] are the most commonly used for gene therapy purposes. Importantly, the chemical structure of these molecules influences the final properties of the product [45,46] being, hydrophilic-lipophilic balance (HLB) [38], the critical packing parameter, which affects the vesicular-forming ability of niosomes [47], or the gel liquid transition temperature, which impacts on the drug entrapped efficiency [48], the most relevant parameters that should be considered to elaborate niosomes.

Meanwhile, cationic lipids provide the niosome with positive charges that interact electrostatically with the negative charges of the genetic material making it possible to form complexes and promote the fusion with the negatively charged cell membranes. The structure of cationic lipids has three domains: a cationic head, a hydrophobic tail and a linker between these two. Any chemical modification made to these individual domains influences the physicochemical characteristics of the final lipids [49-51]. Some cationic lipids are commercially available, such as 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) [38,52], 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [53,54] or 2,3-dioleyloxy-N-[2(sperminecarboxamido)

ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) [50,55]. But the most interesting aspect of these lipids is that they can be tailor-synthesized to increase the delivery and transfection efficiencies, optimize targeting and improve biocompatibility [51,56].

Another component that can be part of the niosome formulation is the "helper" component. This molecule provides fluidity and stability to the lipid bilayer and supports transfection processes, including cell penetration and intracellular trafficking [27,57]. Typically, these compounds have been neutral lipids, for example, dioleoylphosphatidylethanolamine (DOPE) [53], cholesterol, squalene, squalane [58] and lycopene [59]. However, recently researchers in the field have included other compounds with specific properties to overcome some disadvantages of niosome formulations.

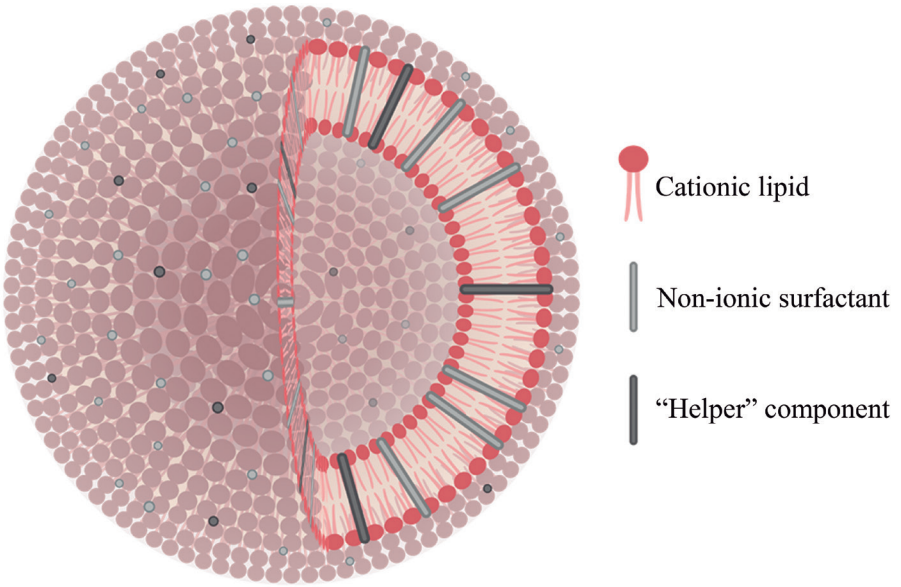


Figure 4. General scheme of the structure of niosomes.

The chemical composition has an impact on the biophysical properties of the niosomes, so does the elaboration method, which influences transfection efficiency and cytotoxicity, among others [39,60,61]. The main techniques for niosome elaboration include thin layer-evaporation, oil in water (o/w) emulsion technique, melted amphiphile injection, solid amphiphiles/hot water,

micellar solution/enzymes, ether injection, transmembrane pH gradient method, reversed-phase evaporation, bubbling or microfluidization [17,18,62]. Once the positively charged niosomes are formed, they need to be complexed with the negatively charged genetic material to elaborate nioplexes through electrostatic interactions. In this step, the mass ratio between the cationic lipid of the niosome and the genetic material plays a pivotal role in the biophysical properties of the complexes and affects their transfection efficiency and cytotoxicity [27].

Among all the possible combinations of molecules to elaborate niosomes for genetic material delivery to cells, selecting the most effective and safe one in each case also depends on the application, which involves the target cells, the administration route and the respective biological barriers that the particle will encounter. Conveniently, niosomes can be optimized and customized to address each application.

Considering the biological barriers that can prevent the niosome from reaching its target, we can classify them into extracellular and intracellular (Figure 5). Regardless of the method of administration, it is unavoidable that niosomes come into contact with the extracellular environment. Here, the major disadvantages to overcome are rapid clearance, non-specific interactions with cells and proteins, and degradation by digestive enzymes. In order to avoid these problems, genetic materials need to be complexed on niosome surface to form nioplexes or to introduce cover techniques [20,27]. In addition, to reach target cells, formulations need to be modified to cross specific extracellular barriers, such as the blood-brain barrier (BBB) or the blood-retinal barrier (BRB).

Once niosomes are close to the cellular target, the next barrier is the cellular uptake and the internalization processes. In this sense, niosomes can have positive charges that interact non-specifically with the negative charges of cell membranes, or they can be coated with appropriate ligands to establish specific interactions [20,63]. The main cell internalization strategies are fusion with cell membrane and endocytosis. The predominance of each strategy depends on both the type of chemical composition of the niosome and the target

cell [63,64]. Cells have four principal endocytosis pathways: clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), macropinocytosis and phagocytosis [50,63,64]. After this step, endosomal escape is fundamental, before the fusion of endosomes with lysosomes, to avoid degradation of the niosomes. This is possible with the introduction of compounds that promote the endosomal escape via membrane fusion, pore-forming peptides or proton sponge effect [64]. Good examples are cell-penetrating peptides (CPPs) [65,66] and the chloroquine molecule [67,68], but CPPs stability is worse and their formulation and production are more complex and expensive than chloroquine. Once in the cytoplasm, the niosome unpacking (pDNA release) and intracellular distribution are necessary to reach the cell nucleus, which can only be accessed through the nuclear pore complex or during the mitotic phase, when the membrane is temporarily disassembled [57,63].

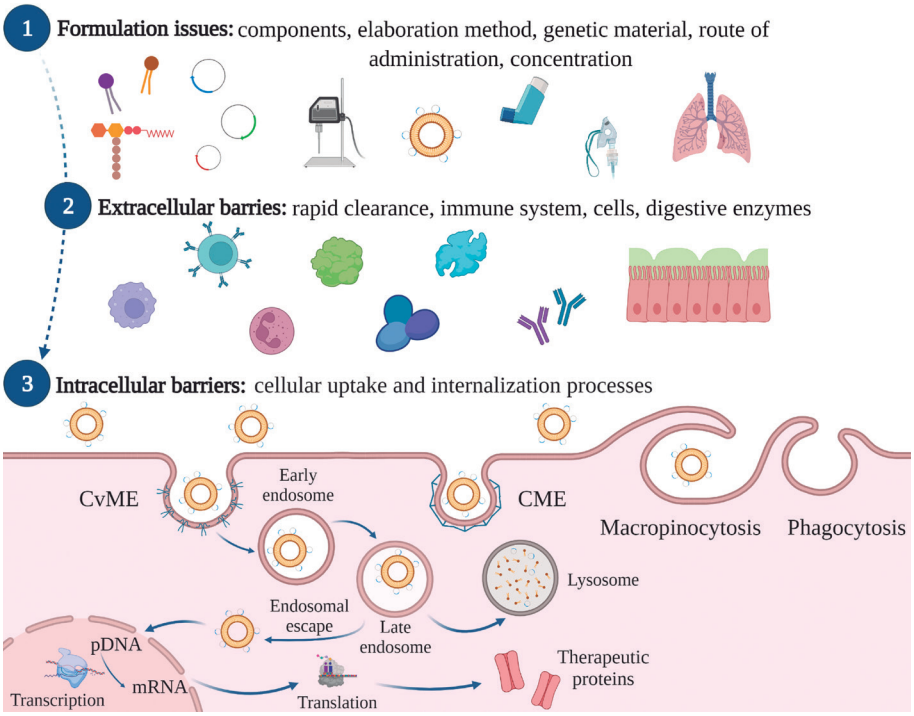


Figure 5. Schematic representation of the main general hurdles for niosomes to reach the cell nucleus: from formulation issues to biological barriers, both extracellular and intracellular. CME: clathrin-mediated endocytosis. CvME: caveolae-mediated endocytosis. pDNA: plasmid DNA. mRNA: messenger RNA.

Regarding the administration route, intravenous injection is the most commonly used. However, alternative routes may be of interest depending on the characteristics of the target pathology. For example, local administration produces localized action, minimizes adverse effects and often employs non-invasive methods [14].

Gene therapy can be an excellent treatment option for many disorders, including neurodegenerative, ocular, neuromuscular or cancer [6,69,70], among others. In this regard, inherited and monogenic diseases, such as cystic fibrosis (CF), are particularly attractive for this treatment. CF is an autosomal monogenic recessive disease caused by different mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The CFTR gene encodes the CFTR protein, a transmembrane channel located in the apical membrane of secretory epithelial cells, that regulates the flow of chloride and bicarbonate, principally.

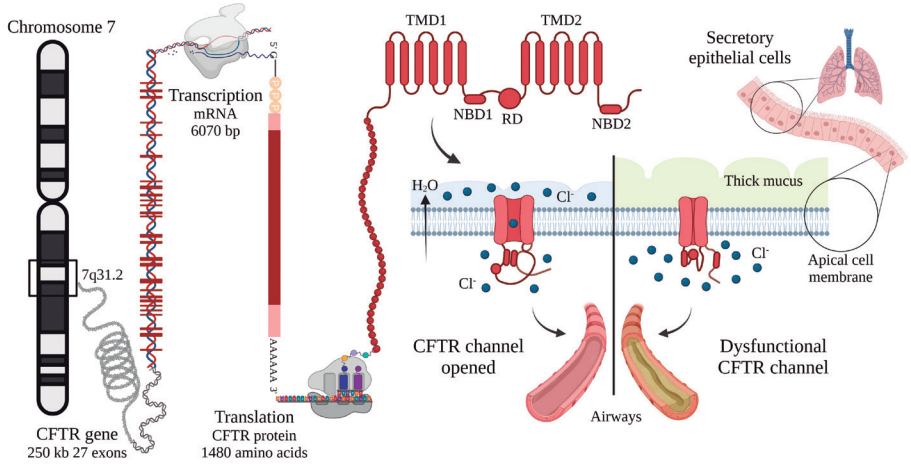


Figure 6. Genetic background of cystic fibrosis (CF) disease. Chromosome 7 of the human genome contains the cystic fibrosis transmembrane conductance regulator (CFTR) gene on its long arm. The messenger RNA (mRNA) obtained from DNA transcription is translated into the CFTR protein. The CFTR protein is a transmembrane protein with two transmembrane domains (TMD1 and TMD2), two nucleotide-binding domains (NBD1 and NBD2) and one regulatory domain (RD). The CFTR channel is located in the apical membrane of secretory epithelial cells, and its dysfunctionality leads to increased and thickened mucus in the airways.

In CF patients, CFTR dysfunction results in impaired epithelial mucus secretion in numerous tissues, such as airways, pancreas, liver, digestive and reproductive tract (Figure 6) [71,72]. The most common symptoms stem from these tissues and include salty-taste skin, cough, frequent lung infections, wheezing or shortness of breath, infertility or digestive problems [72,73]. CF is considered a "rare disease" due to its low prevalence being the number of patients related to ethnicity, with the highest prevalence in Caucasian people (one in 2,000-3,000 newborns) [71,74]. The CFTR gene contains more than 1,900 mutations divided into mainly six groups, according to the type of CFTR protein synthesis disorder they cause. The most frequent mutation affects approximately 60-65 % of CF patients and involves the deletion of a phenylalanine at position 508 (F508del), resulting in a protein folding problem [72,75].

The choice of the most suitable treatment for CF strongly depends on the type of CFTR mutation that causes the disease in each case. Over the years, the scientific community has developed and incorporated different drugs to mitigate the effects of this pathology. These strategies range from symptom-reduction drugs, such as pancreatic enzymes, medicines for airway clearance and antibiotics, to drugs with the ability to modulate the CFTR protein expression, which include potentiators and correctors [71,76,77]. Potentiator molecules increase the functionality of the CFTR channel, with a mechanism based on the opening of the chloride channels and keeping them open, to increase the transport of chloride ions [71,73]. Corrector molecules facilitate the adequate folding of the CFTR protein, its traffic to the cell membrane and its permanence there [71,73]. The first successful molecule developed to correct the inadequate CFTR protein folding was the potentiator ivacaftor (Kalydeco®). However, only about 5 % of CF patients are candidates for this treatment [78]. In order to be able to treat a larger number of patients, many new drugs have emerged, as well as combinations of potentiators and correctors, which improve the chloride efflux and reduce CF symptoms [73,77]. In this regard, regulatory agencies approved Orkambi® for CF patients with two copies of the F508del mutation. Its composition is a mixture of the molecules lumacaftor (corrector drug) and ivacaftor [77]. A few years later, another

combination of tezacaftor (corrector drug) and ivacaftor, named Symdeko® also reached the clinic, with fewer adverse effects and a broader target population [71,73]. Besides, the latest treatment (Kaftrio®) was approved last year and consists of a first triple combination of CFTR modulators, with improved results and broader applicability. This therapy includes ivacaftor, tezacaftor and elexacaftor (a next-generation corrector that also improves an additional flaw in the F508del CFTR protein) [73,79]. Currently, clinical trials are undergoing for several new treatments that could reach the market and the clinic in the upcoming years, notably improving the life of the patients [73,80]. However, despite the clear benefits that these personalized therapies entail, there are still a few disadvantages that should be taken into account, such as their high cost, which also limits their access in lower-income countries, and their lifelong treatment condition [78].

All the above-mentioned strategies are able to improve the symptomatology of the patients affected by CF, but nowadays, there is still no cure for this disease. As a monogenic recessive condition, in which the correction of only one of the CFTR alleles is sufficient to reverse the disease, CF is an excellent candidate for gene therapy approaches. Regardless of the exact mutation affecting the CFTR in each case, a single gene therapy strategy could be applied in most of these patients [74].

The most important breakthrough for CF was in 1989 when the CFTR gene was identified and cloned [81]. Since then, more than fifty CF clinical trials using gene therapy approaches have been initiated, of which thirty-five were already completed [80,82]. The first clinical trial used AdV vectors, followed by nine others using the same approach, with the conclusion that these vectors are not suitable for the CF treatment due to their low gene transfer and the induction of immune responses [75,83]. Since 1998, researchers showed a preference for AAV vectors in clinical trials and evaluated different routes of administration with safer results but limited efficacy [75,84]. On the other hand, the efficacy and safety of non-viral vectors for the treatment of CF have also been evaluated in nine clinical trials [75]. The UK CF Gene Therapy Consortium conducted the most prominent clinical trial with non-viral vectors in 2013. They combined a cationic liposome called GL67A and a CpG-free

plasmid DNA named pGM169, which encodes the CFTR protein, in CF patients using nebulized administration into the lungs. Nevertheless, the achieved efficacy was not considered enough [85,86].

In order to make further progress in the field of gene therapy for the cure of CF, it could be interesting to develop non-viral vectors, especially niosomes. These niosomes have some key aspects that we should take into account (Figure 7). The first step, and in accordance with the main objective of the research, includes the selection of the chemical compounds and the preparation method of the niosome formulations. Secondly, an exhaustive physicochemical and biophysical characterization is necessary, followed by their *in vitro* evaluation [87,88]. These *in vitro* studies, which are performed on cell lines chosen in relation to the characteristics of the disease and the drugs mechanism of action, describe the ability of the niosomes to complex, protect and release the genetic material, causing the expected therapeutic effect. The last step before taking the technology to clinical trials is *in vivo* evaluation, which requires approval by the ethics committees. *In vivo* assays use animal models chosen according to the principle of the 3Rs (Replacement, Reduction and Refinement of animals in research) [89].

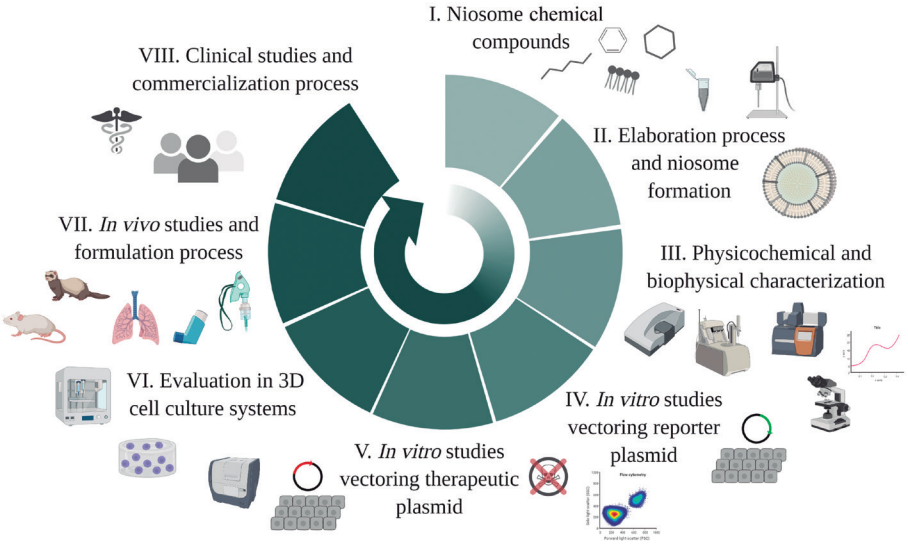


Figure 7. Schematic representation of the different steps needed to develop niosome formulations to address human disease, as applied to this work. 3D: three-dimensional.

Currently, there is no good correlation between the results obtained in *in vitro* and *in vivo* studies and, to overcome this issue, new three dimensional (3D) *in vitro* models that better mimic the *in vivo* environment are emerging. In this regard, the most commonly used 3D cell cultures are scaffolds (matrices or hydrogels), 3D microfluidic cell cultures, bioreactors, organ-on-a-chip models, spheroids or organoids. These structures allow cell-to-cell and cell-to-extracellular matrix (ECM) interactions to create a more realistic environment [90-92]. Many techniques can be employed to produce 3D cultures, but recently 3D bioprinting technology is gaining prominence to elaborate functional tissue constructs for multiple applications. This method allows to control the architecture of the constructs, obtain high reproducibility, automate the processes, and monitor every stage [93-95]. The method requires the development and bioprinting of a bioink, which consists of a mixture of biomaterials and cells. There are many different approaches to this technique, such as laser-assisted, inkjet (or droplet) or extrusion-based bioprinting. Its great versatility allows the production of customized bioprinting and even simultaneous and subsequent printing of multiple materials. The composition of the bioink depends on the final application and the 3D bioprinting technique used in each case [94,96,97].

To sum up, gene therapy has emerged as a realistic tool to treat genetic disorders. The rise of nanomedicine opens the opportunity to develop and optimize non-viral vectors, such as niosomes, to address CF disease. To this end, the inclusion of novel compounds in formulations and the choice of the most appropriate niosome elaboration method, as well as an extensive biophysical characterization and *in vitro* evaluation in a more realistic environment, may open new perspectives and opportunities to improve the treatment of this disease by gene therapy approach.

2. Methodology

2.1. Preparation of niosomes and nioplexes

There are many well-described techniques for niosome elaboration. Among them, one of the most widely used methods is the oil in water (o/w) emulsion technique, and it is the one we have used to prepare the formulations in this doctoral thesis. This technique involves the mixing of two phases, the aqueous (AP) and the organic phase (OP) [48,62]. Normally, the active compounds remain dissolved in one of the two phases, depending on their solubility profile: hydrophilic compounds in the aqueous phase and lipophilic compounds in the organic phase. In addition, the HLB of non-ionic surfactants and the elaboration method are also determinant factors. After mixing, both phases are emulsified by sonication, and the organic solvent is evaporated under magnetic stirring. The final product is the niosomes suspended in the aqueous medium.

The chemical reagents utilized in this doctoral thesis are the following. The selected cationic lipids were DOTMA -a commercially available lipid- and 2,3-di(tetradecyloxy)propan-1-amine(hydrochloride salt) (DTPA) -a tailor-synthesized lipid [98]-, which were always dissolved in the organic phase. Non-ionic surfactants can be combined and more than one can be included in the same formulation. The surfactants chosen for these formulations were poloxamer 188, poloxamer 407, polysorbate 20 and polysorbate 80. Chloroquine and squalene were chosen as "helper" components but, in some formulations, were not used.

In the first experimental work, we evaluate the effects of the niosome composition on the biophysical characteristics of the formulations, more concretely, the role of the non-ionic surfactants and the chloroquine as the "helper" component. In order to evaluate the effects of the chloroquine molecule, two niosome formulations were prepared under the same conditions, but only formulation **1** (F**1**) had chloroquine as the "helper" component, formulation **3** (F**3**) did not. To determine the effects of the addition in different phases of non-ionic surfactants on the elaboration method and the substitution of

poloxamer 407 by poloxamer 188, two different formulations, named formulation **1** and formulation **2** (F**2**), respectively, were also evaluated. In the second experimental work, we evaluate different formulations to find the most efficient one to transport the CFTR gene. To this end, two more formulations were elaborated, which contained DOTMA as cationic lipid and included polysorbate 20 and 80 as non-ionic surfactants, to form formulation **4** (F**4**) and formulation **5** (F**5**), respectively. Table 1 describes all formulations and their composition.

Table 1. Description of the components and their addition phase in the elaboration method of the different niosome formulations used along these experimental works. OP: organic phase; AP: aqueous phase.

	Components							
	Cationic lipids		Non-ionic surfactants				"Helper" component	
			Poloxamer		Polysorbate			
Niosomes	DTPA	DOTMA	188	407	20	80	Chloroquine	Squalene
F**1**	OP			OP		OP	AP	
F**2**	OP		AP			AP	AP	
F**3**	OP			OP		OP		
F**4**		OP			AP			OP
F**5**		OP				AP		OP

In order to perform the transfection assays, it is necessary to mix the niosomes with the genetic material and incubate them for 30 min at room temperature to form the nioplexes by electrostatic interactions. Different volumes of the niosome formulations and DNA stock solution yield different mass ratios of cationic lipid/DNA. The ratios used in these experimental works were 2/1, 5/1 and 10/1.

2.2 Genetic material

As mentioned before, many types of genetic material can be used for gene therapy purposes. Throughout the experimental works of this doctoral thesis, we employed four different DNA constructs. The reporter plasmid used

encodes for the enhanced green fluorescent protein pCMS-EGFP called pEGFP (5.5 kb). For tracking studies, the previous plasmid was labeled with fluorescence isothiocyanate (FITC), creating the FITC-pCMS-EGFP (FITC-pEGFP). In addition, two of the plasmids encode for the therapeutic protein CFTR. The first also encodes the reporter EGFP, called pEGFP-CFTR (9.1 kb, kindly provided by Unit of Viral Infections and Comparative Pathology at University of Lyon 1). The therapeutic CFTR plasmid used is a CpG-free plasmid with fewer bases than conventional therapeutic CFTR plasmids, called pGM169 (6.5 kb, CpG-free CFTR plasmid kindly provided by UK Cystic Fibrosis Consortium).

Plasmid amplification can be used to obtain large amounts of DNA. Both pEGFP and pEGFP-CFTR were propagated in *Escherichia coli* DH5α and One Shot™ Stbl3™ chemically competent *Escherichia coli*, respectively. Maxi-prep kit was then used for plasmid purification, while the resulting pDNA was quantified at an absorbance of 260 nm.

2.3 Biophysical characterization of niosomes and nioplexes

2.3.1. Nano DSC studies

Differential scanning calorimetry (DSC) is designed to characterize the molecular stability of biomolecules in diluted solutions, by a linear time-dependent temperature scan. The difference in heat efflux between the sample and the reference is the amount of heat absorbed or removed during the molecular transitions produced. Therefore, Nano DSC equipment was used to determine the niosome thermostability. To perform the analysis, niosome formulations were diluted to a concentration of 0.5 mg/mL and degassed before their introduction into the sample chamber. MilliQ® water was used as reference buffer scan in both reference and sample chambers. Samples were scanned from 4ºC to 100ºC with a scan rate of 1ºC/1 min.

2.3.2. Morphology, size, dispersity and superficial charge

Morphology, size, dispersity (Đ) and superficial charge are some of the critical parameters that influence the performance of the niosomes in their different applications. These factors compromise the interactions between

the niosomes with both the cells and the environment, as well as the gene delivery process, which determines the success of the transfection [32,99]. Niosome morphology can be explored by transmission electron microscopy (TEM), scanning electron microscopy (SEM) or atomic force microscopy (AFM). For better results, we used negatively stained niosome samples in TEM. Size, dispersity and superficial charge were obtained in a Zetasizer Nano ZS, which uses Dynamic Light Scattering (DLS) to measure particle size and dispersity, and Lasser Doppler Velocimetry (LDC) for zeta potential.

2.3.3. ITC studies

Isothermal titration calorimetry (ITC) assays allow quantitative determination of the binding enthalpy of a molecule or molecular complex by the released or absorbed heat measurement at constant pressure. In this regard, we used a MicroCal PEAQ-ITC microcalorimeter to observe the interactions between the niosomes and the genetic material (pEGFP) during nioplexe formation. Measurements were performed injecting repeated shots of niosome formulations at 1 mg/mL into the reaction cell, which was previously loaded with a solution of genetic material at 0.0166 mg/mL. To measure the heat produced only by the dilution of the niosome formulations, the same measurements were carried out but loading the cell with MilliQ® water. Subsequently, the result was subtracted from the total heat in the data analysis.

2.3.4. Membrane packing studies

The proper order or packing of lipids is crucial for membrane functionality. Lipid packing can be quantitatively studied with sensitive fluorescent lipophilic probes, such as laurdan (6-Dodecanoyl-2-Dimethylaminonaphthalene) [100]. This compound displays different excitation and emission spectra depending on the ordering of the lipid environment, namely gel or liquid-crystalline phases. The emission between the different order states of the membrane is used to calculate the general polarization (GP), a relative quantitative measure of lipid packing. The GP value is in the range between +1 (most ordered) and −1 (least ordered) and is obtained from the following equation, where I_{440} and I_{490} are the intensity values obtained at 440 and 490 nm, respectively: $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$ [100,101]. Laurdan molecule was used to measure the

lipid packing of niosomes and their corresponding nioplexes vectoring pEGFP. In more detail, the assay was carried out at room temperature, in a 96-well black plate, that contained 0.5 μM of laurdan probe reagent and 0.25 μM of formulation per well. The plate was measured in a TECAN plate reader at 340 nm (excitation wavelength), and an emission spectra from 400 to 500 nm.

2.3.5. Buffer capacity assay

Buffer capacity assay measures quantitatively the resistance of a solution to change its pH with the addition of H^+ or OH^- ions. In this case, formulations were prepared in 150 mM NaCl at a concentration of 0.1 mg/mL, and the pH was adjusted to 10 with 0.1 M NaOH. Finally, the solution was titrated with 0.1 M HCl, and the pH values were measured with a Crison pH-Meter GLP21.

2.3.6. Vulnerability assay of complexes in the late endosome

The escape of nioplexes from endosomes is a critical step in transfection as these vectors are entrapped, with subsequent degradation, in the acidic compartments of the endo/lysosomal pathways [102,103]. The endosomal escape ability of the nioplexes was evaluated with phosphatidylserine (PS) micelles, analogous to the lysosomal compartments. After micelle formation, the nioplexes and PS were incubated for 1 h at room temperature at a mass ratio of genetic material/PS of 1/50. Both the samples and the control, naked DNA, were included in a 0.8 % agarose gel (200 ng DNA per sample), which was immersed in tris-acetate-EDTA buffer. After 30 min exposure at 100 V, the bands were revealed with GelRed™. Once the images were captured with a ChemiDoc™ MP Imaging System, the DNA bands were quantified with the Image Lab™ software. The percentage of DNA released from each complex was calculated with the following equation, where SC is supercoiled DNA:

$$\% \text{ DNA released} = [(\text{SC band})/(\text{total DNA})] \times 100$$

2.4 *In vitro* assays

2.4.1. Cell culture and transfection protocol

For the *in vitro* assays, we selected a human CF airway epithelial cell line derived from lung patient, homozygous for the F508del mutation (CuFi-1 cell

line from ATCC®). These cells were seeded on collagen type IV pre-coated plates or flasks and incubated at 37ºC, and 5 % CO_2 atmosphere.

Transfection assays were performed on different plates depending on the application of each technique. 96-well plates were used for kinetic analysis and channel functionality assays, at a cell density of 3×10^4 cells per well; 24-well plates were used for flow cytometry analysis, at a cell density of 1.6×10^4 cells per well; and 6-well plates were used for CFTR protein expression assays, at a cell density of 8×10^5 cells per well. After seeding, cells were incubated overnight to reach 70 % cell confluence at the time of transfection. For transfection purposes, nioplexes were formed at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1 in Opti-MEM™ transfection medium. DNA dose depends on the kind of plate: 0.21 µg, 1.25 µg and 6.31 µg of DNA per well in 96-, 24- and 6-well plates, respectively. Cells were incubated with nioplexes for 4 h at 37ºC. The next step was to remove nioplexe solution and add fresh medium to the cells. Transfection efficiency was evaluated after 48 h. Opti-MEM™ medium without nioplexes and Lipofectamine™ 2000-transfection reagent were used as negative and positive transfection controls, respectively. Each condition was evaluated in triplicate.

2.4.2. Analysis of pEGFP expression and cell viability

After the transfection process, EGFP-expressing cells are those that have been able to incorporate the nioplexes vectoring pEGFP and perform the intracellular processes. Currently, there are many methods to perform the intracellular EGFP signal, both qualitatively and quantitatively. Quantitative data of pEGFP expression (percentage of EGFP-positive live cells and mean fluorescence intensity (MFI)) and cell viability (obtained after addition of propidium iodide at a 1:300 dilution) were revealed with a flow cytometry system. In the case of kinetic analysis, transfection efficiency and biocompatibility values were assessed with the Cytation™ 1 equipment, monitoring for 7 days post-transfection the quantitative measurements of green fluorescence intensity and cell absorbance at 600 nm. In addition, fluorescence and brightfield images were captured every 24 h for qualitative analysis.

2.4.3. Cellular uptake

Cellular uptake assay shows which cells incorporate the nioplexes from the medium. In this case, cellular uptake was evaluated with FITC-labeled pEGFP, which marks the nioplexes with a green signal (another option would be to label the niosomes instead of the genetic material). Subsequently, cells were incubated with these fluorescent nioplexes and quantitatively analyzed by flow cytometry at 2 and 4 h. The results were expressed as percentage of cells containing FITC-labeled pEGFP after removal of dead cells.

2.4.4. Intracellular trafficking

In order to study the most common endocytic pathways by which formulations are internalized into cells, nioplexes can be monitored intracellularly by fluorescently labeling niosomes or genetic material. In this case, as in the cellular uptake assays, cell internalization processes were performed with FITC-labeled pEGFP, which marks the nioplexes with green signal. The cell staining procedure was as follows. Cells were seeded on coverslips in 24-well plates and incubated with nioplexes vectoring FITC-pEGFP for 3 h. Endocytic vesicle markers were then added and incubated for 1 h more. Finally, cells were fixed with 4 % paraformaldehyde and mounted with Fluoroshield™ with DAPI. Transferrin-Alexa Fluor™ 594, Cholera toxin B-Alexa Fluor™ 594 and Dextran-Alexa Fluor™ 594 were used as markers for CME, CvME and macropinocytosis, respectively, whose signal is red. The samples were captured with an inverted fluorescence confocal microscope and co-localization of green and red signals was quantified with a cross-correlation analysis and ImageJ software [38,104].

2.4.5. CFTR protein quantification

After the transfection procedure, the amount of produced CFTR protein was quantified and compared to the base-level production of non-transfected cells. In this experimental work, we evaluated the CFTR protein expression with a Western blot assay. Cells were seeded on 6-well plates and transfected with nioplexes vectoring pGM169. 48 h post-transfection, cells were detached and treated with lysis buffer to remove total protein, which was quantified by

a BCA protein assay kit. Here, 30 µg of proteins of each sample were loaded with Laemmli buffer on an SDS-PAGE 7 % acrylamide electrophoresis gel and processed for 75 min at 130 V. The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane with a semi-dry system. The membrane was incubated at room temperature in a blocking solution for 1 h. The next incubation included the exposure to a monoclonal mouse anti-human/-mouse against CFTR (1:500) overnight at 4ºC. To reveal the protein bands, a peroxidase-conjugated goat anti-mouse IgG (1:10,000) was added to the membrane and incubated for 1 h. A rabbit polyclonal anti-β-actin antibody (1:400) and peroxidase-conjugated secondary antibody (1:20,000) were used as loading controls (β actin). At the end of the process, specific protein expression was detected by chemiluminescence with ECL Plus and observed with a ChemiDoc System. Densitometric quantification of the bands was performed with Image Lab™ software and the data were expressed as arbitrary densitometric units (ADU) relative to β-actin expression.

2.4.6. CFTR channel functionality

The quantification of the CFTR protein shows the amount of protein present in the cells but it is also necessary to assess whether these channels are able to perform their function properly. This means that it is necessary to evaluate the biological functionality of this synthesized protein to observe the degree of functional recovery of the cells. The biological activity of the chloride channel can be detected by measuring iodide efflux progression over time (usually chloride efflux is replaced by iodide efflux [105]) (Figure 8). To this end, cells were seeded on 96-well plates and transfected with nioplexes vectoring pGM169. 48 h post-transfection, cells were incubated with the fluorescent molecule 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) for 45 min. To monitor the efflux, cells were washed and incubated with NaI buffer, which was subsequently changed to nitrate buffer that included both CFTR channel agonists forskolin (FSK, 10 µm) and 3-isobutyl-1-methylxanthine (IBMX, 200 µM). The last medium replacement was with NaI buffer to return to basal levels. The fluorescent signal of SPQ was detected with a microplate reader at 443 nm over time.

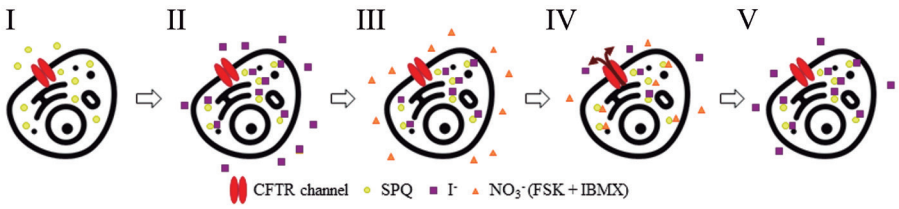


Figure 8. General scheme of the functionality assay performed: (I) SPQ loading into human CF cells; (II) quenching of SPQ by I^- from NaI buffer; (III) addition of nitrate buffer containing NO_3^- and both CFTR channel agonists forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX); (IV) exit of I^- from human CF cells across the CFTR channel and dequenching of SPQ; and (V) addition of I^- from NaI buffer and secondary quenching of SPQ.

2.5. 3D bioprinted scaffolds

2.5.1. Elaboration

3D bioprinting technique can be used to obtain suitable scaffolds for diverse applications, if some parameters, including pressure and speed of the printing, structure and size of the scaffold, or composition of the bioink, are carefully optimized. In our case, scaffolds based on biocompatible components, such as hyaluronic acid, alginate, gelatin, fibrin and A-proteinin, were selected as the most adequate. Human CF airway epithelial cells were introduced into this bioink at a density of 5×10^6 cells/mL. Extrusion-based bioprinting with Cellink® BioX bioprinter equipment was used to produce the scaffolds in 24-well plates. The scaffold shape was optimized to a 1 cm square. Once bioprinted, they were covered with growth medium, renewed every 2 days, and incubated at 37ºC and 5 % CO_2 atmosphere.

2.5.2. Characterization

In order to characterize and evaluate the cells embedded in the scaffolds, three different cell viability assays were performed on days 1, 3, 7, 14 and 21 after bioprinting. For the qualitative assay, a Live/dead™ viability/cytotoxicity kit was used, as a stain to track cell status, and images were captured with an inverted microscope equipped with an accessory for fluorescence observation. Cell metabolic activity was measured in a 96-well plate using a Cell Counting Kit-8 (CCK-8) with a microplate reader at 450 nm, and a wavelength of 650

nm was used as a reference. Scaffolds without cells were used as a control. Cell cytotoxicity was monitored with a lactate dehydrogenase (LDH) activity assay, measured in a 96-well plate with a microplate reader at 490 nm, and 680 nm was used as a reference. Acellular scaffolds were used as a negative control, scaffolds with cells and lysis buffer as a control for spontaneous LDH expression and LDH solution as a positive control.

2.5.3. Transfection

Transfection assays applied to scaffolds followed the same pattern as two-dimensional (2D) cultures. However, 3D cultures can also be used as a more advanced system to simulate the schedule of administration. The DNA used to transfect them was the reporter plasmid encoding for EGFP. In fact, two different approaches were evaluated in the scaffolds: external transfection and bioink transfection. External transfection consists of multiple additions of nioplexes (2.5 µg DNA per scaffold) into the scaffold medium every 72 h (4 times). In the bioink transfection, the nioplexes were included into the bioink using three different doses: 7.5, 15 and 22.5 µg DNA per scaffold. Transfection efficiency was monitored with Cytation™ 1 equipment for 13 days.

2.6. Statistical analysis

The experimental data were statistically analyzed with the IBM® SPSS® Statistics computer software. Normal distribution and homogeneity of variances were assessed by the Shapiro-Wilk test and the Levene test, respectively. Under parametric conditions, the Student´s t-test or the ANOVA followed by the post-hoc HSD Tukey test were performed. The Kruskal-Wallis test and the Mann-Whitney U test for two to two comparisons were executed under non-parametric conditions. In all samples, p-value ≤ 0.05 was considered as statistically significant. Data were represented as mean ± standard deviation (SD).

3. Hypothesis and objectives

In recent years, non-viral vectors have become increasingly important in gene therapy approaches. Although their transfection efficiencies are low, their high genetic cargo packing capacity, low immunogenicity, and easy and cost-effective production make non-viral vectors a promising alternative to viral vectors, which show higher transduction efficiencies but relevant biosafety issues. Indeed, the scientific community has made great strides in the field of non-viral vectors to overcome their main concerns. In particular, niosomes have emerged as biocompatible non-ionic surfactant vesicles that are effective as gene delivery nanoparticles in many disorders located in the retina and central nervous system (CNS), among others. Basically, niosomes developed for gene delivery purposes consist of a non-ionic surfactant, a cationic lipid and a "helper" component.

The progress, discovery and incorporation of new "helper" components in niosome formulations have enabled the development of these nanoparticles, whose physicochemical and biophysical properties depend on the composition of the formulation as well as its method of preparation. Therefore, an in-depth evaluation of niosomes is essential to reveal how formulation composition affect transfection efficiency and cytotoxicity. The characterization of niosomes brings them closer to their application in clinical practice. Many diseases can be treated with gene therapy approaches, but CF stands out because of its autosomal recessive monogenic nature. In order to achieve this goal, niosome formulations have to overcome different barriers associated with the route of administration and the extracellular and intracellular environments, to reach the nucleus of target cells and demonstrate their transfection efficiency and low immunogenicity.

Taking into account this information, the main objective of this doctoral thesis is the development and in-depth biophysical and biological characterization of non-viral vectors based on cationic niosomes to face CF disease by gene therapy approach. To accomplish this purpose, five specific objectives are considered:

1. To understand the characteristics, strengths and weaknesses of gene therapy based on non-viral vectors in order to develop effective and safe niosome formulations for translation into clinical practice. (Appendix 1).

2. To analyze the applications of non-viral vectors to address CF disease through gene therapy approach, and consider both biological barriers and formulation development deficiencies that need to be overcome in order to reach clinical relevance. (Appendix 2).

3. To study the effects of both chloroquine and chemical composition and phase incorporation of non-ionic surfactants on the biophysical properties of niosome formulations by thorough evaluation, including physicochemical characterization, transfection efficiency and biocompatibility. (Appendix 3).

4. To evaluate the behavior of niosomes in human CF airway epithelial cells to understand their transfection process and accomplish sufficient *in vitro* transfection efficiency to recover the CFTR channel functionality. (Appendix 4).

5. To explore and develop a 3D scaffold that mimics the *in vivo* environment and can be used to evaluate new treatments and simulate different schedules of administration for CF in order to bridge the gap between *in vitro* and *in vivo* studies. (Appendix 5).

4. Results and discussion

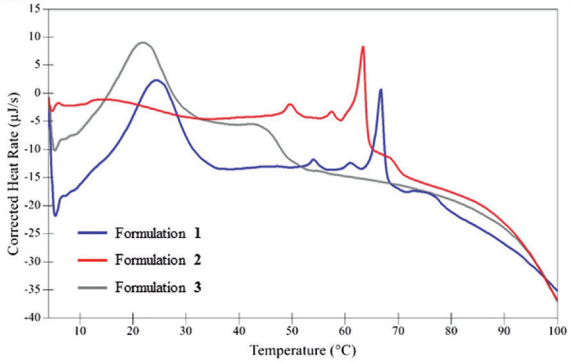
Despite all the efforts made by the scientific community in the last years, there are still no treatments available for genetic disorders that in the future will probably be addressed by gene therapy. As described before, the aim of this therapy is the incorporation of genetic material into target cells to modulate their protein expression [106]. One of the most challenging aspects to complete the clinical translation of gene therapy is the lack of safe viral vectors and the low transfection efficiency of non-viral vectors [35,36,62]. In this regard, niosomes have recently emerged as a promising non-viral vector for gene delivery purposes.

In the first experimental study of this doctoral thesis, we aim to determine the effects of, on the one hand, the inclusion of chloroquine as a biophysical performance enhancer agent and, on the other hand, the impact of non-ionic surfactants on the biophysical performance of niosomes, both chemical composition and incorporation phase in the elaboration. For this purpose, we elaborated three different niosome formulations (Table 1): formulation **1** and **3** were based on the same chemical compounds but "helper" content changed (formulation **3** lacked chloroquine), and formulation **2** differed from formulation **1** in non-ionic surfactant content (formulation **2** diverged in one of the non-ionic surfactants and its protocol of incorporation into the formulation). In order to analyze the differences between the formulations, many biophysical assays were carried out.

The thermal stability of niosomes, based on formulations **1**, **2** and **3**, was studied by DSC and the thermograms obtained from each formulation are displayed in Figure 9A. Formulation **1** (blue line) showed higher thermal stability compared to its counterparts, due to the slight displacement of its thermogram to the right [107]. Different endothermic events are depicted in the thermograms and, the maximum peak of each curve represents the thermal melting (Tm) temperature (Figure 9B): the thermograms of formulations **1** and **2** (red line) presented five Tm and formulation **3** thermogram (grey line) showed two Tm. Furthermore, formulations **1** and **3** matched in Tm1, which would be a consequence of the presence of poloxamer 407 in both

formulations, confirmed by the fact that formulation **2**, without poloxamer 407, did not show it. Formulations **1** and **2** matched in Tm2, Tm3, Tm4 and Tm5, being the region between 48ºC and 70ºC an effect of chloroquine behavior since it was not possible to detect it in formulation **3**.

A



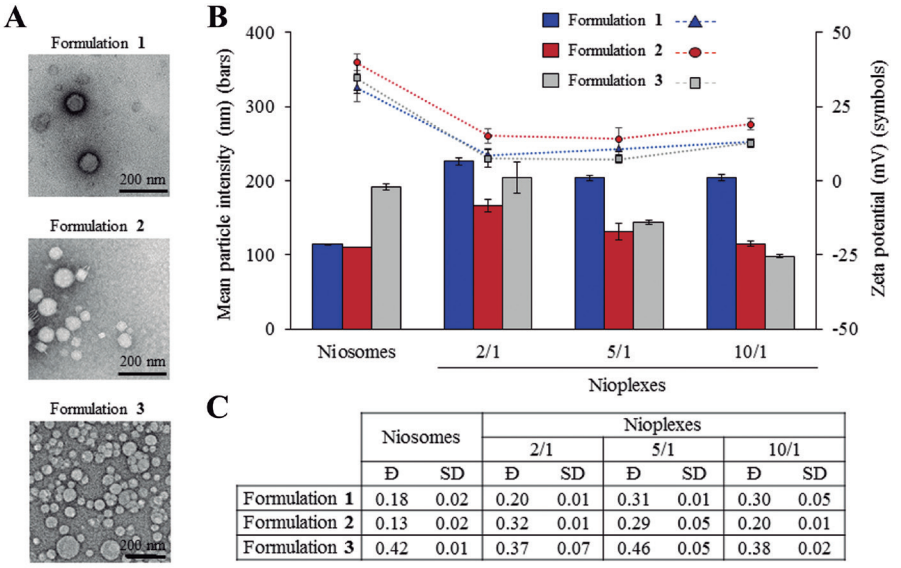
B

Formulation	1	2	3
Tm1 (ºC)	24.47	15.57	21.76
Tm2 (ºC)	54.24	49.59	44.28
Tm3 (ºC)	60.82	57.5	--
Tm4 (ºC)	66.66	63.17	--
Tm5 (ºC)	75.40	69.01	--

Figure 9. Niosomes evaluation by differential scanning calorimetry (DSC). **A**. Thermograms obtained from formulations **1** (blue line), **2** (red line) and **3** (grey line). **B**. Thermal melting (Tm) points as midtransition temperature of the niosome formulations.

Following with the physicochemical analysis, parameters such as morphology, size, dispersity (Đ) and zeta potential, which are closely related to the transfection capacity of niosome formulations, were evaluated, in this case, on niosomes based on formulations **1**, **2** and **3**, and their corresponding nioplexes vectoring the reporter pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1. All niosome formulations showed a spherical shape, as seen in the images captured by TEM in Figure 10A. Regarding size measurements (Figure 10B, bars), all niosome formulations and their corresponding nioplexes obtained diameters between 100 and 230 nm, which are considered appropriate values to improve the cellular uptake of nanoparticles [108]. The mean particle size of niosome formulations **1**, **2** and **3** was 114.43 ± 0.64 nm, 110.40 ± 0.40 nm and 191.73 ± 4.11 nm, respectively. After complexing niosomes with pEGFP at cationic lipid/DNA mass ratio 2/1, size values increased 2-fold in formulation **1**, 1.5-fold in formulation **2**, and there was no change in formulation **3**. In the case of mass ratios 5/1 and 10/1, all formulations followed the same pattern: size values gradually decreased when increasing the mass

ratio. Nioplexes based on formulation **1** had the highest size values at all mass ratios.



C

	Niosomes		Nioplexes					
			2/1		5/1		10/1	
	Đ	SD	Đ	SD	Đ	SD	Đ	SD
Formulation **1**	0.18	0.02	0.20	0.01	0.31	0.01	0.30	0.05
Formulation **2**	0.13	0.02	0.32	0.01	0.29	0.05	0.20	0.01
Formulation **3**	0.42	0.01	0.37	0.07	0.46	0.05	0.38	0.02

Figure 10. Characterization of niosomes based on formulations **1**, **2** and **3**, and their corresponding nioplexes vectoring pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1 in terms of (**A**) morphology by transmission electron microscopy (TEM) images (scale bar: 200 nm), (**B**) particle size (bars) and zeta potential (symbols) and (**C**) dispersity (Đ). Each value represents the mean ± standard deviation (SD) of three measurements.

As for zeta potential values (Figure 10B, symbols), the three niosome formulations showed a positive superficial charge (+31.37 ± 4.78 mV, +39.93 ± 2.64 mV and +34.63 ± 5.28 mV of niosome formulations **1**, **2** and **3**, respectively), which promotes electrostatic interactions with the genetic material, avoids particle aggregation and enhances cellular internalization [109]. The zeta potential values of nioplexes, at a cationic lipid/DNA mass ratio 2/1, declined and rose again as the ratio increased to 5/1 and 10/1 without reaching the original values of niosomes. Formulation **2** reached the highest data at any mass ratio without exceeding +20 mV. Concerning dispersity (Đ) (Figure 10C), the lowest values were achieved by niosome formulations **1** and **2** (below 0.35), and the highest values were recorded for formulation **3** (around 0.40), both with and without DNA. These results show a more heterogeneous particle size distribution of formulation **3** derived from the lack of chloroquine.

To further investigate nioplexe formation, interactions between niosomes based on formulations **1**, **2** and **3**, and genetic material (pEGFP) were monitored by ITC analysis. Figure 11A illustrates the titration profiles represented by the heat released from DTPA -cationic lipid- (per gram) contained in niosomes, resulting from the increase of the cationic lipid/DNA mass ratio. For niosomes based on formulations **1** and **2**, the saturation point of the heat evolved at DTPA/pEGFP was reached at a mass ratio of approximately 2/1-3/1, whereas in formulation **3** it was reached at a mass ratio of 9/1. These results determined that formulations **1** and **2**, which contained chloroquine, showed a higher affinity for DNA molecules than formulation **3**. This can be explained by the higher DNA binding affinity due to the presence of chloroquine in the formulation composition, which is consistent with previous studies [10]. In addition, these profiles showed that differences in transfection efficiency above the 2/1 mass ratio in niosome formulations **1** and **2** could be caused by a redistribution of DNA leading into nioplexes, with a decrease in the fraction of plasmid molecules bound to niosomes when the ratios are increased.

In order to observe how these interactions influence lipid membrane packing, the laurdan lipophilic probe was used to calculate GP values. These measurements, shown in Figure 11B, were performed on niosomes based on formulations **1**, **2** and **3**, and their corresponding nioplexes. All GP values of the niosomes were negative and close to zero, but the lowest value was obtained by formulation **2** (–0.12 ± 0.03). For nioplexes based on formulations **1** and **2**, these GP values showed an increase at cationic lipid/DNA mass ratios 2/1 and 5/1 up to 0.7 and 0.5, respectively. At a mass ratio of 10/1, the GP values approached 0.1. In the case of nioplexes based on formulation **3**, their GP values were close to zero at all mass ratios studied. Therefore, the highest ordered membrane packing was achieved with the nioplexes based on chloroquine-containing formulations (formulations **1** and **2**). These results indicated that some ordering occurs in membrane packing produced by interactions between pDNA and niosome components in nioplexe formation, reflecting a significant role of chloroquine in such interactions, possibly because of its ability to interact with DNA [110,111].

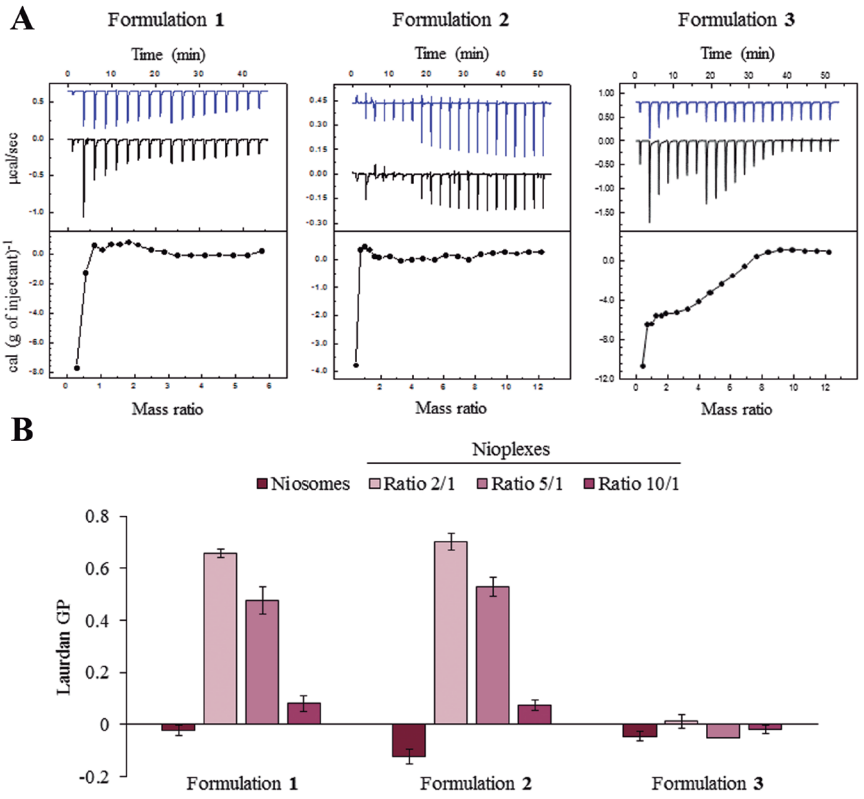


Figure 11. Interactions between niosome formulations and genetic material. **A**. Isothermal titration calorimetry (ITC) studies of niosomes based on formulations **1** (1:1 dilution), **2** and **3** with pEGFP. The upper panels show the raw data of the injection of the respective formulation into the plasmid solution (black line) or the blank solution (blue line). The lower panels show the dependence of the evolved heat per gram of injected cationic lipid as a function of the DTPA/pEGFP mass ratio in the sample cell. **B**. GP values obtained with laurdan probe in niosomes based on formulations **1**, **2** and **3**, and their corresponding nioplexes vectoring pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1.

As already mentioned, one of the handicaps of non-viral vectors is the ability to release genetic material from the endosomal compartments to avoid degradation [102]. In order to analyze the ability of niosomes to escape from intracellular endosomes, we evaluated the buffering capacity of niosomes based on formulations **1**, **2** and **3** (Figure 12A), and the DNA release profile of their corresponding nioplexes vectoring pEGFP from artificial endosomes (Figure 12B). Formulation **3** obtained the lowest pH buffering capacity and the lowest values of DNA release from artificial endosomes. Therefore, formulation **3**

elicited the worst endosomal escape ability. Formulations **1** and **2** showed a higher buffering capacity that can be explained by the inclusion of the chloroquine molecule, enhancing endosomal escape mainly by the proton sponge effect [35,112]. Concerning the DNA release profiles from the PS micelles, formulation **1** obtained the highest values (more than 70 %) at all cationic lipid/DNA mass ratios studied.

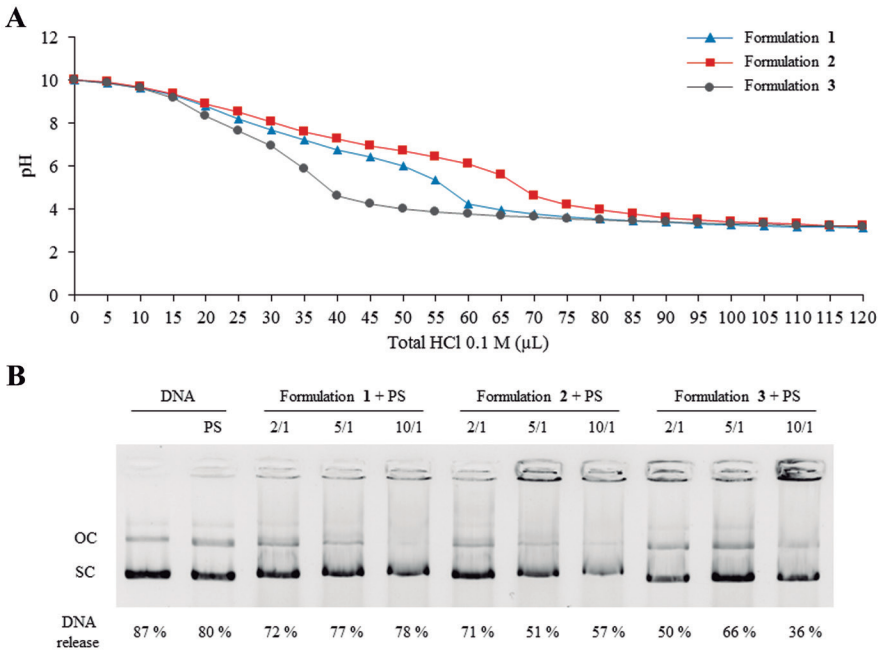


Figure 12. Buffer capacity and endosomal escape studies. **A**. pH buffering capacity of niosomes based on formulations **1** (blue line, triangles), **2** (red line, squares) and **3** (grey line, dots). **B**. DNA release profiles of nioplexes based on formulations **1**, **2** and **3** vectoring pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1 from phosphatidylserine (PS) micelles. OC: open circular. SC: supercoiled.

All the information obtained from previous assays was used to set up *in vitro* studies in human CF airway epithelial cells to evaluate nioplexes based on formulations **1**, **2** and **3** vectoring pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1. For this purpose, a kinetic study was performed in which both transfection efficiency (Figure 13A) and cell growth (Figure 13B) were evaluated for 7 days. Moreover, representative microscopy images of the

process are shown in Figure 13C. In some cases, the 10/1 mass ratio showed higher transfection efficiency than the other ratios, but the cytotoxicity values were raised. Therefore, to keep the balance between transfection efficiency and cytotoxicity, the 5/1 mass ratio was considered as the most suitable one in all formulations. The fluorescence intensity results reached a maximum peak at 72 h post-transfection and remained close to the maximum for, at least, 7 days. Formulations **1** and **2** showed quite similar results, with elevated fluorescence intensities, whereas formulation **3** obtained rather lower values. Herein, the presence or absence of chloroquine is critical to understanding the differences in transfection efficiency between formulations **1** and **2** with formulation **3**. The highest transfection efficiency values were obtained for nioplexes based on niosome formulations with a chloroquine concentration that promotes endosomal escape and avoids cytotoxic effects [113].

In this case, gene addition therapy consists of the introduction of a pDNA, considered as a non-integrative system, whose protein expression is reduced caused by not integrating into the cell genome. Even so, and as shown in this *in vitro* study, the intracellular expression of EGFP was remained until, at least, 168 h after nioplexes exposure. To quantify this expression, Cytation™ 1 equipment allows continuous monitoring throughout the days to assess the period in which EGFP expression is kept. This issue is relevant because data on the duration of cell transfection can be obtained, which enables the design of the drug administration patterns in subsequent *in vivo* studies.

To further evaluate this transfection behavior, flow cytometry assays were performed at 48 h post-transfection with nioplexes based on niosome formulations **1** and **2** vectoring pEGFP in terms of transfection efficiency and cytotoxicity. In this regard, formulation **1** achieved significantly higher values of the percentage of EGFP positive live cells at cationic lipid/DNA mass ratios 5/1 and 10/1 (21.02 ± 2.68 % and 28.22 ± 0.76 %, respectively), than formulation **2** (13.94 ± 0.56 % and 20.75 ± 1.68 %, respectively) (Figure 14A, bars). The live cells percentage values of formulation **1** were also significantly higher than formulation **2**, although the percentage of both formulations decreased with increasing mass ratio (Figure 14A, symbols). MFI values showed that formulation **1** reached significantly higher values at cationic lipid/DNA

mass ratios 5/1 and 10/1, around 410 A.U., than formulation **2**, around 300 A.U. (Figure 14B). On the other hand, formulation **2** presented higher values at pEGFP expressing cells and MFI only at the 2/1 mass ratio, but these values were lower than those obtained at mass ratios 5/1 and 10/1.

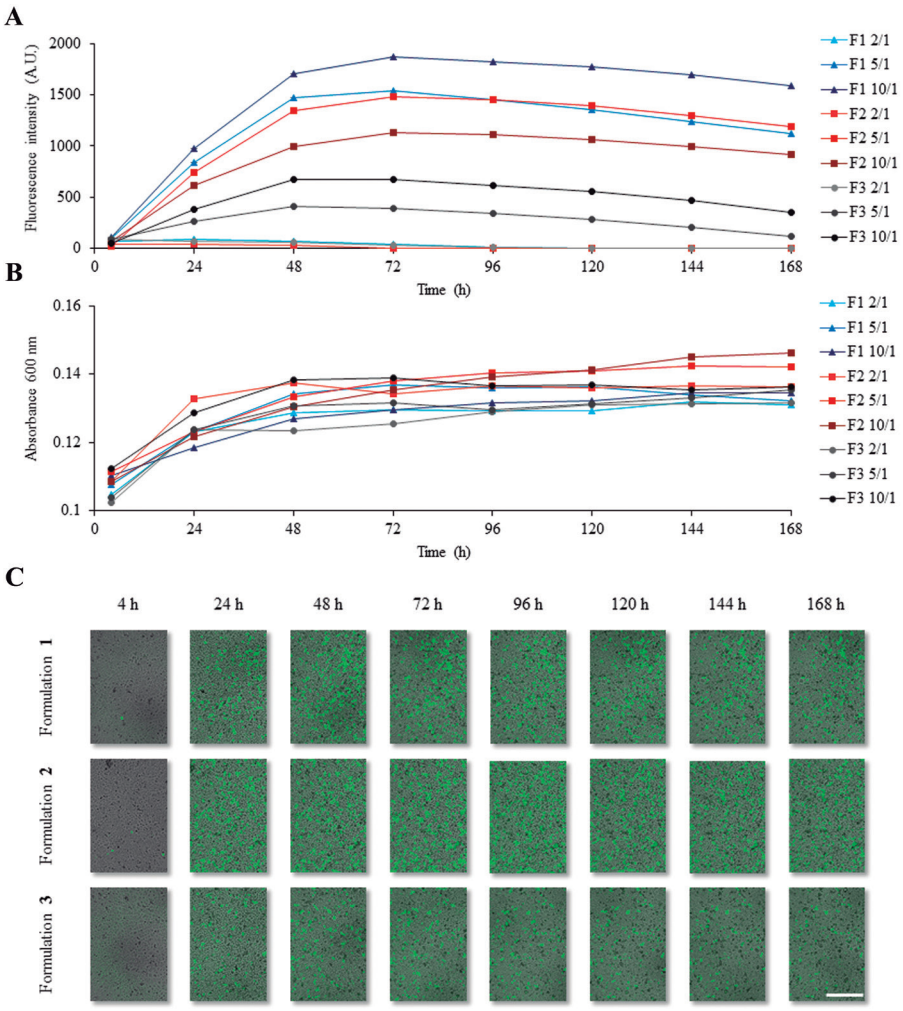


Figure 13. Kinetic study of transfection efficiency evaluated with the Cytation™ 1 equipment. (**A**) Fluorescence intensity and (**B**) mean absorbance curve showing EGFP expression and cell viability over 7 days in human CF airway epithelial cells transfected with nioplexes based on formulations **1** (F1), **2** (F2) and **3** (F3) vectoring pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1. **C**. Representative brightfield merged fluorescence images of transfection with nioplexes based on formulations **1**, **2** and **3** vectoring pEGFP at a mass ratio of 5/1. Scale bar: 200 μm.

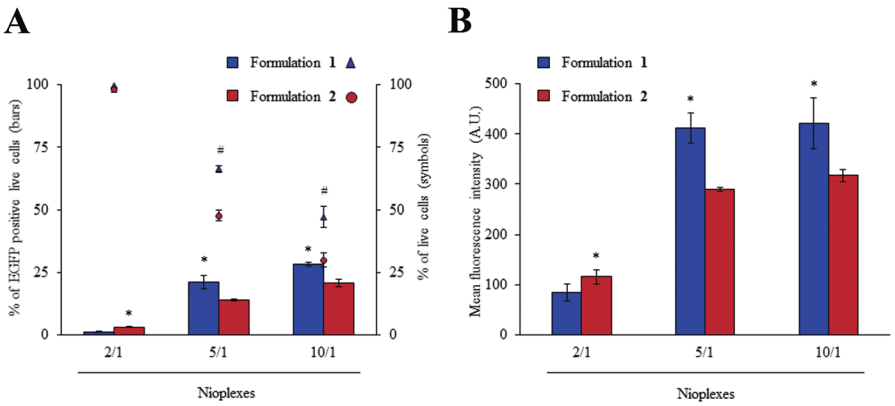


Figure 14. Transfection efficiency assessed by flow cytometry at 48 h post-transfection. (**A**) Percentage of live cells expressing EGFP (bars), percentage of live cells (symbols) and (**B**) mean fluorescence intensity (MFI) data of transfection with nioplexes based on formulations **1** and **2** vectoring pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1. Each value represents the mean ± SD of three measurements. Statistical significance: $*p \leq 0.05$ in transfection and MFI; $\#p \leq 0.05$ in cell viability. A.U.: arbitrary units.

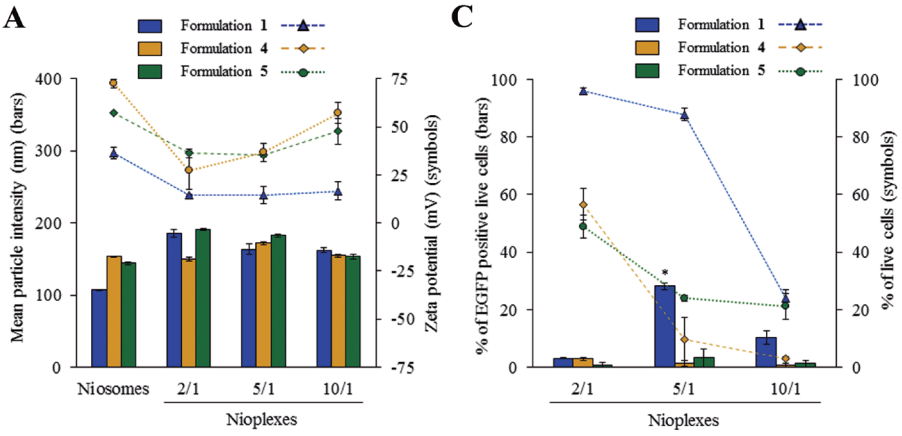
To sum up, the biophysical evaluation of the different niosome formulations determined, on the one hand, the effect of the presence of chloroquine in niosomes and, on the other hand, the impact of chemical composition and incorporation protocol of non-ionic surfactants. The differences observed in formulations **1** and **3** established the importance of chloroquine content between niosome vesicles, since it affected not only physicochemical parameters but also the biological performance of the niosomes. These results revealed that chloroquine increased thermal stability, decreased particle size and dispersity, enhanced affinity for DNA molecules, improved ordered membrane packing in nioplexes, increased buffering capacity of niosomes, and enhanced endosomal escape ability. Improvements in these parameters could explain the raised transfection efficiency of niosomes that include chloroquine in their composition. The effects of non-ionic surfactants were determined, both in terms of chemical composition and incorporation protocol, by comparing niosome formulations **1** and **2**. And, although the differences in biophysical properties were slight, formulation **2** showed worse thermal stability, higher zeta potential values and increased buffer capacity in the niosomes. However, formulation **2** resulted in a lower nioplexes ability to escape from artificial endosomes, which was related to a lower transfection efficiency with higher

cytotoxicity. In this case, niosome formulation **1** stood out with better biophysical properties than its counterparts for gene delivery purposes.

In the second experimental part of this doctoral thesis, new niosome formulations were incorporated into the study of non-viral vectors for gene delivery purposes to face CF disease. In this case, three different niosome formulations **1**, **4** and **5** were developed and physicochemically characterized. The corresponding nioplexes were formed and *in vitro* assays were carried out including cellular uptake, intracellular trafficking, transfection efficiency and cell viability. In addition, the CFTR transgene was quantified and channel functionality was assessed in order to determine the transfection efficiency of the incorporated therapeutic genetic material. Formulation **1** was also used in the first experimental part and was made of DTPA as the cationic lipid, poloxamer 407 and polysorbate 80 in the organic phase as the non-ionic surfactants and chloroquine in the aqueous phase as the "helper" component. Niosomes based on formulations **4** and **5** consisted of DOTMA as the cationic lipid, polysorbate 20 or 80 as the non-ionic surfactant, respectively, and squalene as the "helper" component. Table 1 shows the chemical composition of formulations.

First, niosomes based on formulations **1**, **4** and **5**, and their corresponding nioplexes vectoring pEGFP at different cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1 were physicochemically characterized in terms of mean particle size, zeta potential and dispersity (Đ). The mean particle sizes (Figure 15A, bars) obtained from all niosome formulations and their corresponding nioplexes, were between 100 and 200 nm in diameter, a suitable size to enhance the nanoparticle cellular uptake, as mentioned before [108]. As for the niosome formulations, formulation **1** showed the lowest value, close to 100 nm, while formulations **4** and **5** had values around 150 nm. Once complexed with pEGFP, the size values of the nioplexes, based on formulations **1** and **5**, increased at mass ratio 2/1 up to 190 nm and slightly decreased with increasing mass ratio. Meanwhile, nioplexes based on formulation **4** maintained a similar mean particle size at all mass ratios studied. Concerning zeta potential values (Figure 15A, symbols), all formulations obtained a positive superficial charge (+36.40 ± 3.00 mV, +57.03 ± 0.51 mV and +72.43 ± 2.16 mV of niosome formulations **1**, **4** and **5**, respectively), which, as commented previously, promotes

electrostatic interactions with the genetic material, prevents particle aggregation and enhances cellular internalization [109]. Zeta potential parameters decreased, at a cationic lipid/DNA mass ratio of 2/1, and rose again with increasing mass ratios to 5/1 and 10/1 but without reaching niosome values. In all cases, formulation **1** obtained the lowest superficial charge, around 15 mV in its corresponding nioplexes. Regarding dispersity results (Figure 15B), all measurements were below 0.4. The most heterogeneous niosome formulation, in terms of particle size distribution, was formulation **5**, as it reached the highest dispersity values.



B

	Niosomes		Nioplexes					
			2/1		5/1		10/1	
	Đ	SD	Đ	SD	Đ	SD	Đ	SD
Formulation **1**	0.16	0.01	0.36	0.02	0.37	0.01	0.32	0.01
Formulation **4**	0.14	0.01	0.21	0.03	0.26	0.01	0.18	0.01
Formulation **5**	0.21	0.01	0.34	0.01	0.39	0.03	0.32	0.02

Figure 15. Physicochemical characterization of niosomes based on formulations **1**, **4** and **5**, and their corresponding nioplexes vectoring pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1 in terms of (**A**) mean particle size (bars) and zeta potential (symbols) and (**B**) dispersity (Đ). **C**. Percentage of EGFP positive live cells (bars) and percentage of live cells (symbols) evaluated by flow cytometry. Each value represents the mean ± SD of three measurements. Statistical significance $*p \leq 0.05$ in terms of transfection efficiency.

Once the niosomes were physicochemically characterized, *in vitro* studies were initiated to analyze the behavior of nioplexes based on formulations **1**, **4** and **5** vectoring the pEGFP reporter plasmid at cationic lipid/DNA mass ratios

2/1, 5/1 and 10/1. Transfection assays were performed in human CF airway epithelial cells and assessed by flow cytometry at 48 h post-transfection. The percentage of live cells expressing EGFP (Figure 15C, bars) after exposure with nioplexes based on formulations **4** and **5** was less than 5 % at all mass ratios, with no significant differences between them. In the case of transfection with nioplexes based on formulation **1**, higher values were obtained at all cationic lipid/DNA mass ratios, specifically, at a 5/1 mass ratio. The results of cell viability (Figure 15C, symbols), in the transfection process with nioplexes based on formulations **4** and **5** at a 2/1 mass ratio, were around 50 %, while at mass ratios 5/1 and 10/1, cell viability declined noticeably to values below 20 %. Cell viability values after transfection with nioplexes based on formulation **1** at mass ratios 2/1 and 5/1 were around 90 %, decreasing considerably at mass ratio 10/1. Data showed that nioplexes based on formulations **4** and **5**, with low transfection efficiencies, were worse tolerated by human CF airway epithelial cells, possibly due to higher positive superficial charge values [114].

Taking these results into account, niosomes based on formulations **4** and **5**, both with DOTMA and squalene, showed worse gene therapy features than formulation **1**, with DTPA and chloroquine. In fact, and as mentioned in the first experimental study, these improvements in transfection efficiency may be due to the chloroquine content in niosome formulation **1**. For the next steps of the study, we selected niosomes based on formulation **1** at a cationic lipid/DNA mass ratio of 5/1.

To better understand the transfection process of nioplexes based on formulation **1** in human CF airway epithelial cells, cellular uptake and intracellular trafficking assays were carried out. Remarkably, not only cell physiology but also the composition and size of the nioplexes are key factors in achieving successful transfection efficiencies [115-117]. Among the different endocytic pathways, CME is probably the most efficient for uptake. However, disruption of the endosome is necessary to avoid degradation of the genetic material in the endo/lysosomal environment, as provided by chloroquine via the proton-sponge effect. CvME has a non-acidic and non-degradative environment, and macropinocytosis is more involved in the endocytosis process of large complexes [116,118,119]. For these studies, niosomes based on formulation

1 were complexed with FITC-labeled pEGFP, at a cationic lipid/DNA mass ratio of 5/1. Cellular uptake assay (Figure 16A) revealed that approximately 80 % of live cells incorporated nioplexes after 4 h of incubation. Thereafter, a cellular internalization study in human CF airway epithelial cells suggested that the endocytic pathways CvME and macropinocytosis were more predominant than CME. In agreement with these results, the intracellular trafficking assay showed the cross-correlation function (CCF) values (Figure 16B) based on the co-localization analysis of nioplexes (green signal) with the respective endocytic vesicle-specific markers (red signal). The CCF values were higher in the CvME (0.36 ± 0.01) and macropinocytosis (0.39 ± 0.06) pathways than in the CME pathway (0.13 ± 0.02). Representative merged microscope images were captured and shown in Figure 16C.

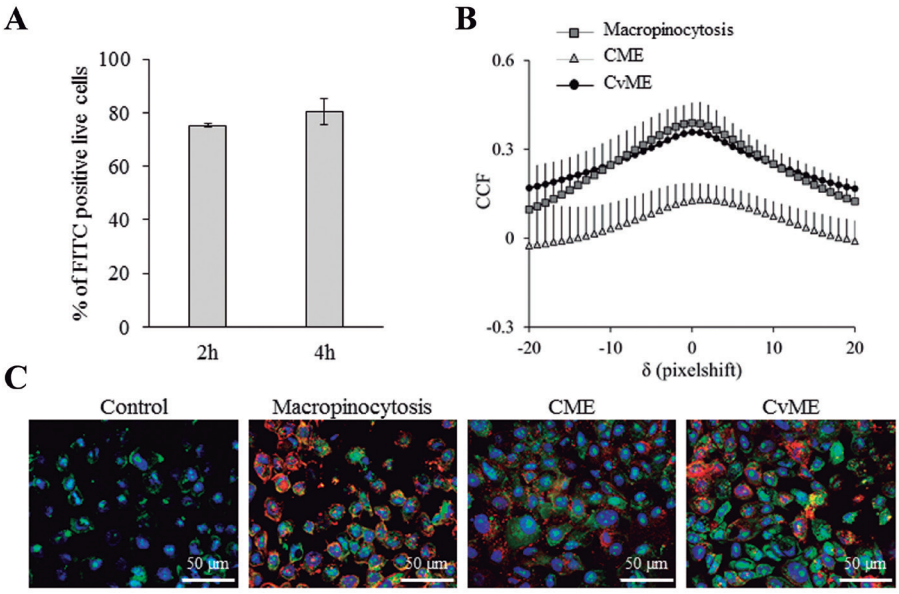
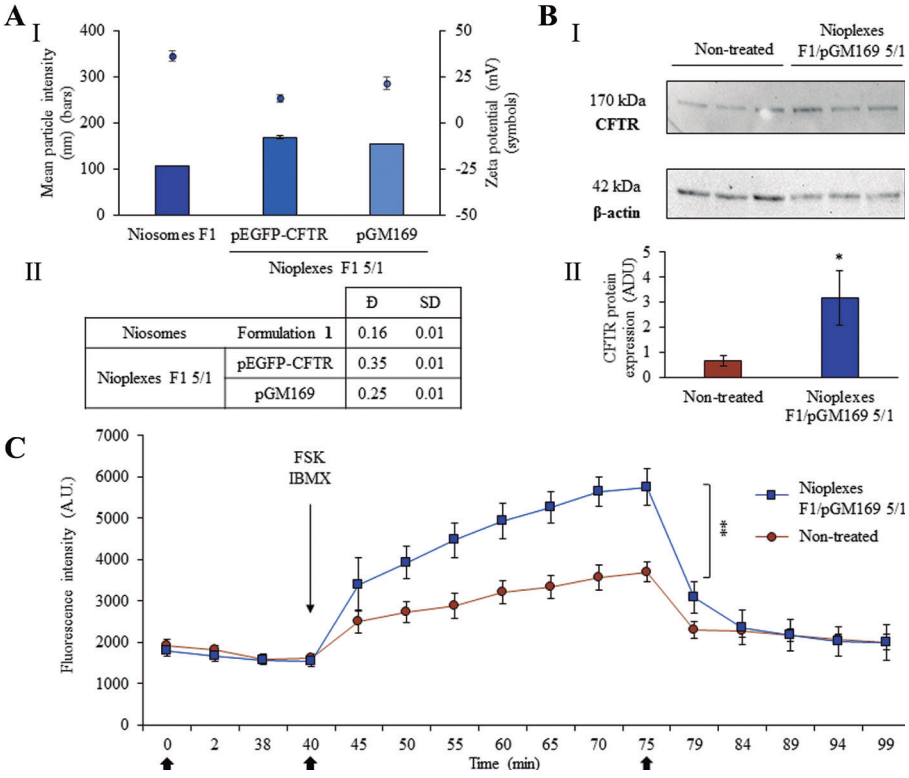


Figure 16. Cellular uptake and intracellular trafficking assays in human CF airway epithelial cells co-incubated with nioplexes based on formulation **1** vectoring the FITC-labeled EGFP plasmid at a cationic lipid/DNA mass ratio of 5/1. **A**. Percentage of live FITC-positive cells after 2 and 4 hours of co-incubation analyzed by flow cytometry. **B**. Co-localization values of FITC-labeled EGFP plasmid and endocytic pathway signals determined by cross-correlation function (CCF) in each case. Each value represents the mean ± SD, n=3. **C**. Representative merged images of intracellular trafficking obtained by confocal microscopy. Nioplexes in green (FITC-pEGFP), cells nuclei in blue (DAPI) and endocytic vesicles in red (Dextran-Alexa Fluor™ 594 for macropinocytosis, Transferrin-Alexa Fluor™ 594 for clathrin-mediated endocytosis (CME) and Cholera toxin B-Alexa Fluor™ 594 for caveolae-mediated endocytosis (CvME)). Scale bar: 50 µm.

Once the characterization of nioplexes vectoring the reporter plasmid, the cellular uptake and internalization monitoring assays were completed, niosome formulation **1** was evaluated for CFTR therapeutic gene constructs delivery, at the established cationic lipid/DNA mass ratio 5/1. Figure 17A shows the physicochemical characterization of niosomes based on formulation **1** and their corresponding nioplexes vectoring pEGFP-CFTR and pGM169 at a mass ratio of 5/1, including mean particle size (Figure 17A-I, bars) (168.47 ± 3.17 nm and 155.10 ± 0.43 nm, respectively) and superficial charge (Figure 17A-I, symbols) (+13.6 ± 2.01 mV and +21.77 ± 3.42 mV, respectively). As expected, these results were in agreement with plasmid weight (pEGFP, 5.5 kb; pEGFP-CFTR, 9.1 kb; pGM169, 6.5 kb): the higher the weight, the larger the particle size and the lower the zeta potential. As for dispersity (Đ) values (Figure 17A-II), all of them were less than 0.4. Therefore, these results confirmed the suitability of nioplexes based on formulation **1** for transfection purposes.

For *in vitro* assays, human CF airway epithelial cells were exposed to nioplexes based on formulation **1**, vectoring pEGFP-CFTR at a cationic lipid/DNA mass ratio of 5/1. Their transfection efficiency was evaluated by flow cytometry and the results obtained were as follows: the percentage of live cells expressing EGFP was 16.34 ± 0.41 %, and the percentage of live cells was 99.48 ± 3.24 %. Moreover, the transfection efficiency with nioplexes based on formulation **1** vectoring pGM169 at a cationic lipid/DNA mass ratio of 5/1 was evaluated, in terms of CFTR protein expression by immunoblot assay (Figure 17B). This assay revealed an intense band of approximately 170 kDa (Figure 17B-I), which corresponds to the glycosylated CFTR protein [13,120], and the band intensity was normalized to β-actin. Quantification of the bands showed a 5-fold increase of CFTR protein levels expressed in transfected cells compared to non-transfected cells (Figure 17B-II). Subsequently, the biological activity of the CFTR channel was monitored over time with the mean fluorescent signal emitted by the SPQ molecule in iodide efflux (Figure 17C). Herein, the addition of the cyclic adenosine monophosphate (cAMP) agonists FSK and IBMX caused a steady increase in the fluorescent signal to 1.5-fold higher values, in transfected cells relative to non-transfected CF cells. In the last step, NaI buffer recovered the baseline fluorescence values.



		Đ	SD
Niosomes	Formulation 1	0.16	0.01
Nioplexes F1 5/1	pEGFP-CFTR	0.35	0.01
	pGM169	0.25	0.01

Figure 17. Characterization and *in vitro* assays of nioplexes based on formulation **1** (F1) vectoring therapeutic DNA constructs at a cationic lipid/DNA mass ratio of 5/1. **A**. Physicochemical characterization in terms of (A-I) particle size (bars) and zeta potential (symbols) and (A-II) dispersity (Đ) of niosomes based on formulation **1** and their corresponding nioplexes vectoring pEGFP-CFTR and pGM169. Each value represents the mean ± SD of three measurements. **B**. Evaluation of protein expression of human CF airway epithelial cells transfected with nioplexes based on formulation **1** vectoring pGM169 compared to non-transfected cells: (B-I) Western blot bands of CFTR and β-actin (loading control) and (B-II) CFTR/β-actin protein expression ratio. ADU: arbitrary densitometric units. Each value represents the mean ± SD, n=3. Statistical significance $*p \leq 0.05$. **C**. CFTR chloride channel biological activity of human CF airway epithelial cells transfected with nioplexes based on formulation **1** vectoring pGM169 and non-transfected cells, determined by iodide efflux rate monitored with the fluorescent SPQ molecule. Each value represents the mean ± SD, n>3. Statistical significance $**p \leq 0.01$. A.U.: arbitrary units.

According to the literature, 5 % of wild-type CFTR mRNA is sufficient to correct chloride ion transport defects in mice to ameliorate the severity of CF pulmonary disease [121-123]. Likewise, other works in the same field revealed an increment of about 25 % of CFTR channel activity in HEK-293 transfected with the commercial liposome formulation Lipofectamine™ 2000 vectoring

pGM169 [13], and an increase of around 36 % in CF cells transfected with lipid nanoparticles (LNPs) vectoring modified CFTR mRNA [124]. Notably, in this experimental work, a 5-fold increase in CFTR protein expression corresponded to a 1.5-fold increment (approximately 55 %) in CFTR channel functionality. These data would far exceed the values needed to provide therapeutic benefits, using for the first time cationic niosomes as non-viral vectors in gene therapy approach for CF disease.

The aim of the final experimental study of this doctoral thesis was to develop new scaffolds to improve the correlation between *in vitro* and *in vivo* studies. Cells behave differently in 2D and 3D cultures, as 3D environments are far more complex and allow cell-to-cell and cell-to-ECM interactions. Therefore, 3D constructs were developed based on 3D bioprinting technology that combines biomaterials with human CF airway epithelial cells that mimic the *in vivo* environment to evaluate niosome behavior for gene delivery purposes. Once the bioink composition, scaffold design and cell density were optimized, the scaffolds were characterized in terms of cell viability, metabolic activity and cytotoxicity effect. Subsequently, to explore the 3D constructs for gene therapy purposes, human CF airway epithelial cells embedded in the scaffolds were exposed to nioplexes based on formulation **1** vectoring the reporter pEGFP at a cationic lipid/DNA mass ratio of 5/1, where different doses and administration schedules were evaluated.

Figure 18A shows fluorescence microscopy images of scaffolds stained with the Live/dead™ viability/cytotoxicity kit. The green signal dots represent live cells, which increase in number as days go by. The red signal depicts dead cells, which appear scarcely in all pictures, being more numerous on days 7 and 14. The metabolic activity assay revealed very similar values on all days, except on day 7, where higher values were obtained without statistical significance (Figure 18B-I). In addition, the measure of cytotoxicity effect showed the highest value on day 1. Stable and minimum values were obtained on the following days (Figure 18B-II). The values described are consistent with the cellular stress caused by the 3D bioprinting process, but these cells seem to fully recover after the process.

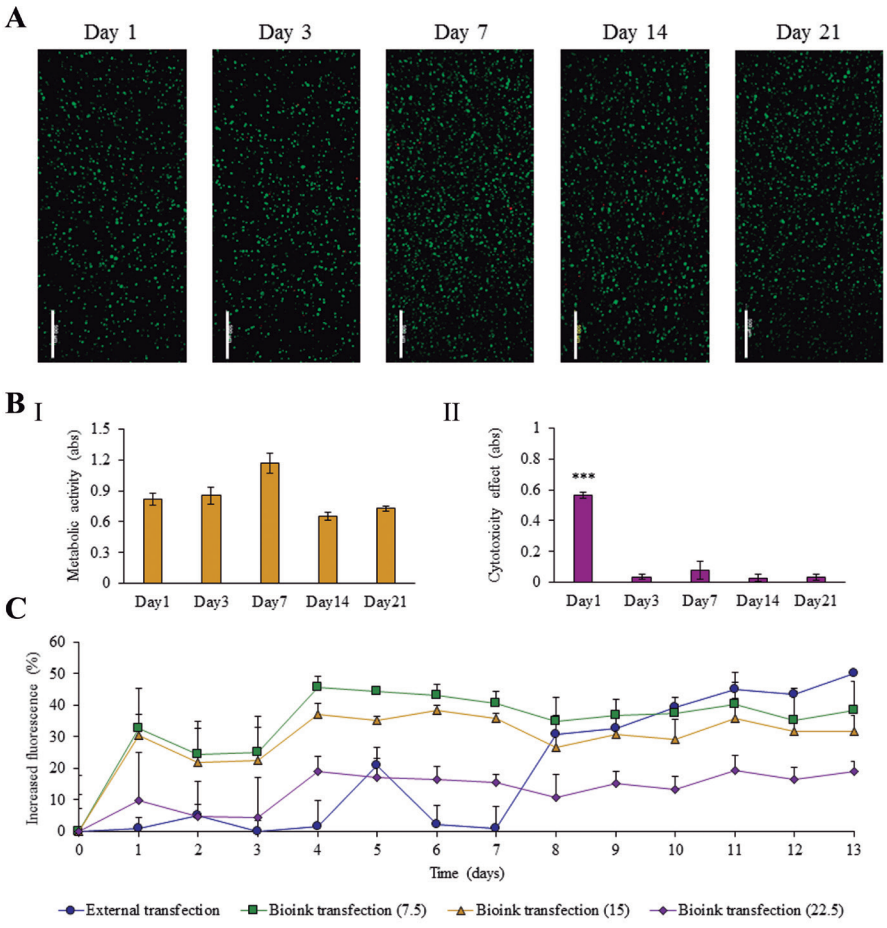


Figure 18. Characterization and transfection assays in human CF airway epithelial cells embedded in bioprinted scaffolds. **A**. Representative fluorescence images of Live/dead™ stained scaffolds. Scale bar: 500 μm. **B**. Evolution of absorbance in (B-I) metabolic activity and (B-II) cell cytotoxicity assays over 21 days. Each value represents the mean ± SD, n=4. Statistical significance $***p \leq 0.001$. **C**. Increase in fluorescence signal of bioprinted scaffolds treated with different transfection approaches containing nioplexes based on formulation **1** vectoring pEGFP at a cationic lipid/DNA mass ratio of 5/1 for 13 days. Each value represents the mean ± SD, n=4.

Transfection efficiency assays were performed in human CF airway epithelial cells, embedded in bioprinted scaffolds, with nioplexes based on formulation **1** vectoring pEGFP at a cationic lipid/DNA mass ratio of 5/1 (Figure 18C). Two different transfection approaches were followed: external and bioink transfection. In external transfection, the administration of repeated doses of nioplexes was simulated (every 72 h). In this case, nioplexes have to pass

more barriers from the culture medium to reach the embedded cells. Results revealed that external transfection obtained the lowest fluorescence values until day 7, when they started to rise. In the case of the bioink transfection, nioplexes were administered in a single dose included into the bioink, and all three groups followed the same pattern: the increment of fluorescence until day 4 that becomes stable in the following days. Dose 7.5 obtained the highest values, followed by dose 15 and ending with dose 22.5. In conclusion, human CF airway epithelial cells embedded in bioprinted scaffolds are viable and metabolically active after 21 days in culture and recovered from the first-day cellular stress after bioprinting. Furthermore, in the endpoint, external multi-dose transfection showed higher increase in fluorescence values than any dose of bioink transfection. However, the relevance of this technique is to simulate different administration strategies, so that single and repeated dosing patterns with different timing can be evaluated. In other words, both the administration interval and drug concentrations can be specified in order to implement drug schedules of administration for *in vivo* studies.

The importance of 3D systems lies in their ability to mimic *in vivo* environments and simulate administration patterns more accurately than *in vitro* assays. In addition, 3D bioprinting technology has gained momentum and is being used to design and elaborate scaffolds that are employed for a wide range of pathologies and conditions that are currently untreatable. Applied to CF disease, these new scaffolds may be the beginning of a strategy that still needs optimization and improvement of its features, but could one day become a realistic *in vitro* lung epithelial model to evaluate both drugs and their administration guideline.

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Chapter 2

CONCLUSIONS

Based on the results obtained in the research studies, the main conclusions of this doctoral thesis are the following:

1. The inclusion of the chloroquine molecule in the niosome formulations decreased both particle size and dispersity values, and enhanced thermal stability and DNA affinity. Furthermore, chloroquine also increased membrane packing of the nioplexes and elicited endosomal escape properties. As a result, *in vitro* studies in human CF airway epithelial cells showed higher EGFP protein expression.

2. The chemical composition of the non-ionic surfactants and their incorporation protocol into the niosome formulation resulted in subtle biophysical variations between the formulations, but affected the *in vitro* studies in human CF airway epithelial cells. Specifically, niosomes elaborated with DTPA, poloxamer 407 and polysorbate 80 in the organic phase, and chloroquine in the aqueous phase obtained the highest transfection efficiency and cell viability values.

3. The intracellular trafficking studies revealed that the niosome formulation made with DTPA, poloxamer 407 and polysorbate 80 in the organic phase, and chloroquine in the aqueous phase, predominantly used the CvME and micropinocytosis cell internalization pathways. When these niosomes vectored CFTR plasmids, a 5-fold increment in CFTR protein expression was obtained, which resulted in a 55 % increase in chloride channel functionality in human CF airway epithelial cells, compared to non-transfected control cells.

4. The 3D bioprinting technology was used to obtain a bioink consists of biocompatible materials and human CF airway epithelial cells in order to elaborate scaffolds. The cells embedded into the bioprinted scaffolds remained viable and metabolically active for, at least, 21 days and were transfected under exposure to nioplexes. This novel strategy based on 3D bioprinting technology could represent an attractive gene delivery *in vitro* model before evaluation under *in vivo* conditions.

Chapter 3

APPENDICES

Published articles

Appendix 1

How far are non-viral vectors to come of age and reach clinical translation in gene therapy?

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How far are non-viral vectors to come of age and reach clinical translation in gene therapy?

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ABSTRACT

Efficient delivery of genetic material into cells is a critical process to translate gene therapy into clinical practice. In this sense, the increased knowledge acquired during past years in the molecular biology and nanotechnology fields has contributed to the development of different kinds of non-viral vector systems as a promising alternative to virus-based gene delivery counterparts. Consequently, the development of non-viral vectors has gained attention, and nowadays, gene delivery mediated by these systems is considered as the cornerstone of modern gene therapy due to relevant advantages such as low toxicity, poor immunogenicity and high packing capacity. However, despite these relevant advantages, non-viral vectors have been poorly translated into clinical success. This review addresses some critical issues that need to be considered for clinical practice application of non-viral vectors in mainstream medicine, such as efficiency, biocompatibility, long-lasting effect, route of administration, design of experimental condition or commercialization process. In addition, potential strategies for overcoming main hurdles are also addressed. Overall, this review aims to raise awareness among the scientific community and help researchers gain knowledge in the design of safe and efficient non-viral gene delivery systems for clinical applications to progress in the gene therapy field.

Keywords: non-viral vectors • gene therapy • nanotechnology • gene delivery • clinical translation

1. Introduction

The main concept of gene therapy is quite simple and overall relies on the delivery of exogenous genetic material into target cells to modulate the expression of an altered genome. Basically, three different approaches can be identified (Figure 1). In the case of *gene addition* therapy, a "healthy" copy of the gene is administered to recover the functionality of the affected cells. This strategy can be suitable to face diseases caused by mutations with loss of function [1]. For instance, the autosomal recessive cystic fibrosis disease caused by a deletion of the phenylalanine at the position 508 of the CFTR (cystic fibrosis transmembrane conductance regulator) protein [2]. However, in the case of a mutation that overexpresses genes, the aim is to administer an inhibitory sequence to knock out the expression of the mutated gene [3]. This strategy is referred to as *gene inhibition* therapy and can be applied, for instance, to face autosomal dominant retinitis pigmentosa secondary to specific mutations in the pre-mRNA splicing-factor gene PRPF31 [4]. The third approach, named as *genome editing*, incorporates specific genome editing tools to repair mutations in the genome with gain or loss of function [5]. This strategy has been successfully used in combination with iPSC technologies to combat human β-thalassemia disease in mice [6].

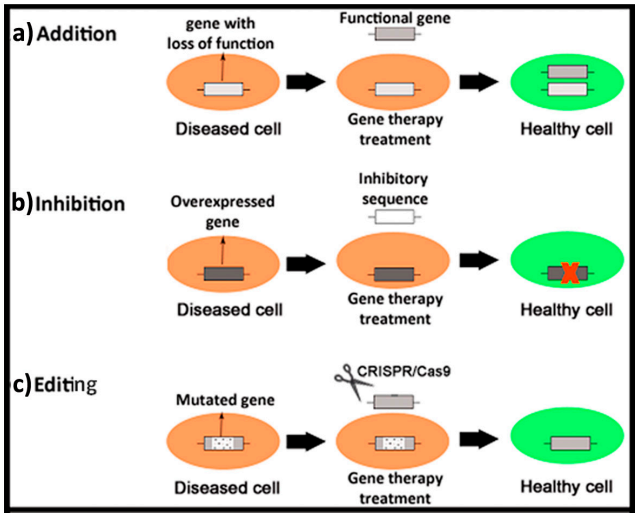

Figure 1. Brief schematic representation of three different genetic material-based approaches to face human diseases. (**a**) *Gene addition* therapy. (**b**) *Gene inhibition* therapy. (**c**) *Genome editing*.

The main characteristics and composition of the genetic cargo strongly depend on the gene therapy approach used (Table 1). For instance, in the case of the *gene addition* approach, which is classically used to face genetic disorders that follow an autosomal recessive inheritance pattern, the most common polynucleotide used is referred to as plasmid (pDNA). Such a plasmid is a circular and double-stranded DNA construct, typically between 1.5 and 20 kbs, that drives the transient transgene expression in the nucleus of target cells, encoding the protein of interest [7]. Typically, a transfection mediated by conventional pDNAs is moderated and only active during 1-2 months. However, smaller versions of a conventional pDNA, known as minicircle DNA (mcDNA, 2-6 kbs) or micro-intronic plasmid (2-4 kbs), can improve transgene expression by 10- to 100-fold and prolong the effect for some years [8,9]. Another commonly used polynucleotide in the *gene addition* approach is the single strand messenger RNA (mRNA). Nowadays, this strategy holds great promise, especially in the vaccine research area, since two vaccines produced by Moderna and Pfizer/BioNTech companies have been recently approved by the European Medicines Agency (EMA) to fight against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and the resulting coronavirus disease (Covid-19). As in the case of pDNA, the effect mediated by mRNAs is also transient and the stability in plasma is even lower, around 1 h. The less tight conformation of RNA, which allows an easier access of the degradative enzymes, and also the presence of hydroxyl groups in the main structure, which enhances hydrolyzation of RNAs [10,11], significantly contribute to decrease the stability. However, the main advantages of RNA-based gene therapy compared to pDNA include a safer profile, since it decreases the risk of mutagenesis and immunogenicity and a more efficient modulation of target gene expression because the place of action of this genetic cargo is in the cytoplasm [12]. Therefore, there is no need to get access into the nucleus of cells, which is classically considered as one of the main bottlenecks of plasmid-based expression systems [13].

Table 1. Scheme of essential characteristics and properties of main polynucleotides used in oligo-based gene therapeutics and gene therapy.

Mechanism of action	Polynucleotide	Chemical structure	Lasting Effect	Place of action
Gene addition	Plasmid (dsDNA)		Transient	Nucleus
Gene addition	mRNA (ssRNA)		Transient	Cytoplasm
Gene inhibition	miRNA (ssRNA)		Transient	Cytoplasm
Gene inhibition	siRNA (dsRNA)		Transient	Cytoplasm
Gene inhibition	AON (RNA/ssDNA)		Transient	Cytoplasm/ Nucleus
Genome editing	CRISPR/Cas9 plasmid (dsDNA)		Permanent	Nucleus
Genome editing	CRISPR/Cas9 mRNA (ssRNA)		Permanent	Cytoplasm/ Nucleus
Genome editing	CRISPR/Cas9 ribonucleoprotein		Permanent	Nucleus

In contrast to *gene addition* strategy, the *inhibition* approach can be used when the main goal is to silence the expression of an altered gene that has a gain of function mutation. This scenario is common in genetic disorders that follow an autosomal dominant inheritance pattern [14]. In this case, the inhibitory sequence can also have a single strand RNA-based structure, such as the microRNA (miRNA), or a double strand RNA structure, such as the small interfering RNA (siRNA). Typically, these structures inhibit the translation of mRNA in the RNA-induced silencing complex (RISC) of the cytoplasm in a transitory way to avoid the expression of the target gene [15]. Interestingly, other synthetic and smaller single strand RNA/DNA structures, known as antisense oligonucleotides (AON), can also inhibit mRNA translation by a different mechanism of action. In this case, the oligonucleotide sequence interferes with pre- and mRNA in the nucleus or cytoplasm through a complementary hybridization mechanism that enhances specificity but with lower knockdown efficiency [16].

Apart from *gene addition* and *gene inhibition* strategies, another approach consists of the permanent correction of the mutated gene with the use of specific genome editing tools such as those developed by the game changer CRISPR/Cas9 technology [17]. In this case, once the mutated gene is identified, different RNA guides (gRNA), which recognize 20 nucleotides of the mutated allele, can be designed and synthetized to be delivered alongside the Cas9 protein, which will cut the genome 3 nucleotides to the left side in the 5'-3' direction of the protospacer adjacent motif (PAM) region. In this scenario, a single strand DNA sequence with the corrected mutation, typically 100 nucleotides long, can be supplied as a donor template to be incorporated in the cell genome by the homologous recombination mechanism [18]. Such CRISPR/Cas9 editing tools can be delivered in different genetic constructors such as pDNA, mRNA or ribonucleoprotein (RNP) complex, acting in different cell places [19].

Unfortunately, all the previously described genetic cargoes need to overcome both extracellular and intracellular barriers to reach the place of action. In the case of *in vitro* conditions, which is the simplest scenario, only intracellular barriers need to be considered. However, in the case of *in vivo*

experimentation, the delivery process to the place of action can also be affected by additional extracellular barriers, which strongly depends on the route of administration and the organ to be treated [20]. To overcome such biological barriers, gene delivery systems are necessary, since genetic cargo by itself, in most of the cases, is not effective. Classically, gene delivery systems are divided into viral and non-viral vectors. Viral vectors are recognized by their high gene delivery efficiency. In fact, viruses have evolved along many years to infect efficiently different kinds of cells with their genetic cargo, and currently, such viruses can be easily modified in the laboratory to deliver the genetic cargo of interest into target cells, reducing their pathogenic effect [21]. As a result, most of the clinical trials, and the great majority of gene therapy drugs approved for human use by regulatory agencies, are based on viral vectors. Some examples of marketed gene therapy products that use viral vectors include Luxturna, Zolgensma, Oncorine and Imlygic, to name just a few [22,23]. However, relevant concern still remains in the research community related, over all, to their potential immunogenicity and oncogenic capacity [24,25]. In addition, previously mentioned approved drugs are highly expensive, mainly due to the intrinsic characteristics of biologic drugs [26]. Therefore, interest in non-viral gene delivery systems has recently gained momentum. A brief schematic representation of both physical and chemical methods for non-viral gene delivery is summarized in Figure 2.

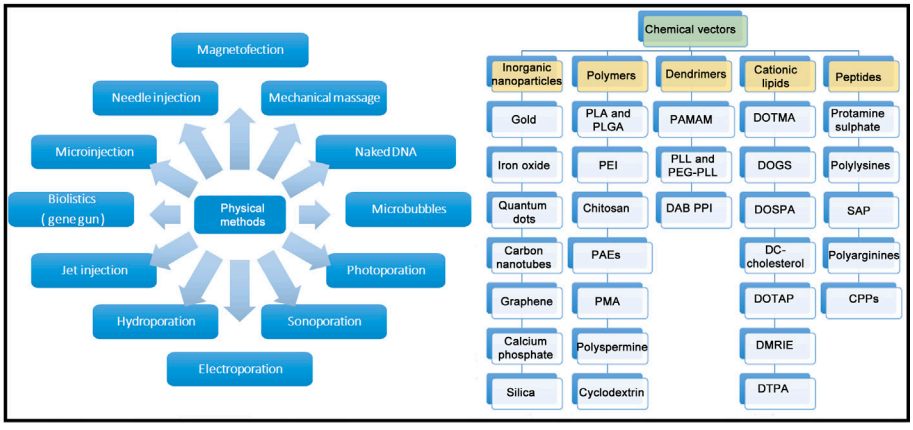

Figure 2. Overview of main physical and chemical methods of non-viral vectors.

Compared to their viral counterparts, non-viral vectors show some appealing properties such as lower immunogenicity, safer profile and higher genetic cargo packing capacity. In addition, non-viral vectors are cheaper and easier to manufacture and scale up [27]. Due to these obvious advantages, the gene delivery mediated by non-viral vectors is nowadays considered the cornerstone of modern gene therapy, especially for CRISPR/Cas9 delivery, where non-viral vectors predominate over viral vectors at the preclinical level [28]. In any case, although with few exceptions, this strategy has been poorly translated into clinical success. However, some promising clinical trials based on gene therapy treatments, summarized in Figure 3, are ongoing.

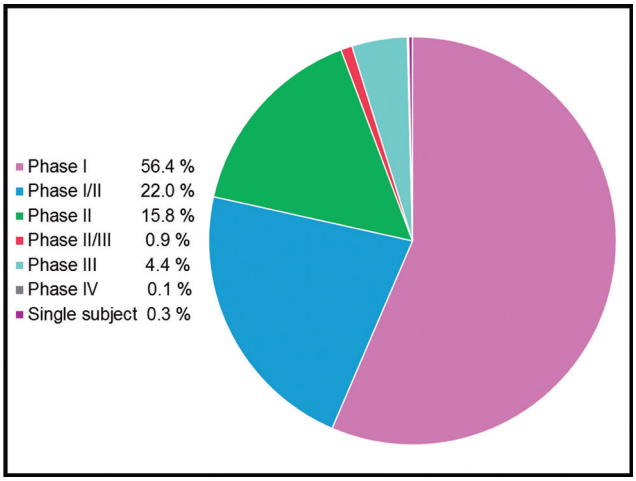

Figure 3. Clinical phases of 3180 ongoing trials based on gene therapy (adapted from http://www.genetherapynet.com/clinical-trials.html; accessed 1 June 2021; gene therapy clinical trial database).

In this review, some critical issues in the way to clinic application of non-viral vectors (Figure 4) and potential strategies to overcome such hurdles have been addressed. More specifically, special attention has been paid to the gene delivery efficiency and biocompatibility of non-viral vectors. Additionally, the duration of the transgene expression, along with the route of administration, the design of experimental conditions and some concerns related to the commercialization process, has also been discussed.

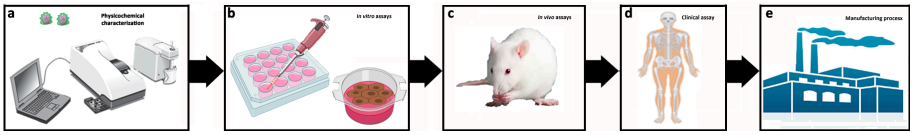

Figure 4. Journey of non-viral vectors from lab to bench. Initially potential non-viral vectors are physicochemically characterized (**a**) before performing both *in vitro* (**b**) an *in vivo* (**c**) biological studies. Most promising formulations are further evaluated in clinical trials (**d**). In case of success, the manufacturing process for commercialization starts (**e**).

2. Gene delivery efficiency

A critical issue that hampers the regular application of non-viral vectors into regular medical practice is their low gene delivery efficiency [29]. In this sense, viral systems clearly surpass non-viral counterparts, probably due to their continuous evolution along millions of years, which has allowed them to get better access into the genome of the target cell, overcoming both extracellular and intracellular barriers [30]. Nowadays, most of the commercially available gene therapy-oriented drugs use recombinant viruses modified in the laboratory, such as retroviruses, lentiviruses, adenoviruses or adeno-associated viruses to shuttle their genetic cargo. However, their overall safety concerns related to the biological origin and the low genetic cargo packing capacity, along with the difficulties associated to scaling up their production and high cost of development, have contributed to exploring different gene delivery approaches based on the design of novel non-viral vectors [31]. Research on this area has quickly captured the attention of the scientific community, and, currently, this strategy represents a safer and more affordable alternative, although the gene delivery efficiency of these systems needs to be improved to reach a regular clinical practice.

Gene delivery efficiency of non-viral vectors is typically evaluated at a preclinical level once such systems have shown appropriate physicochemical and biophysical properties, for instance, in terms of particle size, superficial charge, polydispersity, morphology and capacity to bind and protect genetic material to release it without suffering any degradation, since all these parameters can affect the transfection process [32]. Initially, and as a proof of concept, gene delivery capacity is evaluated in culture cells and, for that purpose, the expression of different reporter plasmids that encode fluorescent proteins

[33] or enzymes such as luciferase [34] or galactosidase [35], is employed to be quantitatively evaluated by different techniques. In this sense, it is worth mentioning that each reporter plasmid and corresponding assays have different sensitivity and their own metrics [36]. For example, the reporter plasmid that encodes green fluorescent protein is a good descriptor of the transfection efficiency at a single cell's level, since results are typically expressed as the percentage of live cells that show green signal by flow cytometry [37]. However, luciferase expression provides information related to the plasmid expression in a whole population of cells, since the luminescence is normalized by the quantity of proteins in cell lysates.

When a therapeutic genetic material, instead of the reporter one, is used in *in vitro* conditions, it is also important to consider, from a practical point of view, the transfection efficiency value required to reach a therapeutic effect, which highly depends on the particular application and disease. For instance, in the case of cystic fibrosis, an autosomal recessive disorder caused by the dysfunction of the CFTR gene, 28 % of living human cystic fibrosis airway epithelial cells (CuFi-1) were transfected with the pEGFP reporter plasmid, using a lipid-based non-viral vector, named as N**3** [38]. Such formulation reported a 5-fold increase of CFTR protein expression in transfected versus non-transfected cells with the pGM169 therapeutic plasmid, which led to 1.5-fold increment of the chloride channel functionality, exceeding the value required to get a therapeutic benefit (Figure 5).

Such *in vitro* studies are typically used as a screening methodology to select the non-viral vector candidates that show better performance before conducting *in vivo* studies, in accordance with the principle of the three Rs (replacement, reduction and refinement of animal labs). This sequential approach is aimed to reduce the number of animals used in *in vivo* experiments. However, it should also be borne in mind that a direct correlation between *in vitro* and *in vivo* results in terms of gene delivery efficiency does not always exist, essentially because experimental conditions in each scenario are quite different [37]. As a consequence, some readjustments in terms of the composition of the formulation, preparation methods, doses or volumes to be administered need to be performed to succeed in *in vivo* experiments [39].

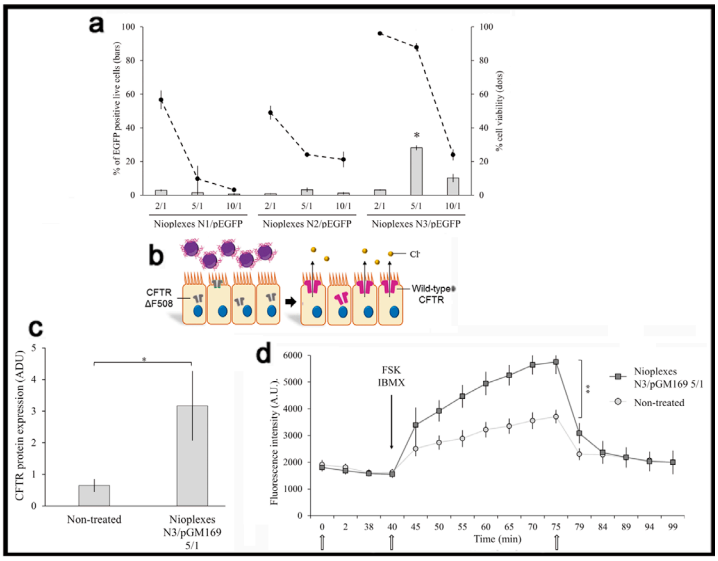

Figure 5. Transfection efficiency and therapeutic effect. (**a**) Percentage of live transfected CuFi-1 cells with the pEGFP reporter plasmid. (**b**) General scheme of transfection process with pGM169 therapeutic plasmid. (**c**) CFTR/β-actin protein expression determined by Western blot. (**d**) CFTR chloride channel activity determined by SPQ analysis. Reproduced with permission of [38].

In any case, the transfection efficiency of non-viral vectors is highly related to their cytotoxic effect, which is also a highly cell-dependent process [40]. Therefore, a suitable balance between the transfection efficiency value required to obtain a therapeutic effect and the cytotoxic effect needs to be acquired for each clinical application to enhance translation of non-viral vectors to the regular medicine practice. Such a toxic effect of non-viral vectors depends on many physicochemical parameters that affect the gene delivery process such as particle size, morphology and zeta potential of complexes [41]. In addition, the elaboration method, along with the intrinsic properties of the materials used to obtain the different kinds of non-viral vectors, can impact on the final cytotoxic effect, depending, for instance, on the degradation rate or the persistence along the time in organs and tissues [42]. It should also be kept in mind that the persistence and accumulation of metabolites that come from the degradation of different compounds present in non-viral vector formulations can also induce an inflammatory process and, therefore, cause toxicity. Nevertheless, the cytotoxic effect does not only depend on the non-viral vector's compounds. The genetic material that is aimed to be delivered can also

be toxic, considering, for instance, the bacterial origin of many plasmids that can enhance the induction of undesired immune responses and the secretion of proinflammatory cytokines [43]. Interestingly, small plasmidic cassettes as mcDNA have been recently developed to mitigate some disadvantages associated with the use of conventional plasmids [31]. Such mcDNAs contain a minimal expression cassette, where the bacterial backbone DNA has been eliminated, which reduces the unwanted immunogenic responses and enhances the transfection efficiency due to the reduced size of this CpG-free genetic material [8,44]. The cytotoxic effect of the non-viral vectors can be qualitatively evaluated by different techniques based on microscopy analyses [45]. However, normally, quantitative analysis of toxicity is assessed by means of a broad spectrum of cell viability/cytotoxicity colorimetric available kits, such as, for instance, CCK-8, MTT assay and Alamar Blue™, or by mean of flow cytometer analysis [46-48]. In this sense, it is worth mentioning that many chemical compounds that are present in non-viral vector formulations can interfere with the previously described colorimetric assays, providing confusing results. In the case of flow cytometer analysis, fluorescent dyes such as ethidium homodimer-1, propidium iodide or 7-Amino-actinomycin D (7-AAD) are normally used to stain and analyze dead cells, which should be excluded from the final transfection efficiency results [49].

To reduce the cytotoxic effect of non-viral vector formulations, many natural compounds, such as cholesterol [50], lycopene [39] or squalene [33], can be incorporated into lipid vesicles as "helper" components. In addition, some non-ionic surfactants, such as polysorbate 80, can also reduce the toxic effect of cationic lipids [51]. Although the use of cationic materials, such as the mentioned cationic lipids or polycationic polymers, facilitates the complexation with the negatively charged nucleic acids for gene delivery as well as cellular internalization, an excess of positive charge can have detrimental effects on cell viability. Hence, other strategies to avoid cationic vectors, and thus cytotoxicity, have been developed for nucleic acid delivery [52]. In the case of polymeric-based non-viral vector formulations, stimuli responsive polymers, also knowns as intelligent polymers, represent an appealing approach to enhance not only biocompatibility of the formulation but also the specificity and

the duration of the gene expression [53]. These particular polymers can modify their biological performance in response to small environmental changes of physicochemical parameters such as pH value, temperature or ionic strength to name just some of the most relevant ones [54].

3. Duration of gene expression

Another main reason that limits the clinical application of non-viral vectors into regular medical practice is the loss of transgene expression over time in clinical trials. A transgene expression can decrease with time because of many causes such as the inactivation of the genetic material by nucleases, the loss of activity by recombination processes, the ineffective distribution into intracellular vesicles, or even the recognition and subsequent silencing of foreign DNA by the host immune system [55]. In this sense, while the retroviral and lentiviral vectors do integrate into the host cell genome, providing a long-lasting effect [56,57], the main reason for adeno-associated viruses (AAV) vectors to provide sustained transgene expression is not integration. AAV vectors barely integrate into the genome unlike wild-type AAV. In contrast, an AAV vector genome persists in the host cell nucleus as episomal concatemers that are highly resistant to nucleases. The fact that AAV genomes are diluted over time as the cell undergoes repeated rounds of replication, with the rate of transgene loss dependent on the turnover rate of the transduced cell [58], is a proof of the occurrence. For instance, commercially available Luxturna drug delivers by means of an AAV type 2 a healthy copy of the RPE65 gene into the subretinal space of patients affected by retinitis pigmentosa and Leber congenital amaurosis. Despite the high cost of the treatment, around $ 850,000, only one injection is required to complete the treatment, due to the long-lasting effect obtained, in slow dividing cells of the retina. This fact is particularly relevant in the case of invasive routes of administration, such as intravitreal or subretinal administrations, into the cerebral cortex after craniotomy. In this scenario, repeated administrations could increase the after-care cost due to additional hospital visits [59], and, in many cases, jeopardize the acceptance of these aggressive gene delivering routes because of the cumbersome approach and related side effects.

Most of the strategies that have been developed by the research community to enhance the lasting effect of transgene expression are mainly focused on modifications of the genetic material to be delivered rather than on modification on the components of the non-viral vector formulation. For example, in the case of the *gene addition* approach, the previously described mcDNA technology not only reduces the cytotoxic effect but also represents a promising approach to prolong the therapeutic effect when this genetic cargo is combined with non-viral vectors [8]. The lack of bacterial backbone sequences, along with the low content of unmethylated CpG dinucleotides, reduces the activation of nuclear transgene silencing mechanisms, which finally results in a sustained transgene expression effect (Figure 6, [60]).

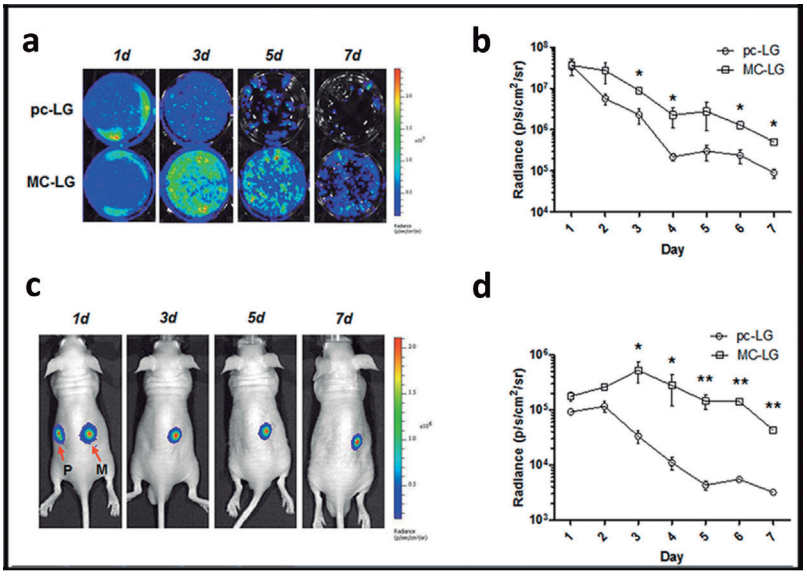

Figure 6. Minicircle approach to transfect mesenchymal stem cells (MSCs). (**a**) Bioluminescence images of MSCs transfected with pcDNA3.1-fLuc-2A-EGFP (pc-LG) or McCMV-fLuc- 2A-EGFP (MC-LG). (**b**) Quantification of bioluminescence signal emitted by the MSCs transfected with pc-LG (circle) or MC-LG (square). (**c**) Bioluminescence images of nude mice subcutaneously injected with MSCs transfected with pc-LG (P) and MC-LG (M) into left and right back. (**d**) Quantification of bioluminescence emitted from the injected area of mice. Reproduced with permission of [60].

In addition, to prolong the effect, the transgenes of interest can be incorporated into the host genome by means of viral integrase or site-specific

recombinase enzymes, or by the addition of transposable elements, such as transposons or "jumping" genes [55]. In any case, the translation into the clinic of these promising approaches to enhance the transgene expression effect is clearly conditioned by relevant safety issues such as the possible induction of insertional mutagenesis in the host cells with permanent consequences. Another approach that can be used to prolong the transgene expression effect consists of the addition of viral DNA sequences that allow plasmid replication outside the chromosomes. However, again, safety concerns can arise due to the viral DNA nature that is associated with immune responses and the risk of oncogenesis. As a safer alternative to the aforementioned viral sequences, mammalian scaffold/matrix attachment regions (S/MARs) can also be incorporated into pDNA to enhance the episomal replication of plasmids [61]. Episome sequences autonomously replicate plasmids that do not need to be integrated into the host genome to express the transgene, which minimizes the mutagenesis risk [62]. Episomal replicating plasmids are especially interesting to face tumor cells by gene therapy, where a vertical transfer of the therapeutic plasmids is particularly relevant in fast-dividing malignant cells [63]. Gene expression can also be prolonged by specific inhibition of gene silencing mechanisms of cells that hampers transgene expression [64]. Interestingly, artificial transcriptional activators can also be incorporated into therapeutic plasmids with appropriate promoters to modulate gene expression. In the case of the *gene inhibition* approach used to silence the expression of an altered gene with inhibitory sequences such as miRNA, siRNA or AON delivered by non-viral vectors, the effect is also transient, which requires repeated administrations [32]. In this case, a permanent correction of the altered gene can be achieved with the use of CRISPR/Cas9 technology. Such genome editing tools can be delivered by non-viral vectors complexed to different genetic constructs such as pDNA, mRNA or RNP complexes [19]. In any case, when time is a crucial factor, differences in anatomy, physiology, development and biological phenomena between animal labs and human beings should be also considered to extrapolate experimental results. For instance, it has been estimated that one lived day for rats is comparable to 30 lived days for humans [65].

4. Administration route

The administration route of non-viral vectors affects not only the previously described gene delivery efficiency and duration of gene expression but also the design of the formulations. To be active at the place of action, non-viral vectors need to overcome different biological barriers that strongly depend on both the administration route and the target organ (Figure 7).

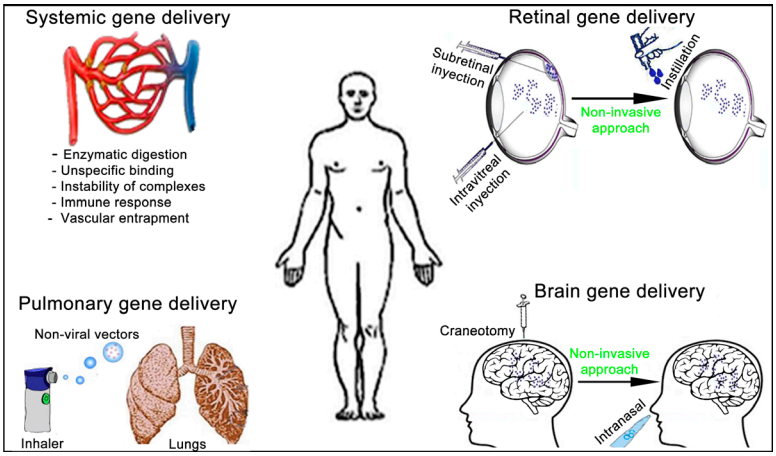

Figure 7. Schematic representation of the main administration routes to face systemic and tissue-specific human diseases by non-viral vectors gene therapy approach.

The main and most studied administration route of non-viral vectors to face disseminated cancer or infectious diseases is the intravenous route [66]. The idea is to transport the genetic material to as many cancerous and infected cells as possible. However, efficiency of this interesting and ambitious approach is strongly limited by the enzymatic degradation that a genetic material can suffer from the point of entry [23]. In addition, positively charged non-viral vector complexes can interact by non-specific electrostatic attractions with negatively charged biological compounds such as serum proteins and blood cells, which limit their final performance [67]. Another concern to be aware of is the possible instability of such complexes in the extracellular biological medium at physiological conditions, where pH value, temperature and ionic stench, among many other factors, can result in the formation of aggregates along the exposure time [18]. Furthermore, the systemic administration at high volumes of non-viral vector complexes can trigger the host

immune responses against some of their components, resulting in an inflammatory response, which can be more pronounced if administrations are repeated [68]. Finally, it is worth mentioning that endothelial cells of the vascular system constitute a relevant and effective biological barrier. This barrier can limit the size and number of non-viral vector complexes that can pass through it, therefore, reducing the transfection efficiency in the targeted tissues after a systemic administration [69].

To overcome the previously described systemic barriers, non-viral vectors can be structurally modified. For instance, the addition of positively charged protamine into non-viral vectors protects the genetic material from enzymatic digestion and, therefore, increases the transfection efficiency. This approach has also been successfully used with other lipid formulations such as solid lipid nanoparticles [70] or liposomes [71]. Other commonly used strategy to increase the stability of non-viral vectors complexes in biological fluids and reduce the immune response consists of the addition of poly(ethylene glycol) (PEG) chains or other hydrophilic polymers, such as poly(N-vinyl-2-pyrrolidone) (PVP), into the outer surface of complexes [72]. The neutral PEG chains produce a steric barrier against enzymatic degradation and avoid aggregation of non-viral vector complexes in systemic circulation [73]. In addition, PEG chains reduce the quantity of cationic lipids required to deliver a genetic material, which enhances biocompatibility of lipid formulations [74].

Another main route of administration to face devastating diseases that affect the lungs, such as cancer, cystic fibrosis or asthma, is the pulmonary route. In this case, intratracheal intubation can be used for gene delivery of pulmonary disease-oriented treatments. However, the non-invasive nature of the inhalation approach is preferable for clinical applications. Another advantage of this administration route includes the use of small doses, which in turn, reduces side effects by increasing drug concentration at the area of interest. In addition, inhalation can also be used for gene delivery to treat systemic diseases due to the quick absorption on the alveolar region of lungs [75]. In any case, the effectiveness of the inhalation process depends mainly on the amount of the genetic material that will finally reach the targeted region and the deposition pattern, which is deeply conditioned by the composition of

the material to be delivered and by the device used for inhalation [76]. In this sense, relevant issues such as the characteristics of the drug delivered, the most adequate pattern for its delivery, the design of the device and its effectiveness need to be considered in detail to enhance the effectiveness of this appealing administration route. In the specific case of genetic material-based formulations, the main difficulty to reach the nucleus of target cells is related to the susceptibility of such molecules to be degraded by the hydrodynamic shear forces generated during aerosolization process, which result in a clear decrease of the efficiency compared to *in vitro* conditions [77]. Some interesting approaches to protect DNA during the aerosolization process consist of the incorporation of compounds such as bovine serum albumin (BSA), which stabilizes the supercoiled DNA [78], or the design of efficient aerosol delivery systems for genetic material, such as nebulizers, dry powder inhalers (DPIs), pressurized metered dose inhalers (pMDIs) or mechanical metered dose inhalers (mMDIs) [79].

Another relevant issue that hampers the delivery of genetic material to the lung of affected patients is the presence of specific biological extracellular barriers at this level such as the alveolar macrophages, the mucus and alveolar fluids, which make the diffusion of genetic material difficult due to mechanical obstructions and non-specific interactions [80]. In this case, excipients such as mucolytic agents, which improve the mobility of genetic material, or cell penetrating peptides (CPP), which enhance the cell internalization process, can be incorporated into non-viral vector formulations to enhance gene delivery efficiency into the lung [81].

However, there are other relevant organs such as the brain and the eye that, due to the essential functions that they perform, have developed additional extracellular barriers over many years of evolution to protect them against foreign agents such as the blood brain barrier (BBB) and the blood retinal barrier (BRB), respectively, in each organ [82,83]. Consequently, effective gene therapy approaches to treat both inherited and acquired diseases of the brain and the eye rely, currently, on the in situ administration of genetic material by invasive routes or in the systemic administration of non-viral vectors decorated with appropriate ligands to overcome such additional extracellular barriers

[23]. In the case of the brain, most commonly used strategies to cross the BBB include the incorporation of receptor-mediated uptake of ligands such as insulin, transferrin or lactoferrin, since endothelial cells of the BBB express high quantity of receptors for such molecules [84]. Other interesting approaches to cross BBB consist of the addition of specific monoclonal antibodies that are recognized by receptors of the BBB into the non-viral vector formulations, or the transient mechanical disruption of the BBB permeability [84]. In the case of the eye, some studies have demonstrated that submicron-sized formulations based on liposomes or chitosans, with appropriate ligands such as annexin A5 or transferrin, can target the retina (which can be damaged in many inherited disease that affect eyesight), after non-invasive topical application into the conjunctival/scleral tissues [85,86]. Interestingly, non-invasive approaches have also been described to circumvent BBB. In this case, non-viral vectors based on 10 kDa polyethyleneglycol (PEG)-substituted lysine30-mers (CK30PEG10k) were able to deliver EGFP plasmid into the brain after intranasal instillation [87]. All these findings raise reasonable hope to treat human diseases that affect the eye and the brain by a safe and effective gene therapy approach based on non-viral vectors administered by non-invasive routes in the future.

5. Design of experimental conditions

To become clinically relevant, non-viral vectors must first meet some critical physicochemical and biophysical parameters that affect the transfection efficiency, such as size, morphology, superficial charge, thermal stability and rheological properties, to name just the most relevant ones [88,89]. Normally, this is the first set of experiments that are performed when designing non-viral vector formulations for gene delivery purposes. Even in this preliminary step, the design of experimental conditions to be evaluated needs to be considered in detail [48]. For instance, preferably, the nanometric particle size of non-viral vectors should be reported as hydrodynamic diameter by cumulative analysis [90] rather than by the area of the predominant peak measured by dynamic light scattering (DLS), normally in a Zetasizer instrument. However, to use this approximation correctly, and for comparative purposes, particle

size distribution must follow a monomodal distribution, with a polydispersity index below 0.3. In addition, to be more sensitive to small numbers of aggregates or dust, such a particle size must preferably be reported as an intensity correlated function of the scattered light rather than by volume or number of particles distribution [91]. The particle size of formulations, along with the presence of possible aggregates, can also be analyzed with appropriate staining reagents such as uranyl acetate by different microscopic techniques, including transmission electron microscopy (TEM), cryo-TEM, scan electronic microscopy (SEM) or atomic force microscopy (AFM) [92]. However, with these approximations, the number of particles analyzed is more limited and not always provides a pretty correlation with the DLS technique, mainly due to the different sample manipulation processes between both approaches. Therefore, such microscopy-based approaches are normally used to provide evidence of the nanoparticles and evaluate their morphology, since this is another relevant parameter that affects the gene delivery process [93].

In addition to particle size, the superficial charge of non-viral vectors is another commonly studied physicochemical parameter that affects the electrostatic interactions with the genetic material [45]. Superficial charge value can also affect the interaction of complexes with cell membranes and the stability of colloidal dispersions. In this sense, high zeta potential values (either positive or negative) prevent aggregation among particles by electrostatic repulsions [90]. Such a parameter is normally reported as a function of the zeta potential value, which can also be measured in a Zetasizer instrument by laser doppler velocimetry (LDV). In this case, the approximation model used to calculate zeta potential value is another relevant parameter to be considered, with the Smoluchowski equation being the most commonly one used [94].

Other parameters that directly affect both particle size and zeta potential values of non-viral vectors and make the profitably replication of these studies difficult for the sake of comparison are the pH value and the ionic strength of the measurement medium [95]. Moreover, the electrostatic interaction between non-viral vectors and the genetic material to obtain the corresponding complexes can be measured at a molecular level through the heat released when such binding occurs by isothermal titration calorimetry [96] or by

agarose gel electrophoresis assays [48]. When this last technique is used, the protection capacity of genetic material by non-viral vectors from enzymatic degradation can also be evaluated. In any case, again, standardized protocols should be implemented to make such evaluations as unbiased as possible, since many parameters, such as the exposition time to the enzyme, the temperature, the composition of the buffer or the potential applied to run the electrophoresis assay, can impact the final results obtained.

Once non-viral vectors show appropriate physicochemical parameters for gene delivery purposes, normally, and before performing any *in vivo* biological study, the gene delivery capacity is preliminarily evaluated in *in vitro* conditions. However, such *in vitro* studies that usually also consider the biocompatibility of the formulations, along with the cellular uptake and posterior intracellular trafficking analysis, are typically validated in experimental conditions that not always represent the *in vivo* environment [97]. For instance, *in vitro* results are normally obtained in homogenous cell populations that are exposed to non-viral vectors for long incubation times. Therefore, positive outcomes obtained in such simplified tests do not always guarantee success in further animal model validation assays [37]. A more realistic scenario, which better resembles an *in vivo* environment, is obtained when immortalized cell lines are substituted by primary culture cells [98]. In this case, due to the intrinsic characteristics of primary culture cells, transfection efficiency values normally decrease when compared to immortalized cell lines. Consequently, studies performed in primarily culture cells or in other difficult-to-transfect cell lines such as neurons are normally used to report the kind of cells that have been transfected by immunohistochemistry techniques rather than the transfection efficiency in quantitative terms [23,44].

Interestingly, more sophisticated *in vitro* scenarios, that better predict the *in vivo* performance of non-viral vectors, can be obtained using, for instance, microfluidic technology that resembles extracellular barriers of immune-privileged organs such as the eye or brain [99] or by application of the recent game changer 3D-bioprinting technology [100]. In any case, and as occurred with the physicochemical studies, the experimental conditions and protocols of biological assays performed in *in vitro* conditions should also be standardized

for comparative purposes, since there are many variables that can influence the final results obtained [97] (Figure 8).

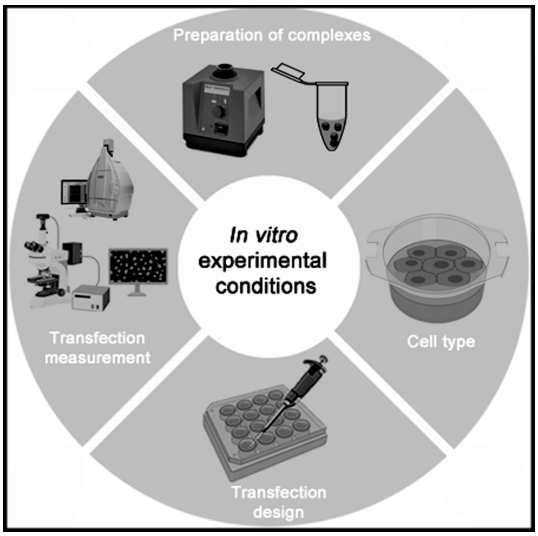

Figure 8. Brief schematic representation of relevant parameters that affect the *in vitro* performance efficiency of non-viral vectors.

For instance, the preparation of complexes plays a pivotal role in the final biological performance and depends on many factors such as the volumes or quantities used, the temperature, the order of components addition or the mixing technique, to name just the most relevant ones. In the case of non-viral vectors based on polymers, a stock aqueous solution is added to an appropriate volume of a solution, which contains the genetic material under vigorous vortex mixing for a short period of time at room temperature. However, normally, in the case of lipid formulations, the electrostatic interaction between the cationic lipid and the genetic material occurs under gentle mixture with an appropriate pipette to avoid destabilization of the lipid formulation [33]. In some cases, especially when large volumes of aqueous solutions are required to obtain the complexes, a hypertonic medium, based on the incorporation of appropriate isotonic agents such as mannitol, is added to get a final isotonic medium that will avoid the lysis of cells [37].

Moreover, transfection efficiency is a highly cell-dependent process, which is conditioned by the particular cellular uptake mechanism and the posterior

intracellular trafficking pathway used before reaching the nucleus [45]. In this sense, the low division rate of quiescent cells, such as neurons or primary cells, hampers the entry of exogenous genetic material into the nucleus, challenging the transfection. On the contrary, phagocytic cells can be transfected more easily due to their native biological performance. Therefore, the concept of a unique universal non-viral vector optimized for all clinical applications is currently abandoned. In fact, the most accepted concept relies on the idea that the success of non-viral vectors into clinical practice depends on the development of gene delivery platforms based on multifunctional vectors specifically designed and tailored for each particular purpose [21]. In any case, and as a preliminary proof of concept, the first approach to evaluate transfection efficiency of non-viral vectors can be performed, for instance, in human embryonic kidney (HEK-293) cells, which is a common cell model easy to be transfected due to the specific biological characteristics of these particular cell line [37].

In close relation to the cell type, the transfection experiment conditions can also influence the results obtained. In this sense, some relevant parameters also need to be considered. For instance, the confluence density of the cells, the presence or absence of antibiotics and serum in the culture and transfection medium, or the volume and the quantity of genetic material that is going to be evaluated, which also depend on the area of the cell culture wells, should be standardized for comparative purposes. Finally, and as previously mentioned, transfection efficiency can be measured by different approaches, although normally reporter genetic material is used as a proof of concept before moving to therapeutic genetic material. In any case, slight overall readjustments need to be done in the non-viral vector/genetic material-used ratios due to the impact that both size and composition of the genetic material have on the transfection efficiency process [88].

Once positive outcomes have been obtained in *in vitro* test models, the next step is the evaluation of the performance into first small and later big animal models. Moreover, previously, ethical issues related to the use of animals at preclinical level need to be addressed. Once again, during this step, the complexes administered into *in vivo* studies might suffer slight modifications

related to the volumes, doses or composition of the dilution medium, that depend overall on the administration route to be used [98]. In any case, success in animal models does not necessary guarantee clinical success into humans, since animal testing methods cannot fully address translation to human physiology [97]. In such clinical trials, the delicate balance between the potential benefits of the treatment and the possible associated risks is carefully evaluated under the supervision of a Gene Therapy Advisory Committee that will also deal with ethical issues [101].

6. Commercialization process

Although from a scientific point of view, it is possible to deliver genetic material to specific cells by means of non-viral vector formulations, the regular application into medical practice of this gene therapy approach is highly conditioned by other factors that affect the commercialization process (Figure 9).

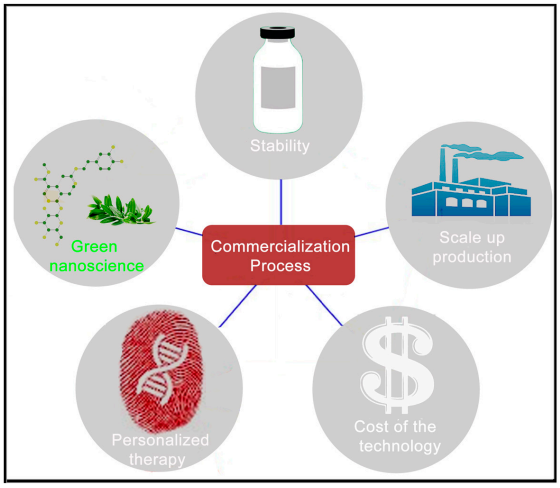

Figure 9. Schematic representation of relevant issues to consider across the commercialization way of non-viral vectors in gene therapy medical field.

For instance, the electrostatic interaction between non-viral vector formulations and genetic material cargo typically occurs in aqueous medium. However, despite the simplicity of this process, batch-to-batch variability can occur due to slight variations during the mixture procedure, which is not

acceptable in a clinical setting [97]. In addition, systems based on aqueous suspensions are highly unstable to allow regular shipping and storage for long periods. In this sense, the recent RNA-based lipid vaccines, developed by both BioNTech and Moderna to face SARS-CoV-2 virus that causes COVID-19 infection represent a clear example of this issue. Interestingly, the development of dry powder formulations, where aqueous medium is removed, represents a promising approach to circumvent such instability issues. In fact, currently, many companies are working on the design and development of stable dry powder RNA-based lipid vaccines against SARS-CoV-2 virus. Such solid forms can be obtained by freeze drying (lyophilization), spray drying or spray congealing techniques [102]. In any case, this technological approach is not an easy matter, since both the components of the non-viral vector formulation and the genetic material can be degraded during the process. In the case of the lyophilization technology, appropriate cryoprotectant agents need to be incorporated into the non-viral vector formulation to avoid the formation of ice crystals during the freezing process. In addition, relevant parameters of the own technology, such as temperature, vacuum pressure and primary and secondary dry periods, need to be optimized to get a homogenous dry powder without residual moisture. The spray dryer technology can be used as an additional or complementary technique to lyophilization to also obtain a product in the form of a dry powder. In this case, the suspensions containing the complexes are absorbed and atomized, forming small vesicles on a stream of hot air, which causes the rapid solidification of these vesicles and their subsequent separation. As in the case of lyophilization, different amounts of excipients, such as mannitol, lactose or trehalose, can be added to improve the flow properties, avoiding collapse and agglomeration of the vesicles during the process, as well as the reconstruction of the vesicles during their rehydration. Likewise, different process parameters require to be adjusted, such as the flow rate of the air stream, the gas temperature or the atomization rate of the sample [103]. In the case of the spraycongealing approach, a colloidal dispersion of complexes with appropriate excipients is atomized into liquid nitrogen with a nozzle [104]. Both the flow rate and the atomizing pressure need to be optimized to generate frozen droplets. Such frozen droplets are quickly transferred into a freeze dryer precooled at a shelf temperature. After

the evaporation of the nitrogen, freeze drying is conducted at a vacuum level of 5 Pa to obtain a homogeneous, porous powder, without residual moisture that will be stored in glass vials for subsequent pulmonary administration by inhalation.

Another relevant step that hampers the commercialization process of gene delivery of advanced therapy medical products based on non-viral vectors is the scale-up production of these drugs, ideally under the Good Manufacturing Practices (GMP) guidelines to avoid any possible modification in their biological performance [105]. In this sense, it is worth mentioning the role that the regulatory agencies such as the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) play to track the manufacturing process. These agencies specify the requirements needed to get the desired bioequivalence of formulations and guarantee the safety and efficacy of the new drugs [106]. Normally, such products are designed and elaborated by small and medium-sized enterprises that collaborate with academic groups. Although these consortiums are highly engaged in preclinical activities, they have limited manufacturing experience at industrial levels [105]. For instance, as commented before, non-viral gene therapy products are normally elaborated in research laboratories when genetic material is mixed with a lipid or polymer aqueous solution, obtaining small volumes of complexes, around 1 mL or even less [107]. In this scenario, the standardization of mixing parameters under GMP and the progress towards clinical application is practically unattainable [108]. In this sense, pilot plants represent an appealing approach to produce small volumes of this technology-based products, to gain knowledge about this technology, and later to design full-scale production systems that can lead in commercial products.

In terms of treatment cost, and contrary to most of the conventional treatment drugs, gene therapy products focus their interest mainly on rare and specific disorders for a small population of patients, which raise relevant ethical questions [109]. In fact, the cost of many gene therapies approved to date is inversely related to the number of patients who could benefit from them, and in some cases, previously approved drugs for commercialization, such as Glybera, have been withdrawn from the market due to the low demand

of patients and high cost of treatments [110]. At present, some of the most expensive marketed drugs, such as Luxturna or Zolgensma, are gene therapy products that use viral vectors to deliver the genetic material to the place of interest. In this sense, the costs to develop non-viral gene delivery vectors is clearly marginal if compared with viral counterparts, since mass production of viral vectors requires the development of expensive scalable and robust processes that affect, for instance, the studies on cell cultures or the amplification and purification steps. [111]. However, although being more affordable from an economical point of view, to reach clinical practice, non-viral vectors require multiple rounds of engineering and many chemical modifications, including the addition of stabilizing components or bioactive targeting ligands that increase their complexity and, therefore, the price of the formulations [105].

In any case, it is worth mentioning that the old idea of a unique conventional treatment for all patients affected by the same pathology is currently out of date. In fact, next-generation sequencing techniques (NGS) have completely revolutionized the genetic diagnosis of rare diseases, which enables the development of increasingly personalized therapies based on the genetic code characteristics of each individual [112]. This methodology allows a fast and accurate analysis of the altered genes at an affordable price. Such genetic information ensures clinical diagnosis, increases prognosis reliability, enables genetic counseling and opens the door to precision treatments. This is the idea of the new medicine that allows the design of personalized therapeutic treatments to correct the initial genetic defect in each patient and restore affected cell function [113]. A clear example of such personalized medicine is the recently developed Milasen product, a gene therapy-based drug created for a single six-year-old patient diagnosed with Batten's disease, an inherited neurodegenerative disorder that leads to retinopathy, seizures and impaired mental and motor skills [114]. In this case, the genome of the patient was sequenced to identify the specific cause of this disease. Researchers found that neither of the two copies of patient's major facilitator superfamily domain containing 8 (MFSD8) gene was functional. In one of them a pathogenic mutation was found and, in the other one, an insertion of a mobile genetic element was found, which affected the processing of the mRNA. Without a

functional MFSD8 gene, the protein necessary for lysosomes to carry out recycling or processing activity of molecules in the cell cannot be produced. As a result, proteins or metabolic substances are accumulated progressively in cells compromising their functionality [114]. From the patient's genetic information, researchers designed and administered specific antisense oligonucleotides to face the patient's disease in a customizable approach. In less than a year and a half, researchers selected the most effective oligonucleotides, carried out all the tests on cells obtained from the patient and, after *ex vivo* evaluation of both efficiency and toxicity, obtained the pertinent institutional authorization to administer the drug. Obviously, the widespread extension of this approach to other human diseases by gene therapy products, based on non-viral vectors, could have a significant impact on the health parameters of the population and, as a consequence, decrease the economic burden that represents for the whole of society.

Finally, an ideal scenario to get this appealing approach would also contemplate the elaboration of personalized non-viral vectors for gene therapy under the umbrella of the Green Chemistry concept (Figure 10).

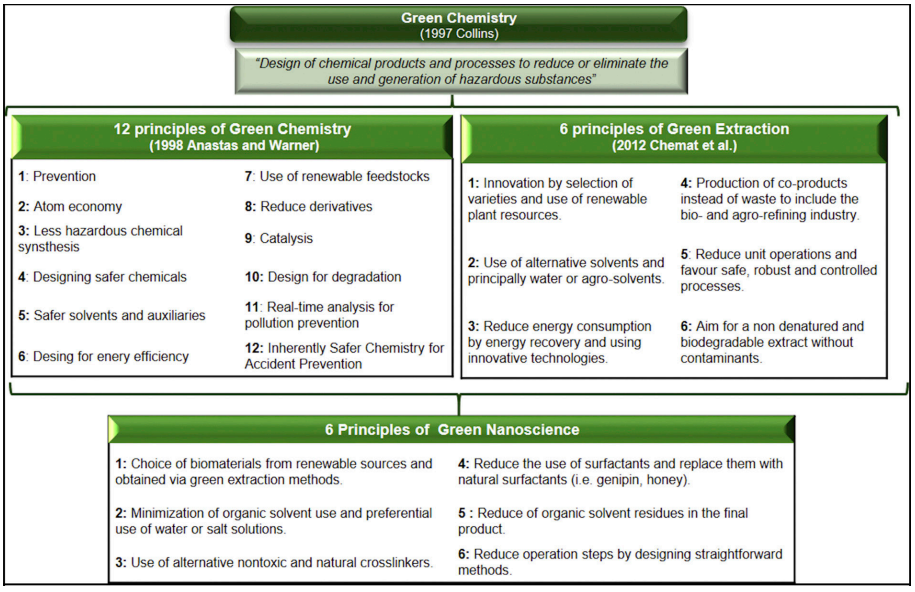

Figure 10. Definitions and principles of Green Chemistry, Green Extraction and Green Nanoscience. Reproduced with permission of [115].

Recent advances in the field of nanotechnology have made a great revolution in different biotechnological sectors, including medicine. Consequently, the intake of pharmaceutical products has increased considerably among the population over the years. However, the clinical and industrial application of this progress has been in the spotlight not only due to the safety risk and possible side effects associated with the use of these advanced drugs but also due to the negative ecological impact that their waste products can have on different aspects of the environment, such as water, soil and air. For instance, the high energy consumption during drug elaboration, along with the use of high amounts of hazardous organic solvents, has a relevant impact in such issues. In addition, most of the drugs and metabolites in wastewater reach the wastewater treatment plants (WWTPs), which are not specifically designed to eliminate these types of compounds. This becomes the main source of entry into the natural environment, which is of utmost relevance due to the possible toxicological risks the drugs can produce [116]. Consequently, the field of Green Nanoscience/Nanotechnology has emerged as a revolutionary strategy to prevent any associated toxic and negative effect on the environment, through the implementation of sustainable and ecologically friendly processes across the whole lifecycle from the extraction of nanomaterials and active compounds (Green Extraction) to the application of the final nanoformulation [115,117]. Some of the Green Nanoscience/Nanotechnology principles include the election of biomaterials from renewable sources obtained by Green Extraction methods, the replacement of organic solvents by water or salt solutions, the use of alternative nontoxic and natural crosslinker, the preferential use of natural tensioactives or the reduction of operating steps designing straightforward methods [118-120]. In any case, notwithstanding the success of Green concepts across inorganic nanoparticles, their application into organic nanoparticles to design and elaborate non-viral vectors for gene therapy is still far away to be a regular practice in the research community.

7. Conclusions

The substantial progress that has been achieved during the past years in different research areas associated with the design of novel gene delivery systems, along with the gain of knowledge acquired in genomics and structural biology, has raised reasonable hope to consider the regular application into medical practice of non-viral vectors as gene delivery systems to face many devastating diseases. Compared to viral vector counterparts, non-viral vectors show relevant advantages. For instance, they are less limited by the genetic packing capacity, are better tolerated by the host immune system and their production is easier and cheaper because they do not have the limitations associated with biological agents. In fact, non-viral vectors are classified as drugs rather than as biological agents by the regulatory authorities, which enhances the clinical translation. However, the reality is that non-viral vectors have been poorly translated into clinical practice. Many concerns that hamper clinical practice of non-viral vectors include low transfection efficiency, short gene expression effect, difficulties to reach the target cells after *in vivo* administration, lack of implementation of standardized protocols for an unbiased evaluation of the performance of non-viral vectors and issues associated to the commercialization process. Although there is no universal non-viral vector for all purposes, in our opinion, lipid non-viral vectors are the closest to reach clinical translation in the gene therapy field. A cutting-edge example is represented by current lipid nanocarriers that have been successfully developed to face the COVID-19 disease by an oligo-based therapeutic approach delivering synthetic mRNA as a nanovaccine. This strategy raises hope to use a gene therapy approach to face diseases with lipid nanoparticles delivering plasmid DNA in the near future. In any case, the success of this appealing approach clearly depends on the development of other game-changing technologies such as big data, tissue engineering or 3D bioprinting to face genetic diseases from a safe, affordable and multidisciplinary point of view.

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Appendix 2

Gene therapy for cystic fibrosis: Hurdles to overcome for successful clinical translation

In vivo and *ex vivo* gene therapy for inherited and non-inherited disorders
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Gene therapy for cystic fibrosis: Hurdles to overcome for successful clinical translation

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ABSTRACT

Cystic fibrosis (CF) is a genetic disease that hampers the lung function. Despite that the main defective gene has been deeply characterized, some relevant concerns still need to be resolved before considering gene therapy as a realistic medical choice. One of the major issues that need to be strongly considered in order to succeed in the search for an effective gene therapy approach for CF is the design of the appropriate genetic material to be delivered. Other relevant factors to take into consideration include the design of safe and effective gene delivery systems, the biological barriers that need to be overcome in order to reach the nucleus of the target cells, and the problems related to the design of a drug formulation suitable for lung delivery purposes. Furthermore, some problems related to the commercialization of gene therapy products also need to be resolved. In this chapter, we discuss the up-to-date strategies to overcome such hurdles in order for gene therapy to become a routine treatment modality for CF.

Keywords: cystic fibrosis • gene therapy • drug delivery • biological barriers • drug formulation

1. Introduction

Cystic fibrosis (CF) is a rare disease with low prevalence caused by the dysfunction of the transmembrane conductance regulatory gene (CFTR). The most prevalent CFTR mutation consists of a deletion of a phenylalanine at position 508 [1]. The disease presents a heterogeneous distribution in the world population being more frequent in Northern Europe. According to recent reports, in the European Union, 1 of every 2,000-3,000 newborns is affected by CF. In the USA, the incidence is 1 per 3,500 births. In Asia, the predisposition to CF is low; however, there is evidence to show that this disease is severely underdiagnosed [2]. The basic characteristic of CF is the transport of defective ions in the apical membrane of most secretory cells, which leads to an altered secretion of mucus in the epithelium of the respiratory tract, the digestive tract, the pancreas, the liver, and the reproductive track [1]. The conventional treatments available on market, which include, among others, antibiotics, pancreatic enzyme supplements, high-fat diets, and even physiotherapy [3], afford the consequences derived from CFTR dysfunction and have significantly improved the mean life expectancy of patients affected by the disease up to 34 years [4]. However, their quality of life is severely compromised mainly due to side effects and interactions among such treatments [5]. Therefore, other therapeutic options such as gene therapy, in which the main goal is to restore the function of the mutated CFTR protein acting on the genetic cause of the problem, need to be considered. CFTR gene was cloned more than two decades ago, and the monogenic and autosomal recessive nature of CF disease means that the addition and expression of the corrected gene could reverse the underlying cause of the disease. Therefore, there is reasonable hope to consider gene therapy as a potential realistic medical option, and consequently, some clinical trials have been performed since 1993. However, despite the moderate optimism that emerged with the development of such clinical assays, there are still some hurdles to overcome before considering gene therapy a realistic medical option. Main concerns are related to the intrinsic properties of genetic materials, the development of safe and efficient gene delivery vectors able to deliver genetic materials to the nucleus of target cells, the design of a drug formulation suitable for pulmonary gene delivery applications, and the

hurdles associated with the commercialization of such drugs (Figure 1).

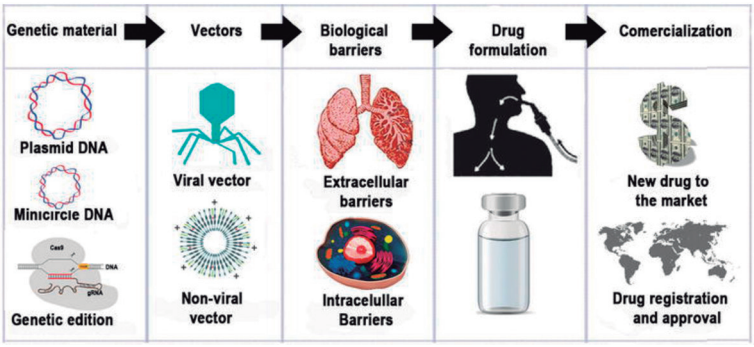

Figure 1. Hurdles that gene therapy should overcome in order to reach clinical practice in the treatment of CF disease.

In the next sections, we will analyze such barriers along with the most relevant approaches developed by the scientific community to circumvent them in order to cure CF with gene therapy.

2. Genetic material

2.1. Plasmid DNA

Bacterial plasmid DNA (pDNA) remains an interesting biomolecule for gene transfer, with several promising reports and clinical trials in progress worldwide [6]. In CF, pDNA has been successfully delivered by nonviral vectors to the sheep lung [7]. Additionally, when administered in multiple-dosage regimen, no loss of activity was observed [8]. In order to be produced in recombinant bacteria and to express their therapeutic gene of interest (GOI), pDNA needs a bacterial origin of replication sequence (bac-ORI). In addition, pDNA backbone includes a sequence with resistance to one/various antibiotics such as kanamycin, which allows to select the clone of bacteria transformed that expresses the plasmid. Finally, a eukaryotic promoter is needed to enhance GOI expression [9]. Usually, when pDNA reaches the nucleus of target cells, it remains in an episomal position, which means that it replicates independently from the host chromosomal DNA, avoiding the undesirable activation of oncogenic genes [10]. The main concerns of pDNA in gene therapy are related to safety issues. Classically, in eukaryotic cells, pDNA has been associated

with the induction of undesired immune responses and secretion of proinflammatory cytokines [11]. For instance, a transient neutrophilic infiltration and an elevation in proinflammatory cytokines have been reported in mouse lung [12]. Although the episomal nature of pDNA could be an interesting advantage, the transfection efficiency remains compromised mainly by the transient and relatively low gene expression. Additionally, the size of the plasmid, determined by the number of base pairs, jeopardizes transfection efficiency [13,14].

2.2. Minicircle DNA

In order to overcome the previously mentioned disadvantages associated with the use of pDNA in gene therapy, small plasmidic cassettes known as minicircle DNAs (mcDNAs) have been recently developed [15]. Cameron and Scheleff first employed mcDNA terminology in 1995. Nowadays, this technology offers a potential alternative to enhance both transfection efficiency and safety of gene delivery [14]. Basically, mcDNAs are circular constructors similar to pDNA but significantly smaller, since mcDNAs contain a minimal expression cassette, of a promoter, a transgene, and a polyadenylation, signal but are devoid of bacterial pDNA elements. Thus, mcDNA technology allows sustained transgene expression mainly due to a lower activation of nuclear transgene silencing mechanisms and reduced immunogenic responses *in vivo* [16,17].

In the lung, some promising results have been obtained with the use of small plasmidic cassettes [18]. In fact, results of a Phase IIb double-blind clinical trial for CF have been recently reported. These trials were performed with a plasmid encoding CFTR gene and lacking CpG bacterial region, known as pGM169 [8]. In such study, treated patients exhibited modest but significant improvements in lung function compared to placebo-treated ones during 1-year follow-up [19]. In any case, despite the optimism generated, there are still some concerns that need to be considered, such as the reproducibility of the results; the intensity of the response, probably conditioned by the degradation of formulation after aerosolization process; or the number of patients that received such treatment.

2.3. Genome editing tools

Both previously mentioned approaches based on pDNA and mcDNA technologies allow to restore the function of the mutated CFTR gene, with the addition of normal copies, but they do not correct the mutation at their local chromosomal location. However, genome editing tools based on zinc-finger nucleases (ZFNs), or transcription activator-like effector nucleases (TALENs), can specifically correct CFTR gene mutations at their natural chromosomal location, and so, the corrected gene can remain under the control of its endogenous promoter [20].

ZFNs are synthetic restriction enzymes, which have three or more zinc-finger DNA-binding motifs linked to the FokI restriction enzyme that recognizes trinucleotides in a specific DNA sequence [21]. When FokI enzyme creates a double-strand break (DSB) near the mutation place, cellular DNA repair mechanisms are activated to maintain cell viability. In these conditions, a donor DNA sequence with high 5′ and 3′ homology with the DNA sequence where DSB has been generated can be exogenously supplemented to enhance the correction of the mutation by homologous recombination (HR) mechanism. This genome editing tool has been successfully used *in vitro* to correct CFTRΔF508 mutation in both human bronchial epithelial cells [22] and CF-induced pluripotent stem (iPS) cells [23].

TALEN technology is very similar to ZFNs. These nucleases were originally characterized in *Xanthomonas* bacteria, in which TALEN proteins are secreted when *Xanthomonas* infect a wide variety of plants, thus activating genes that help to develop the pathogenesis. This genome editing tool also produces a DSB around the mutation site of the target gene and consequently induces cellular DNA repair mechanisms [24]. TALENs are considered as a more efficient and cost-effective alternative to ZFNs [25]. In the case of ZFNs, each finger module recognizes three to four bases of the DNA sequence. However, in the case of TALENs, gene recognition is mediated by a more specific mechanism, where each module of 33-35 amino acid targets a single nucleotide. This technology has been recently applied to correct CFTRΔF508 mutations in CF patient-specific iPS cells [26]. Overall, such study reported correction of

patient-specific iPS cells in less than 3 months, which could allow rapid scaling up for future applications.

Clustered regularly interspaced short palindromic repeats (CRISPR) methodology, originally described as an adaptive immune response in archaea, follows the same rationale described for ZFNs and TALENs, but instead of protein domains, short RNA molecules are used to drive the required homology [27]. In this case, an endonuclease called Cas9 is guided by a single guide RNA (gRNA) to hybridize specifically with the mutated sequence in the DNA; then, as described for ZFNs and TALENs, the resulting DSB triggers cellular DNA repair mechanism [28]. The main advantage of CRISPR technology is that it is an easy-to-synthesize cost-effective tool that is able to correct more than one mutation at the same time, if multiple-gene targeted sgRNAs are delivered to target cell along with the Cas9 protein, which makes it an excellent option [24]. CRISPR technology has been applied to repair CFTRΔF508 mutations in intestinal stem cell organoids of CF patients [29].

This study represents an interesting proof of concept for CFTRΔF508 correction by HR using CRISPR/Cas9 technology in primary adult stem cells derived from patients with a single-gene hereditary defect and offers reasonable hope to be successfully applied to the lungs of patients affected by CF. However, some relevant concerns, mainly related to the frequency of undesirable off targets, still need to be resolved in order to reach clinical practice [19].

3. Vectors

One of the main concerns related to the clinical application of gene therapy is the design and development of safe and effective gene delivery vectors to introduce exogenous genetic material into the nucleus of target cells [30,31]. In the absence of gene delivery vectors, naked genetic material is quickly degraded mainly by exogenous deoxyribonuclease enzymes, which clearly inhibit transfection efficiency [31]. Additionally, the negatively charged genetic material, mainly due to the phosphate groups, hampers the electrostatic interactions with cell membranes, which are negatively charged too. Therefore,

the clinical application of gene therapy demands the design, characterization, and evaluation of efficient and safe carriers to mammalian cells.

3.1. Viral vectors

At present, viral-based carriers are the most appropriate from an effectiveness point of view. The natural evolution that viruses have undergone over many years has allowed them to face different intra- and extracellular barriers and, consequently, infect target cells with high efficiency.

In the CF field, a wide variety of viral-based vectors has been developed in clinical trials. The first one was performed in 1993 with adenovirus in three patients, where partial correction of the chloride transport in nasal epithelium was observed [32]. Some of the main advantages of adenoviruses include their non integrating nature and their natural tropism for the lung. However, despite such favorable properties, and the high transduction efficiency observed in most tissues, gene expression usually remains transient, and these viruses can induce strong immune and inflammatory responses in a dose-dependent manner, which clearly brings up safety issues and, therefore, limits their application in the clinical practice [8].

Initial clinical trials performed with adenovirus allowed the development of adeno-associated viruses (AAV), which have interesting characteristics for their application in gene therapy, such as broad tissue tropism, high transduction efficieny, and persistent episomal expression, which can last for years, even though it is a non integrating vector [33,34]. In addition, recombinant AAV vectors have been shown to be safe in several clinical trials, as they are not related to any known human disease. However, these vectors also present relevant limitations, the main one being their low capacity to load genetic material (<5 kb) [35]. Between 1999 and 2007, six clinical trials were conducted with these kinds of vectors in CF [8]. Phase I clinical trials demonstrated that a single-dose administration of AAV in the respiratory tract of patients affected by CF was safe and well tolerated [36]. Nevertheless, subsequent studies, with repeated doses in more patients, did not report significant improvement in lung function [37]. This lack of efficacy was mainly attributed to the low DNA loading capacity of AAV, which prevented loading the 4.7 kb of the CFTR

gene [8]. In addition, AAV capsid-specific immune responses limited repeated administrations in patients [8]. Nowadays, some interesting strategies are being developed in order to minimize adaptive immune responses after repeated administration, such as the design of hybrid AAV capsids or the removal of CpG bacterial regions from AAV vectors [38,39].

Lentiviruses have an integrative nature and have shown long-term and stable transgene expression when administered in the respiratory tract of mice, which minimizes the need for repeated administration [40]. Additionally, the packaging of full-length CFTR gene and promoters is not limited by size. Therefore, nowadays they are considered promising vectors for the treatment of CF [41]. However, in order to consider its use in clinical practice, some concerns still need to be resolved, such as the scaling in the production of these vectors and the control of the place where the transgene is inserted into the genome of the pulmonary cells, which could increase the tumorigenicity potential of such viral vectors due to random integration [20]. Consequently, such viral vectors could be more suitable for *ex vivo* than for *in vivo* therapy. In any case, a promising study in three newborn CF pigs has recently shown that 2 weeks after lentiviral delivery by aerosolization, the anion channel defect can be corrected in a large animal CF model [42]. Other recent studies assessed with pseudotyped lentivirus vectors in both murine lungs and human air-liquid interface cultures showed that preexisting and acquired immune responses do not interfere with vector efficacy [43]. In such study, at least 14 % of the airway cells were transduced. Interestingly, toxicological results, notably the integration site profile showing absence of integration near oncogenic loci, support further progression toward clinical trials.

3.2. Nonviral vectors

Although the use of viral-based vectors in clinical trials still predominates over that of nonviral vectors, in recent years, there has been a notable increase in preclinical studies using nonviral vectors [44]. The reason is that these systems represent a safer, cheaper, and easier to produce alternative to viral-based vectors [18]. The main advantages of nonviral vectors include, among others, the ability to produce them on a large scale with high

reproducibility and low cost; their relative stability after storage; the possibility of multiple-dose regimen administration due to their low immunogenicity; their high capacity to carry genetic material, independently of the size [45]; as well as the possibility to modify them chemically in order to regulate important physicochemical parameters, such as size, charge, morphology, or polydispersion, which clearly influence their final biological properties. All these important advantages have raised the interest of the scientific community to develop new biocompatible materials of different structures, compositions, sizes, and characteristics to transport therapeutic genes into specific organs or cells, overcoming the different extra- and intracellular barriers [46].

Within the large variety of nonviral vectors developed, most of them are based on peptides as well as on cationic lipids and polymers, which form the corresponding complexes (polyplexes and lipoplexes) after electrostatic binding with DNA [47]. The resulting complexes protect nucleic acids from enzymatic degradation and facilitate cellular uptake by interactions with the cytoplasmic membrane [48]. The PEG-CK30 peptide, due to its low immunogenicity and its ability to be endocytosed by cells, is one of the most widely used, although the formulation must be optimized to allow its administration in aerosol form to reach the lungs [49].

Regarding cationic polymers, polyethylenimine (PEI) is one of the most used, since its chemical structure can be easily modified to increase the efficiency of transfection, for example, by incorporating lactose (Lac-PEI) to improve intracellular trafficking [50]. However, PEI has not yet been used in any clinical trial. The main limitation lies in the difficulty that exists to prepare PEI polyplexes at high DNA concentrations [49]. One of the most promising strategies that have been used to circumvent this problem is the use of ultrafiltration methods, through which PEI/DNA concentrates are prepared.

In the case of cationic lipids, some of the most widely used to develop nonviral formulations in the CF field are N-[1-(2,3-dioleyloxy) propyl]-N, N, N-trimethylammonium, dioleoylphosphatidyl ethanolamine, and dioleoyl trimethyl ammonium [51]. However, currently, the most promising nonviral vector in CF clinical trials is based on the cationic lipid GL67A, which was

synthesized to prevent DNA degradation in the lysosome and to be stable after pulmonary administration by aerosolization [8,51]. In 2011, a preclinical comparative study of the use of PEI, PEG-CK30, or GL67A nonviral vectors in aerosols demonstrated that this last formulation was the best one to transport DNA to sheep's lungs [7]. Moreover, in an extensive preclinical study performed in 2014 [52], it was corroborated that the formulation based on the lipid GL67A, which had already been used for 15 years in CF clinical trials, was still suitable for administration in multiple-dose regimen, without any observed loss of activity [8].

4. Biological barriers

In order to reach the nucleus of target cells and initiate transgene expression, the genetic material must overcome some extracellular and intracellular barriers, which will be discussed in this section, along with the most relevant strategies that have been developed to make the transfection process more efficient.

4.1. Extracellular barriers

Even though intravenous injection is one of the most commonly used administration routes, especially for delivering genetic cargo into cancerous cells, some barriers still hamper its use in clinical practice, particularly in the CF disease [53]. First of all, the genetic material needs to be protected against extracellular enzymatic digestion, since DNA is quickly degraded when administered alone. To avoid such quick degradation, nonviral vectors based on both positively charged lipids and polymers offer the possibility to condense on their surface the genetic material by electrostatic interactions and minimize such undesirable effect [54]. However, the final positive charge of polyplexes or lipoplexes can interact in a nonspecific way, not only with target cell membranes but also with other negatively charged components such as serum proteins that jeopardize transfection efficiency [55]. These interactions could result in the formation of aggregates that classically are eliminated from the blood by the reticuloendothelial system. Interestingly, the incorporation of polyethylene glycol (PEG) motifs into the formulation of some nonviral carriers

enhances the stability of complexes, since the highly hydrophobic nature of PEG chains creates a steric barrier to prevent both aggregation of complexes in blood circulation and extracellular enzymatic degradation by nucleases [55]. In any case, other relevant aspects related with both the length and the degree of PEGylation should be also considered, as they can also decrease DNA condensation efficiency with nanoparticles [56]. In addition to PEG, other polymers with hydrophobic nature such as poly(4-acryloylmorpholine) or poly(N,N-dimethylacrylamide) have recently emerged as interesting and promising alternatives to compensate or ameliorate the negative effects associated with PEGylation [57].

Since intravenous injections present relevant hurdles that hamper the delivery of genetic material into target lung cells, local administration into the lung seems to be a reasonable alternative. In this case, the presence of mucus and the clearance mechanism are the most relevant barriers to overcome [58]. To avoid such barriers associated with pulmonary administration, other interesting noninvasive routes of administration, such as intranasal instillation, can be used to target lung cells. Nevertheless, the main problem is the low amount of genetic material that can be administered by the intranasal route. From a technical point of view, aerosolized nonviral vector/DNA complexes, carefully designed for inhalation in combination with appropriate excipients to enhance both particle flow and aerodynamic diameter, could be an interesting option since they are needle-free systems able to deliver locally high cargo concentrations [53].

To circumvent the diffusion of complexes into lung cells due to unspecific interactions with the biopolymer network of the mucus, some mucolytic agents that hydrolyze mucins can be added [58]. Other strategies include the incorporation of N-acetylcysteine to reduce disulfide bridges between the subunits of mucin, and consequently the viscosity [59], or the functionalization of nonviral vector formulations with mucolytic agents.

4.2. Intracellular barriers

Once extracellular barriers are overcome, there is still a long way full of hurdles before reaching the nucleus of target cells. Firstly, complexes carrying

the genetic material need to be endocytosed by target cells. The interaction between complexes and cell membranes can occur in an unspecific way or can be mediated by a specific ligand, which is the preferred one, especially for *in vivo* applications [60]. Of note, the choice of ligand to be incorporated into the nanoparticle formulation depends not only on the target cell but also on the type of cell entry pathway that will be used once the ligand binds to the desired receptor.

Classically, there are four main pathways of endocytosis: clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), phagocytosis, and macropinocytosis [61,62]. CME is an energy-dependent mechanism widely studied and characterized [63]. Typically, this pathway is directly associated with lysosomes, where the genetic material needs to leave such biological compartment quickly before being degraded by the acidic environment and the enzymes found in lysosomes [63]. To avoid this enzymatic degradation, some interesting strategies can be used, such as the formation of pores in the endosome membrane by incorporation of amphiphilic cationic peptides. This creates strong internal tensions in the membrane and enhances the exit of endosome content through such pores [64]. Another strategy is known as the "proton sponge effect," where the low pH within the endosome allows the protonation of trapped compounds that have a large capacity to absorb protons from the medium (buffer effect). Such protonation causes an important entry of ions (H^+ and Cl^-) and water in the endosome, which produce a swelling effect and its rupture. This effect has been observed in some cationic polymers with high buffering capacity over a wide pH range [64]. In CvME, internalized molecules go to the caveosome instead of lysosome [61] avoiding lysosomal degradation; however, there is still ongoing debate, with some authors claiming that CvME can fuse with lysosomes [65].

Phagocytosis is a special type of endocytosis used mainly by macrophages, monocytes, neutrophils, and dendritic cells, although other cell types can also use this cellular entry pathway [61]. Endocytosis mediated by phagocytosis comprises the formation of membrane extensions with certain forms to capture particles generally greater than 1 μm. In contrast, for endocytosis mediated by macropinocytosis, membrane extensions do not surround particles

but form some kind of protuberances that finally fuse with the cytoplasmic membrane. In many cases, the physicochemical properties of nonviral vector-based nanoparticles, such as particle size, superficial charge, morphology, or polydispersity, directly influence the endocytosis mechanism and consequently the transfection efficiency.

Once the DNA is released into the cytosol of cells, it must enter the nucleus to produce its effect. This is considered a significant barrier that nonviral vectors must overcome in order to mediate a good transfection efficiency. One commonly used strategy to enhance nuclear import of genetic material is to incorporate a nuclear localization signal (NLS), such as polylysine or protamine [66]. NLS contains some amino acids that interact with some proteins of the cytoplasm known as importines. These importins enhance nuclear entry through the nuclear pore complex of the nuclear membrane through an energy-dependent mechanism [67].

5. Drug formulation

The airways seem to be the natural way to treat respiratory diseases and a good alternative to systemic and more invasive procedures. Currently, aerosolization is the prefered method of administration for airway targeting since it is a noninvasive route that induces little stress to patients. Moreover, high quantities of drug can be deposited directly and fast into the lungs, which circumvents the blood circulation and avoids the first-pass effect of the liver. However, the effectiveness of such approach strongly depends on the development of smart drug formulation strategies. One of the critical steps that need to be taken into account for a successful gene delivery approach by inhalation is the formulation of the drug molecules into an appropriate inhalable form with sufficient stability and adequate aerodynamic properties [68]. Highly susceptible molecules, such as nucleic acid, require special attention when delivered by this route of administration. The physicochemical constraints such as the hydrodynamic shear forces generated during aerosolization can induce degradation of the nucleic acids, which will be more or less important depending on their size [69,70]. Therefore, the need to develop a suitable formulation able to protect the material from degradation and at the

same time ensure delivery of nucleic acid to the target cells in the lung needs to be deeply considered. In this sense, the commonly accepted aerodynamic size for pulmonary gene delivery is within the range of 1-5 μm. Larger particles (4-7 μm) tend to deposit in the airways, while smaller particles (1-3 μm) and those in submicron range (< 1 μm) reach the lower airways and deeper lung [68]. The aerodynamic diameter of a particle can be modified not only by changing its size but also by varying its density or shape, which opens new possible strategies for gene delivery to the lung, such as the design of large porous hollow particles [71].

Suitable formulations for pulmonary delivery are mainly prepared either by dissolving or by suspending the therapeutic molecules in a liquid or formulating them into a dry powder for inhalation using liquid inhalers (including nebulizers), dry powder inhalers (DPIs), or pressurized metered dose inhalers (pMDIs); each of them is suitable for different applications. Once the aerosolized droplets or microparticles are deposited next to the target location into the lungs, they need to dissolve in the lung lining fluid for subsequent absorption and cellular uptake [72]. Nowadays, viral gene delivery to the lungs is limited to liquid formulations using a nebulizer [36], and there is no dry powder or metered dose inhaler formulation available for any vector-drug combination. In most cases, the gene transfer efficiency to lung cells using viral vectors is still too low with traditional nebulizer devices, probably due to the degradation of viral envelope by the shear forces caused during aerosolization [73] and the viscous mucus found in obstructive diseases, like CF [74]. Moreover, and as previously highlighted, the use of adenoviral or AAV vectors would likely induce an acute immune response upon the initial administration or result in low efficacy following repeat dosing. This is particularly relevant since aerosolized gene therapy might require repeat dosing because mucus clearance mechanisms and/or phagocytes may engulf and destroy the drug vector before it can be taken up by target cells [75]. In contrast, the simpler composition of nonviral vectors may have, in this case, an advantage over viral vectors, making readministration potentially more successful.

Table 1. Pulmonary gene delivery strategies by aerosolization.

Description	Vector	Aerosolization	References
In vivo aerosol delivery of PEI-DNA comp	PEI	Nebulization	[68]
Randomized, double-blind, placebo-controlled, Phase II trial in CF patients with mild lung disease	AAV2 viral	Nebulization	[32]
Nebulization of receptor targeted nanocomplexes for *in vivo* gene delivery to the airway epithelium	Receptor-targeting peptides and cationic liposomes	Nebulization	[69]
In vivo repeated aerosol delivery of pDNA/PEI complexes with CpG-free plasmids	PEI	Nebulization or instillation	[70]
In vivo aerosol delivery of DNA/liposomes to the lung	GL67A cationic liposomes	Multiple nebulizers	[71]
Randomized, double-blind, placebo-controlled, Phase IIb trial	GL67A cationic liposomes	Nebulization	[14]
In vivo intratracheal administration of pDNAchitosan dry powders, obtained by SFD	Chitosan	Dry powder, obtained by SFP, administered by intratracheal syringe	[72]
Dry powder aerosols for *in vivo* gene delivery to the lung	PEI	Dry powder in insufflator lyophilization/powderization with lactose, sucrose or trehalose	[73]

Although pulmonary gene-based therapies have not yet been granted marketing approval, numerous strategies are being tested both *in vitro* and *in vivo*, and various clinical trials are underway [19,36]. Table 1 summarizes some of the strategies used to date for the pulmonary delivery of nucleic acids by aerosolization.

Nowadays, the most studied approach for gene delivery to the lung involves the nebulization of the selected formulation [76], turning it from a liquid solution to microdroplets. Depending on the aerosolization system used, such as jet, ultrasonic, or mesh nebulizers, the implemented hydrodynamic stress that the therapeutic molecules would be subject to varies [77]. Interestingly, several strategies have been studied to reduce the damage to the genetic material during the aerosolization process, by condensing the nucleic acids with positively charged molecules, such as polyethylenimine (PEI), protamine, or poly-L-lysine (PLL), among others [78].

The elaboration of DPIs, composed of drug-based dry powders and an aerosol-generating device, also presents important advantages such as high physicochemical stability, easy handling, and propellant-free aerosols. In order to transform the therapeutic nucleic acids into stable dry powders, several techniques, such as freeze-drying (FD) [79], spray-drying (SD) [80], and spray freeze-drying (SFD) [81], are being investigated. In addition, the incorporation of suitable stabilizing agents/thermal protectors such as polysaccharides (sucrose [79], trehalose [79], agarose [82], lactose [83], mannitol [81], or chitosan [84]), amino acids (leucine [84] or glycine [82]), or proteins (BSA [85]) is critical.

6. Commercialization

In addition to the above concerns, other relevant issues specifically related to the commercialization of gene therapy medicinal products (GTMP) must also be considered. Commercially available medical products based on gene therapy along with cell therapy and tissue engineering are classified as advanced therapy medicinal products. Although highly promising, their translation into clinical practice is nowadays hampered by major critical issues such as

complex regulatory and ethical aspects, along with the intrinsic difficulties to scale up these products to an industrial level [20].

Regarding the regulatory concerns of GTMP that affect clinical applications, the economical investments, along with their manufacture and control, demand more attention than chemically synthesized small molecules [86]. Therefore, a deep analysis of both costs and benefits needs to be done before considering the commercialization of such therapies [87].

Another relevant concern that jeopardizes the clinical use of GTMP in CF is the ethical aspect of clinical trials. Since the early 1990s, more than 25 Phase I gene therapy clinical trials have been conducted. These trials have been carried out largely to assess the safety and feasibility of gene transfer methods and their expression in the host, reporting variable successes for both viral and nonviral approaches. Gene therapy products designed for the treatment of CF must meet certain requirements in order to become a viable therapeutic option. For instance, their clinical efficacy must be demonstrated by analyzing appropriate variables of the lung function such as the patient´s vital capacity that they are able to expire in the first second of forced expiration (FEV1), their age, sex or body composition, and the therapeutic efficacy which must be maintained with repeated administrations. In addition, the GTMP must demonstrate an acceptable profile when it comes to side effects, and other considerations such as treatment of early versus established lung disease must also be analysed.

Since a high percentage of patients affected by CF are children, clinical trials involving these patients must carefully balance the potential benefits of these therapies and the associated risks [88]. Regarding this controversial issue, the Gene Therapy Advisory Committee recommends that clinical trials on children should only be performed under specific circumstances, whereby: (i) it has been demonstrated that the research is necessary to promote the health of the trial population, (ii) the research cannot be done in adults, and (iii) there is a high potential of therapeutic benefit [88]. In fact, owing to a demonstrated benefit of early gene therapy intervention, the age of enrolment of children in clinical trials has progressively reduced over the years from 18

to 12 years old. However, parents should have legal rights to make the final decision on behalf of their children.

Another critical hurdle that strongly compromises the clinical application of gene therapy products for the treatment of CF is the difficulty to scale up formulations that were originally developed for basic clinical research [89]. Most of these products are usually developed by small- and medium-sized enterprises, in collaboration with academic groups, which are usually highly engaged in preclinical activities, but have limited manufacturing experience at industrial level. For instance, the normal procedure for preparing nonviral-based gene therapy products is by simply mixing and pipetting the negatively charged genetic material and the positively charged polymer -or lipid-based nonviral vector formulations, which are often produced in the laboratory at small volumes that usually oscillate between 1 and 5 mL. However, the standardization of this procedure at industrial level to produce high and stable levels of complexes under GMP conditions represents a great challenge that needs to be overcome for successful clinical application. In this sense, pilot plants, which employ small volumes of the product, represent an interesting option to gain knowledge on the technical process before full scale up production.

7. Conclusion

Despite the fact that the CFTR gene was cloned two decades ago, the current, conventional treatments for CF focus on masking the main symptoms, rather than addressing the underlying genetic cause of the disease. In this sense, gene therapy represents a promising alternative to tackle CF, considering the autosomal recessive nature of the most relevant ΔF508 mutation. Although the main objective of gene therapy seems simple, there are some hurdles that need to be overcome before gene therapy for CF becomes a realistic treatment option. In any case, the increase in knowledge and recent advances in biopharmaceutical technology offer reasonable hope for the treatment of this devastating disease. The minicircle technology, along with the new gene editing tools, offer important advantages compared with classical plasmids used to add functional copies of the gene. Additionally, intense research in novel nonviral vectors functionalized to overcome both extra- and

intracellular barriers and the possibility to aerosolize such formulations without losing activity merit special attention.

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Appendix 3

Correlation between biophysical properties of niosomes elaborated with chloroquine and different tensioactives and their transfection efficiency

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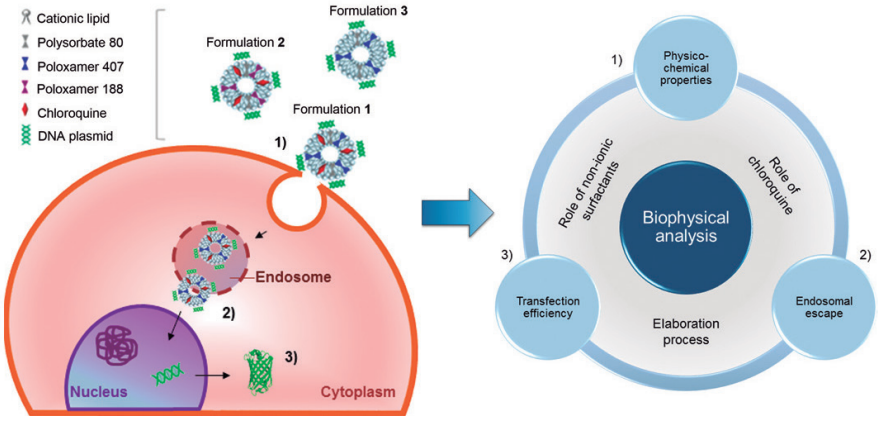
Graphical abstract



Correlation between biophysical properties of niosomes elaborated with chloroquine and different tensioactives and their transfection efficiency

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ABSTRACT

Lipid nanocarriers, such as niosomes, are considered attractive candidates for non-viral gene delivery due to their suitable biocompatibility and high versatility. In this work, we studied the influence of incorporating chloroquine in niosomes biophysical performance, as well as the effect of non-ionic surfactant composition and protocol of incorporation in their biophysical performance. An exhaustive comparative evaluation of three niosome formulations differing in these parameters was performed, which included the analysis of their thermal stability, rheological behavior, mean particle size, dispersity, zeta potential, morphology, membrane packing capacity, affinity to bind DNA, ability to release and protect the genetic material, buffering capacity and ability to escape from artificially synthesized lysosomes. Finally, in vitro biological studies were, also, performed in order to determine the compatibility of the formulations with biological systems, their transfection efficiency and transgene expression. Results revealed that the incorporation of chloroquine in niosome formulations improved their biophysical properties and the transfection efficiency, while the substitution of one of the non-ionic surfactants and the phase of addition resulted in less biophysical variations. Of note, the present work provides several biophysical parameters and characterization strategies that could be used as gold standard for gene therapy nanosystems evaluation.

Keywords: chloroquine • niosomes • gene delivery • biophysical properties • surfactants

1. Introduction

Gene therapy is based on the modification or control of gene expression in order to treat a specific disease [1]. There are two main gene carrier systems, viral and non-viral vectors, which provide genetic material protection and enhance cell internalization [2,3]. Non-viral vectors are usually based on different biocompatible nanoparticles, which represent a safer strategy than viruses and their elaboration process is easier and cheaper. Although in many occasions non-viral vectors are still unable to reach the high transfection levels of viral counterparts, continuous advances in the field bring us closer to this goal [4-7]. In fact, currently some lipid nanoparticles are already commercialized for drug and RNA delivery, such as Onivyde® [8] and Onpattro™ [9], among others. In addition, during the ongoing pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, two lipid nanoparticle-formulated mRNA vaccines have also been developed [10]. However, to date, non-viral vectors have not reached clinical practice for DNA delivery. To this end, it is essential the carefully design and selection of biomaterials to develop efficient nanocarriers with high biophysical performance.

Non-viral vectors can be classified on a wide variety of nanosized materials, including cationic lipids, polymers and carbon-based nanostructures [3,11]. Regarding cationic lipids, niosomes, which can be defined as vesicular structures made of non-ionic surfactants [12], have gained attention over liposomes due to their lower costs, longer stability and lower toxicity [13-16]. Basically, cationic niosome formulations for gene delivery applications [17] contain a cationic lipid, which forms complexes with the negatively charged DNA and promotes the fusion with cell membranes [18], non-ionic surfactants to form stable emulsions and prevent particle aggregations [7] and "helper" components to enhance the biophysical properties [19]. Cationic lipids have three main functional domains: a hydrophilic head-group which enhances electrostatic interactions with the DNA and can be formed by quaternary ammoniums, amines, amino acids, lysine, guanidiniums and heterocycles [20], a hydrophobic domain usually composed of two saturated/unsaturated aliphatic chains [21] and a linker bond which influences in the stability, biodegradability, transfection efficiency and cytotoxicity of the cationic lipid [22]. This linker

bond usually contains an ester, ester amide, carbamate, disulphide, urea or phosphate, among others [22]. Some cationic lipids are commercially available, such as 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) [7] or 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [23], while others can be tailor-synthesized for specific applications. Regarding non-ionic surfactants, the most commonly used are polysorbates (Tween®) [7], sorbitan fatty acid esters (Span®) [24] and polyoxyethylene alkyl ethers (Brij®) [25]. The chemical structure of these surfactants influences on the final product as well as on their hydrophilic-lipophilic balance (HLB), which determines the oil or water solubility. For instance, the surfactants with long alkyl chains usually produce larger vesicles with more rigidity and less deformable membranes [7,26]. The role of the "helper" component is to increase the stability and the fluidity of the lipid bilayer, and it works as an adjuvant of the transfection process since it enhances cellular uptake and intracellular trafficking [19,27,28]. Until now, "helper" components used in non-viral vectors are neutral lipids such as phosphatidylethanolamine (DOPE) [23], cholesterol, squalane, squalene [19] and lycopene [29], but, recently, other compounds have also been explored. In this sense, chloroquine has emerged as an interesting material for gene delivery due to its ability to promote endosomal escape, which constitutes a key limiting step in every transfection process [30]. Although some peptides -such as cell-penetrating peptides (CPPs)- and viruses have also been used to that end, chloroquine presents higher stability than CPPs and its use results less complexity compared to viruses as it is not a biological agent. In addition, the lower cost and easier scale-up production of chloroquine also contribute to increase its attractiveness as a promising material for gene delivery purposes. In this work, we hypothesize that chloroquine not only promotes endosomal escape, but it could also be involved in the modification of different biophysical parameters of the nanoparticle formulations, which might have a direct impact on the transfection process.

Along with the components and their chemical structure, the final biophysical properties of the system also depend on the elaboration method and the molar ratios between the nanocarrier components and the genetic cargo, which ultimately lead to different biological behaviors of the nanocomplexes

[18,31-33]. To elaborate niosomes, many procedures have been described [15,34,35] being the oil in water (o/w) emulsion technique one of the most widely used. In this method, two phases are involved, the aqueous and the organic one. The niosome components are dissolved in the corresponding phase depending on their solubility and, after that, both phases are mixed by sonication to elaborate the emulsion. Thereupon, the organic solvent is evaporated from the emulsion under magnetic agitation which results in the formation of the vesicles suspended into the aqueous medium [34,36]. The solubility of the components is the main factor when choosing the phase in which they will be dissolved: lipophilic and hydrophilic compounds are added in the organic or in the aqueous phase, respectively. Besides, HLB value of non-ionic surfactants normally rules for their addition to organic or aqueous phase: the lower value, the more lipophilic, and the higher value, the more hydrophilic. Additionally, depending on the procedure, the components could be added in different phases, especially the non-ionic surfactants due to their amphiphilic character [35].

Considering all these issues, the aim of this study was to determine, on the one hand, the influence of incorporating chloroquine in niosomes as a biophysical performance enhancer agent and, on the other hand, the effect of varying non-ionic surfactant components and their phase of addition -aqueous or organic- on the biophysical performance of niosomes. For that purpose, three different niosomes, named as formulations **1**, **2** and **3**, were developed. Formulations **1** and **2** differed in one of the two non-ionic surfactants and their phase of addition and both contained chloroquine as a "helper" component. Formulation **3** was elaborated as formulation **1**, and only differed in the "helper" component as it was formulated without chloroquine. The three niosome formulations were prepared by the o/w emulsion technique and were analyzed in terms of differential scanning calorimetry and rheological properties. Niosomes were complexed with the reporter plasmid EGFP (pEGFP) to obtain nioplexes at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1, in order to characterize their mean particle size, dispersity, zeta potential and morphology. The membrane packing capacity of nioplexes was also evaluated, as well as the affinity to bind DNA, release it and protect it from enzymatic digestion.

In addition, the buffering capacity of the formulations and their endosomal escape ability were also studied. Finally, *in vitro* biological studies were performed in order to determine the compatibility of the formulations with biological systems as well as their transfection efficiency and the duration of gene expression over time in human cystic fibrosis airway epithelial (CuFi-1) cells.

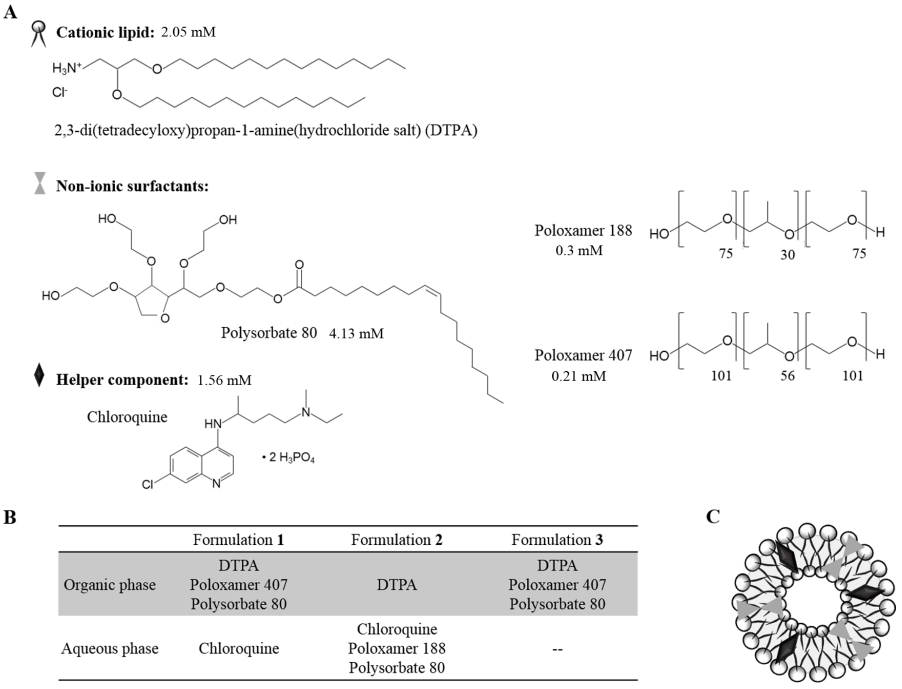
2. Materials and methods

2.1. Preparation of niosomes and nioplexes

Two niosome formulations based on cationic lipid were elaborated using the oil in water emulsion technique as previously described [18]. Both formulations **1** and **2** contained in their organic phase 5 mg of the tailor-synthesized cationic lipid 2,3-di(tetradecyloxy)propan-1-amine(hydrochloride salt) (DTPA) [37], dissolved in 1 mL of dichlorometane (DCM) (PanReac, Barcelona, Spain). The organic phase of formulation **1** also contained 12.5 mg of poloxamer 407 (Merck KGaA, Darmstadt, Germany) and 12.5 mg of polysorbate 80 (PanReac, Barcelona, Spain) as non-ionic surfactants. The aqueous phase of both formulation **1** and **2** contained 2.5 mg of chloroquine (Merck KGaA, Darmstadt, Germany) as "helper" component dissolved in 5 mL of distilled water. Furthermore, the aqueous phase of formulation **2** also contained 12.5 mg of poloxamer 188 (BASF, Ludwigshafen, Germany) and 12.5 mg of polysorbate 80 as non-ionic surfactants. Formulation **3** was prepared as to formulation **1** protocol but with the avoidance of chloroquine. The emulsion was obtained by mixing the organic and the aqueous phases by sonication (Branson Sonifier 250; Branson Ultrasonics Corporation, Danbury, CO, USA) for 30 s at 50 W. The organic solvent was removed from the emulsion by evaporation under magnetic agitation for 1 h at room temperature. The chemical structure of the components, the summary of each formulation components and the general scheme of their disposition in a niosome are represented in Figure 1.

Nioplexes were elaborated by mixing an appropriate volume of DNA stock solution (EGFP reporter plasmid or pEGFP) with different volumes of the niosome formulations to obtain different cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1. The mixture was incubated for 30 min at room temperature to

enhance electrostatic interactions between the niosomes and the genetic material to form the nioplexes.



	Formulation **1**	Formulation **2**	Formulation **3**
Organic phase	DTPA Poloxamer 407 Polysorbate 80	DTPA	DTPA Poloxamer 407 Polysorbate 80
Aqueous phase	Chloroquine	Chloroquine Poloxamer 188 Polysorbate 80	--

Figure 1. Structure and chemical components of niosomes. (**A**) Chemical structure and millimolar quantity of niosome components. (**B**) Description of the components in each formulation. (**C**) General scheme of a niosome.

2.2. Plasmid propagation

Escherichia coli DH5α was used to propagate the CMS-EGFP reporter plasmid (5.5 kb, PlasmidFactory, Bielefeld, Germany), named as pEGFP. Then, according to manufacturer's instructions, the pEGFP was purified using the Qiagen endotoxin-free plasmid purification Maxi-prep kit (Qiagen, Hilden, Germany). The final concentration of pEGFP was quantified by measuring the absorbance at 260 nm using a SimpliNano™ device (GE Healthcare, Buckinghamshire, UK).

2.3. Nano DSC studies

The characterization of the thermostability of niosome formulations **1**, **2** and **3** was performed by differential scanning calorimetry (DSC) using a Nano DSC device (TA Instruments, New Castle, DE, USA). Both in the reference and in the sample cells, MilliQ® water was introduced in order to obtain a buffer line. Measurements of formulations **1**, **2** and **3** were performed with niosomes in the sample chamber at a concentration of 0.5 mg/mL, prior to degassing. The temperature range was from 4°C to 100°C for all the samples and the scan rate was 1.0°C/1 min. Results were collected and analyzed using the DSC Run 4.6 and Launch Nano Analyze 3.11 software (TA instruments, New Castle, DE, USA), respectively. The Nano DSC chambers were cleaned after each run with MilliQ® water, 2 % DECON™ 90 and methanol.

2.4. Rheological studies

The rheological behavior of niosomes based on formulations **1**, **2** and **3** was conducted using the Advanced Rheometer AR1000 equipment (TA instruments, New Castle, DE, USA). A flat plate with 20 mm of diameter was used. The concentration of the niosome formulations was 1 mg/mL and the GAP was settled at 1,200 µm. The shear stress and the viscosity data were obtained at shear rates from 10 to 1,000 s^{-1} with 10 points per decade. Data were collected and processed using the Rheology Advantage™ software (TA instruments, New Castle, DE, USA).

2.5. Morphology, size, dispersity and superficial charge

The morphology of niosomes was determined by transmission electron microscopy (TEM) as previously described [6]. The hydrodynamic diameter, which includes particle size, reported as mean particle intensity, and dispersity (Đ) of niosomes and their corresponding nioplexes was measured by dynamic light scattering (DLS), and the zeta potential was measured by Lasser Doppler velocimetry (LDV) in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). To carry on the measurements, 50 µL of each sample were diluted in 950 µL of 0.1 mM NaCl solution. The particle hydrodynamic diameter was obtained by cumulative analysis. The Smoluchowski approximation supported the

calculation of the zeta potential from the electrophoretic mobility. All measurements were carried out in triplicate.

2.6. Nioplexes membrane packing studies

Lipid packing studies were carried out for niosomes based on formulations **1**, **2** and **3** and their corresponding nioplexes with the lipophilic probe laurdan (6-Dodecanoyl-2-Dimethylaminonaphthalene) (Fisher Scientific, Madrid, Spain). Laurdan was dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 200 μM (stock solution). The assay was performed in a 96-well black plate. The concentration of laurdan fluorescent dye in each well was 0.5 μM and formulations at room temperature were added at a concentration of 0.25 mM. Then, 0.1 mM of NaCl was added to reach a final volume of 200 μL per well. The plate was measured in the TECAN plate reader at an excitation wavelength of 340 nm and the emission spectrum was measured from 400 to 500 nm, increasing 5 nm in each measurement and considering the intensity values at 440 nm (I_{440}) and 490 nm (I_{490}). The general polarization (GP) value was calculated as a relative measure for membrane order using the following formula: $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$ [38]. GP values range from −1 (least ordered) to +1 (most ordered). As a blank, laurdan reagent in NaCl solution was used. Each measurement was performed in triplicate.

2.7. ITC studies

Isothermal titration calorimetry (ITC) was used to monitorize niosome-pEGFP interactions for nioplexes formation using a MicroCal PEAQ-ITC microcalorimeter (Malvern Instruments, Worcestershire, UK). The assays were carried out at 25 °C by stepwise injections of niosome formulations (1 mg/mL DTPA) into the reaction cell loaded with an aqueous solution of pEGFP (0.0166 mg/mL) at the following injection sequences: 1 × 0.4 μL; 8 × 1.7 μL; 9 × 2.5 μL (formulation **1**) and 1 × 0.4 μL; 6 × 1 μL; 14 × 2.3 μL (formulations **2** and **3**). The injections were carried out automatically under 750 rpm stirring. The heat contributed by niosome dilution in MilliQ® water was measured in separate runs using the same injection sequence and subtracted from the total heat produced following each injection prior to the data analysis. The full set of experiments was carried out with the same preparation of pEGFP for the

three formulations, and using the same dilution of niosomes in the binding and dilution runs of each formulation, in order to minimize errors.

2.8. DNA release from niosomes and protection capacity

An agarose (Merck KGaA, Darmstadt, Germany) gel electrophoresis assay was developed to analyze the ability of the niosomes to protect and release the DNA from enzymatic digestion. For DNA release assay, 12 µL of 7 % sodium dodecyl sulfate (SDS) (Merck KGaA, Darmstadt, Germany) was added to the samples and incubated for 10 min at room temperature. For DNA protection analysis, 2 µL of DNase I enzyme (Merck KGaA, Darmstadt, Germany) was added to the samples and incubated for 30 min at 37°C, then 12 µL of 7 % SDS (Merck KGaA, Darmstadt, Germany) was added and incubated for 10 min at room temperature. Before running the gel, 2 µL of loading buffer were added to all samples. Naked DNA was used as a control at each condition. The amount of DNA per well was 200 ng in all cases. The agarose gel (0.8 %) was immersed in a Tris-acetate-EDTA buffer and exposed for 45 min to 100 V. Once running was stopped, DNA bands were stained with GelRed™ (Biotium, Hayward, CA, USA) and images were obtained with a ChemiDoc™ MP Imaging System and analyzed with ImageLab™ Software (Bio-Rad Laboratories, Hercules, CA, USA).

2.9. Buffer capacity assay

Acid-base titration assay was performed to determine the buffer capacity of the niosome formulations, as described previously [39]. Each sample, with 0.1 mg/mL of cationic lipid, was prepared in 10 mL of 150 mM NaCl and adjusted to pH 10 with 0.1 M NaOH. Then, the samples were titrated with 0.1 M HCl solution, added in 5 µL to 5 µL, and pH values were measured by a Crison pH-Meter GLP21.

2.10. Vulnerability assay of complexes in the late endosome

Micelles based on phosphatidylserine (PS) were developed as an analogue of the lysosomal compartment, as described previously [40,41]. PS was dissolved in chloroform at 1.6 mM and, straightaway, the solvent was completely

evaporated under magnetic stirring. Dried sample was resuspended with phosphate buffer solution and a dispersion was obtained by sonication. PS and the nioplexes were incubated for 1 h at a pEGFP/PS mass ratio of 1/50. Naked DNA was used as control. Subsequently, the amount of the released DNA from each complex was determined by agarose gel electrophoresis. Samples (containing 200 ng of pEGFP each well) were loaded onto a 0.8 % agarose gel and exposed for 30 min to 100 V. DNA bands staining was performed as aforementioned. The quantification of DNA bands was obtained using ImageLab™ 4.0.1 software and the percentage of DNA released was calculated applying the equation (1) where SC is supercoiled DNA:

$$\% \text{ DNA released} = (\text{SC band/total DNA}) \times 100 \quad (1)$$

2.11. Cell culture and transfection assays

Human cystic fibrosis airway epithelial (CuFi-1) cells obtained from ATCC® CRL-4013™ were incubated at 37°C and 5 % CO_2 atmosphere in collagen type IV (Merck KGaA, Darmstadt, Germany) pre-treated flasks/plates and were split every 2-3 days to maintain monolayer coverage. The cells were cultivated in bronchial epithelial growth medium -2 bullet kit- (BEGM) (Lonza, Basel, Switzerland).

For transfection assays, cells were seeded in 24 well plates at a density of 1.6×10^5 cells per well (for posterior flow cytometry analysis) or in 96-well plates at a density of 3×10^4 cells per well (for posterior kinetic analysis) and incubated overnight to achieve 70 % of confluence at the time of transfection. The formation of nioplexes at 2/1, 5/1 and 10/1 cationic lipid/DNA mass ratios were performed in serum-free Opti-MEM transfection medium (Gibco, San Diego, CA, USA). The growth medium was removed from the plate and the cells were exposed to nioplexes (1.25 µg and 0.21 µg of pEGFP per well in 24-well and 96-well plates, respectively) for 4 h in the incubator. After the incubation, nioplexes were removed and fresh growth medium was added to the cells. As a negative control for transfection, cells were not exposed to nioplexes but were incubated in Opti-MEM for 4 h. As a positive control for transfection, Lipofectamine™ 2000 transfection reagent (Invitrogen, Waltham, MA, USA) was used. Each condition was performed in triplicate.

2.12. Analysis of EGFP expression and cell viability

Transfection capacity and compatibility with biological systems of nioplexes **1**, **2** and **3** were evaluated over 7 days both qualitatively and quantitatively, using the Cytation™ 1 equipment (BioTek Instruments, Winooski, VT, USA). For quantitative analysis of transfection efficiency and biocompatibility, the green fluorescence intensity and the cell absorbance at 600 nm were measured every 24 h for 7 days. For qualitative analysis of both parameters, bright-field and fluorescent images of cells were also acquired every 24 h for 7 days. Cells were kept alive for the whole experiment and incubated at 37°C and 5 % CO_2 in the Cytation™ 1 equipment, without retrieving or moving the plate between measurements.

Transfection efficiency and cell viability were further quantitatively evaluated 48 h after the exposure to nioplexes by flow cytometry. Specifically, EGFP expression, cell viability and mean fluorescence intensity (MFI) analysis were conducted using a FACSCalibur system flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Transfected cells were washed with Dulbecco´s phosphate buffered saline (DPBS) with calcium and magnesium (Lonza, Basel, Switzerland). Cells were detached using Trypsin/EDTA 0.25 % (Gibco, San Diego, CA, USA) and, then, trypsin inhibitor (DPBS with 1 % of fetal bovine serum (Gibco, San Diego, CA, USA) was added. Cells were centrifuged at 1,100 rpm for 5 min and the resulting pellet was resuspended in culture medium and transferred to specific flow cytometer tubes. In order to evaluate cell viability, propidium iodide (Merck KGaA, Darmstadt, Germany) was added in each sample at 1:300 dilution. The fluorescent signals were measured at 525 nm (FL1) and 650 nm (FL3) corresponding to EGFP positive cells and dead cells, respectively. To establish a collection gate and exclude cells debris, non-transfected cells, used as control samples, were displayed on a forward scatter (FSC) vs. side scatter (SSC) dot plot. Positive transfection control samples containing Lipofectamine™ 2000 transfected cells were used to establish cytometer settings and channel compensations. Cell viability data were normalized in relation to the value of non-transfected control cells. For each sample, 10,000 events were collected. The experiments were carried out in triplicate for each condition.

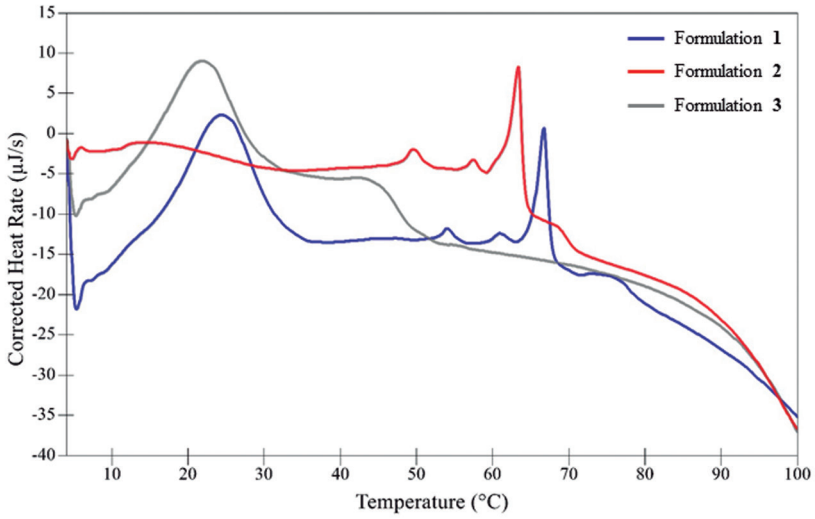
2.13. Statistical analysis

The statistical analysis was carried out using the IBM® SPSS® Statistics 25 software. The Shapiro-Wilk test was used to evaluate normal distribution, and the Levene test was used to evaluate homogeneity of variance. In parametric conditions, Student´s *t* test or ANOVA followed by the post-hoc HSD Tukey test was performed. In non-parametric conditions, the Kruskal-Wallis test and/or the Mann-Whitney *U* test for unpaired comparisons was performed. In all cases, *p* value ≤ 0.05 was considered statistically significant. Data were represented as mean ± standard deviation (SD).

3. Results

3.1. Characterization of the thermostability of niosomes

A



B

Formulation	Tm1 (°C)	Tm2 (°C)	Tm3 (°C)	Tm4 (°C)	Tm5 (°C)
1	24.47	54.24	60.82	66.66	75.4
2	15.57	49.59	57.5	63.17	69.01
3	21.76	44.28	--	--	--

Figure 2. (**A**) Thermograms of niosomes based on formulations **1** (blue line), **2** (red line) and **3** (grey line) in aqueous solution. Scan rate: 1.0°C/1 min. (**B**) Thermal melting temperature (Tm) data for the transitions of niosome formulations.

The thermostability of niosome formulations **1**, **2** and **3** was evaluated by differential scanning calorimetry. As shown in Figure 2, the thermogram of formulation **1** (blue line) was slightly shifted to the right compared to formulations **2** (red line) and **3** (grey line), indicating higher thermal stability than its counterparts (Figure 2A). Regarding their thermal melting temperatures (Tm), formulation **1** showed five well-defined peaks, while in the case of formulation **2** a first wide and weak peak around 15°C followed by four well-defined peaks were obtained. In the case of formulation **3** only two clear peaks were reported (Figure 2B). Remarkably, although slightly shifted to the right, formulation **1** coincided with formulation **3** in Tm1 and with formulation **2** in Tm2, Tm3, Tm4 and Tm5 (Figure 2A,B).

3.2. Rheological properties of niosomes

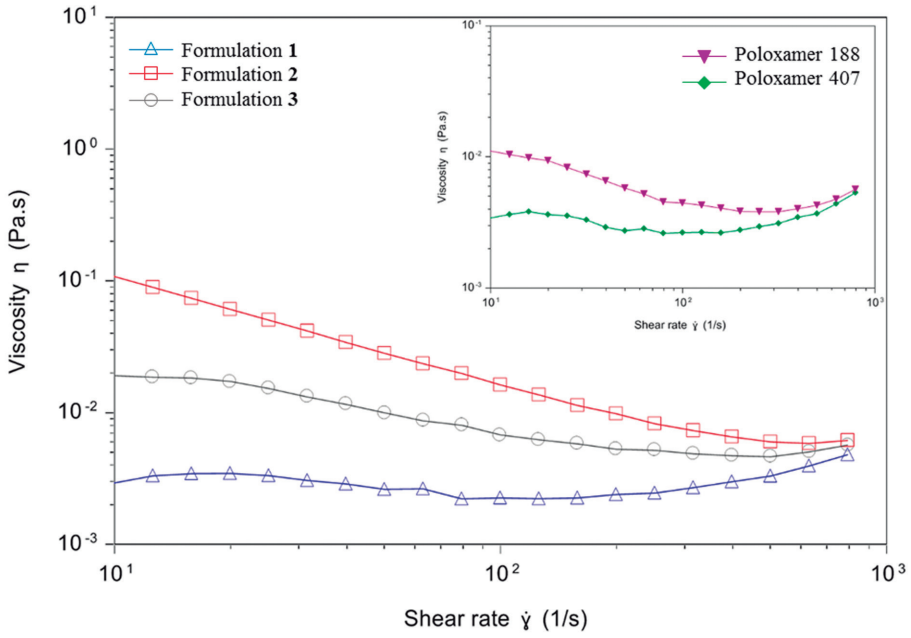
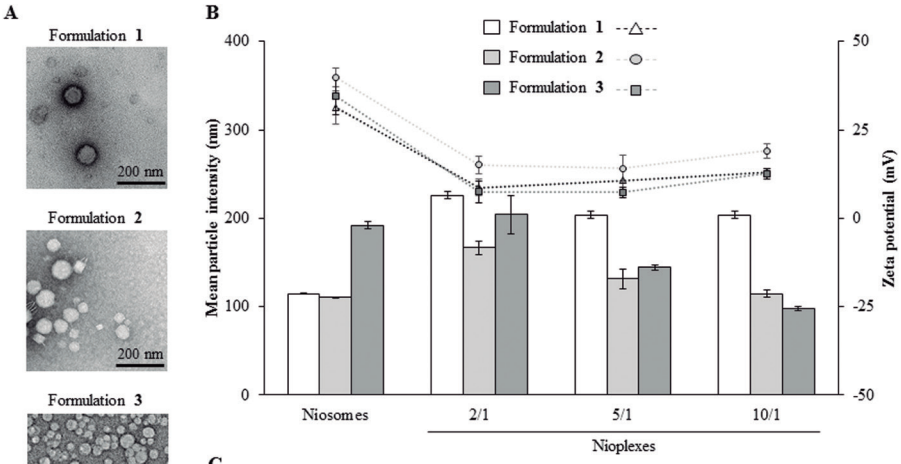


Figure 3. Rheology measurements. Main graph: viscosity curves of niosomes based on formulations **1** (blue line, triangles), **2** (red line, squares) and **3** (grey line, dots) expressed as a function of shear rate. Secondary graph: viscosity curves of poloxamer 188 (violet line, inverted triangles) and poloxamer 407 (green line, rhombus) expressed as a function of shear rate.

Rheological studies were performed in order to analyze the viscosity of niosomes based on formulations **1**, **2** and **3** as a function of the shear rate (Figure 3). Formulation **1** (blue line, triangles) showed the lowest viscosity values among the three formulations, and they remained quite stable when increasing the shear rate, indicating a Newtonian rheological behavior. On the contrary, formulations **2** (red line, squares) and **3** (grey line, dots) showed the higher initial viscosity values which declined when incrementing the shear rate, denoting a pseudoplastic rheological behavior. Regarding the rheological properties of the non-ionic surfactants employed in niosome formulations, poloxamer 188 (violet line), used to elaborate formulation **2**, showed higher viscosity values than poloxamer 407 (green line), used to elaborate formulations **1** and **3**.

3.3. Morphology, size, dispersity and superficial charge

Niosome formulations **1**, **2** and **3** showed a clear spherical shape without particle aggregation (Figure 4A). The mean particle size of niosome formulations **1**, **2** and **3** were 114.43 ± 0.64 nm, 110.40 ± 0.40 nm and 191.73 ± 4.11 nm, respectively (Figure 4B, bars). When complexing to pEGFP at the cationic lipid/DNA mass ratio 2/1, nioplexes based on formulation **1** showed a 2-fold increase in mean particle size, while nioplexes based on formulation **2** presented a more restrained increase of around 1.5-fold and nioplexes based on formulation **3** did not present a significant increase. When increasing the cationic lipid/DNA mass ratios to 5/1 and 10/1, mean particle sizes declined progressively, with a more pronounced slope in the case of nioplexes based on formulations **2** and **3**. In all cases, the mean size values of nioplexes based on formulation **1** were higher than the values based on formulations **2** and **3**. Zeta potential data of niosome formulations **1**, **2** and **3** were +31.37 ± 4.78 mV, +39.93 ± 2.64 mV and +34.63 ± 5.28 mV, respectively (Figure 4B, dots). After the addition of DNA, an initial decrease was observed at the cationic lipid/DNA mass ratio 2/1 and then values increased again with increasing cationic lipid/DNA ratios without reaching the original zeta potential of niosomes in all cases. Regarding dispersity (Đ), formulations **1** and **2** showed low values below 0.35, while formulation **3** had higher values both with and without DNA (Figure 4C).



C

	Niosomes		Nioplexes					
			2/1		5/1		10/1	
	Đ	SD	Đ	SD	Đ	SD	Đ	SD
Formulation 1	0.18	0.02	0.20	0.01	0.31	0.01	0.30	0.05
Formulation 2	0.13	0.02	0.32	0.01	0.29	0.05	0.20	0.01
Formulation 3	0.42	0.01	0.37	0.07	0.46	0.05	0.38	0.02

Figure 4. Characterization of niosomes based on formulations **1**, **2** and **3** and their corresponding nioplexes vectoring EGFP plasmid (pEGFP) at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1. (**A**) Transmission electron microscopy (TEM) images. Scale bar: 200 nm. (**B**) Mean particle intensity (bars) and zeta potential (symbols) values of niosomes and their corresponding nioplexes represented by the mean ± SD of three measurements. (**C**) Dispersity (Đ) values of niosomes and their corresponding nioplexes. Each value represents the mean ± standard deviation (SD) of three measurements.

3.4. Nioplexes membrane packing studies

Membrane GP values were determined in niosomes based on formulations **1**, **2** and **3** their corresponding nioplexes. As shown in Figure 5, formulation **1** niosomes showed negative GP value close to zero that increased at 2/1 and 5/1 cationic lipid/DNA mass ratio to values near 0.7 and 0.5, respectively. Such values decreased again to values around zero at 10/1 mass ratio values. Formulation **2** followed a similar pattern, with the lowest negative value in niosome formulation and, with higher, positive values at ratios 2/1 and 5/1 and a pronounced decrease to values near zero at the cationic lipid/DNA mass ratio 10/1. Formulation **3** presented an intermediate negative value in the niosome formulation and the lowest GP values near zero that switched to negative values at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1.

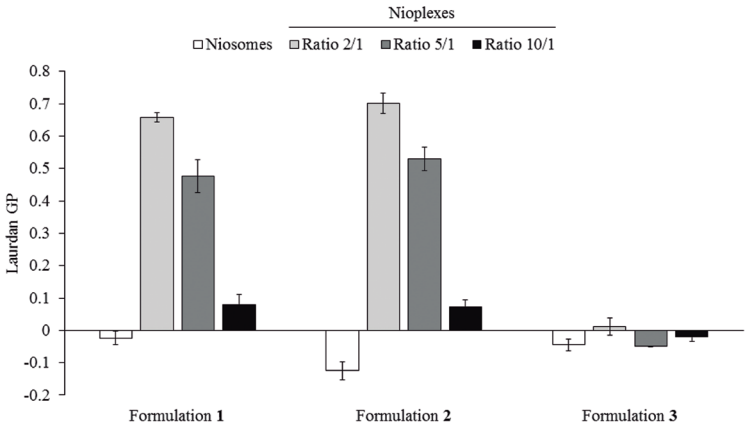


Figure 5. Laurdan general polarization (GP) values measured of niosomes based on formulations **1**, **2** and **3** and their corresponding nioplexes at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1.

3.5. Evaluation of niosome-pEGFP interactions by ITC

The interactions between DNA and niosome formulations **1**, **2** and **3** were followed by ITC, and the heat evolved per gram of DTPA injected as a function of the cationic lipid/DNA mass ratio is shown in Figure 6. Results showed that the titration profiles of niosomes **1** and **2** followed a similar trend, while a clear change was observed in the titration profile of formulation **3**, lacking chloroquine.

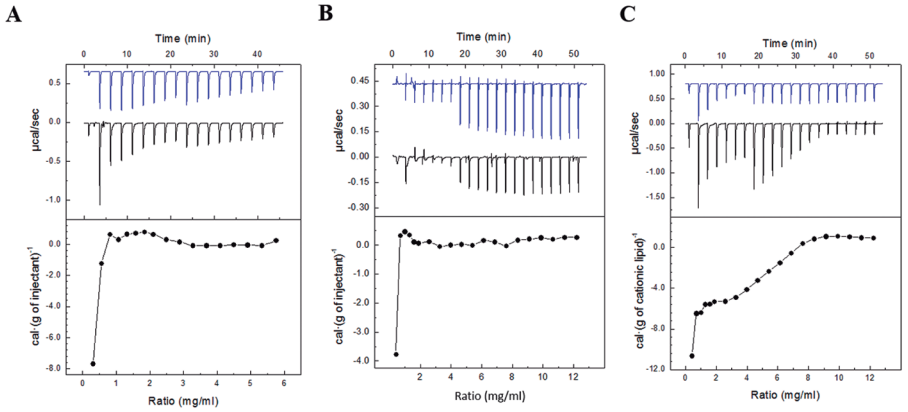


Figure 6. Isothermal titration calorimetry (ITC) of (**A**) formulation **1** (1:1 dilution), (**B**) formulation **2** and (**C**) formulation **3** into pEGFP. Upper panels show the raw data for the injection of respective formula into the plasmid solution (black line) or the blank solution (blue line). Lower panels show the dependence of the heat evolved by gram of cationic lipid injected as a function of the DTPA/pEGFP mass ratio in the sample cell (measures were carried out at 25 °C).

3.6. Buffer capacity and endosomal escape of nioplexes

In order to determine the ability to escape from intracellular endosomes, the buffer capacity and the DNA release profile from artificial endosomes of nioplexes based on formulations **1**, **2** and **3** were evaluated. Among the three niosomes, no significant differences were observed between formulations **1** (blue line, triangles) and **2** (red line, squares), while formulation **3** (grey line, dots) showed the lowest buffering capacity (Figure 7A). Regarding the ability of formulations to escape from endosomal compartment analogues based on PS, results showed that formulations released the DNA after the contact with the lipid membrane of the PS micelles, especially formulation **1**, which revealed the highest ability to escape from artificial endosomes at all cationic lipid/DNA mass ratios evaluated (Figure 7B).

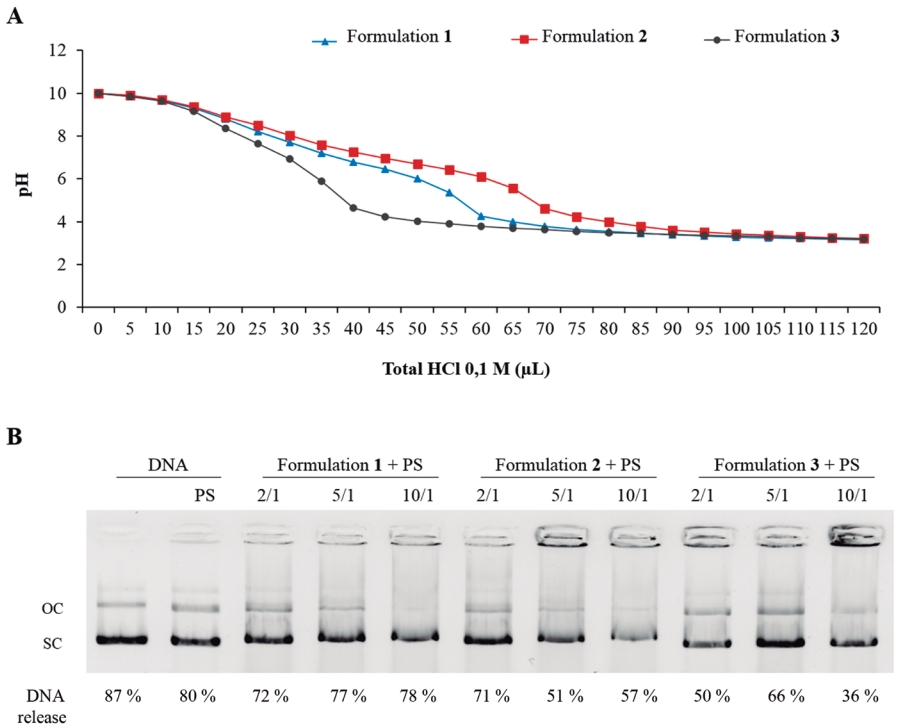


Figure 7. Buffer capacity and endosomal escape evaluation. (**A**) Analysis of pH buffering capacity of niosomes based on formulations **1** (blue line, triangles), **2** (red line, squares) and **3** (grey line, dots). (**B**) DNA release profiles in agarose gel electrophoresis assay of nioplexes based on niosome formulations **1**, **2** and **3** at cationic lipid/DNA mass ratios at 2/1, 5/1 and 10/1 from phosphatidylserine (PS) micelles. OC: open circular. SC: supercoiled.

3.7. Cell viability and transfection efficiency of nioplexes

Transfection and cell viability assays were performed during 7 days in CuFi-1 cells with nioplexes based on formulations **1**, **2** and **3** vectoring pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1. Although the highest ratio 10/1 sometimes achieved higher transfection efficiency, the best results in terms of desirable balance between efficiency and cell viability were obtained with the intermediate mass ratio 5/1 (Figure S1). Focusing on the cationic lipid/DNA mass ratio 5/1, formulations **1** and **2** showed similar fluorescence intensity values, and both reached the maximum fluorescence peak 72 h after transfection (Figure 8A, blue and red lines, respectively). After 72 h, the fluorescence intensity values declined slightly, but were relatively stable until the 7th day. Formulation **3** revealed lower fluorescence intensity values than its counterparts all over the 7 days of the experiment, and reached its maximum value 48 h after transfection (Figure 8A, grey line). Figure 8B shows representative images of CuFi-1 cells transfected with nioplexes **1**, **2** and **3** at the cationic lipid/DNA mass ratio 5/1 over time, with no or little transfection at 4 h and evident increase of EGFP positive cells from 24 h after transfection. Differences on the amount of EGFP positive cells between cells exposed to formulations **1** or **2** and to formulation **3** are also visible in these images. Finally, in order to quantify more precisely the differences in transfection efficiency and cell viability between nioplexes based on formulations **1** and **2**, further flow cytometry assays were conducted. Results showed that, at cationic lipid/DNA mass ratios 5/1 and 10/1, the percentage of EGFP expressing cells was significantly ($p \leq 0.05$) higher with nioplexes based on formulation **1** (21.02 ± 2.68 % and 28.22 ± 0.76 %, respectively) than with those based on formulation **2** (13.94 ± 0.56 % and 20.75 ± 1.68 %, respectively) (Figure 8C, bars). Regarding cell viability, formulation **1** also showed significantly ($p \leq 0.05$) higher percentages of live cells than formulation **2**, although in both cases values decreased when increasing the cationic lipid/DNA mass ratios (Figure 8C, dots). These results were further confirmed by the MFI values, where at cationic lipid/DNA mass ratios 5/1 and 10/1, formulation **1** also showed higher values than formulation **2** with significant differences (values around 400 vs. values around 300, respectively) (Figure 8D). Lipofectamine™ 2000 was used

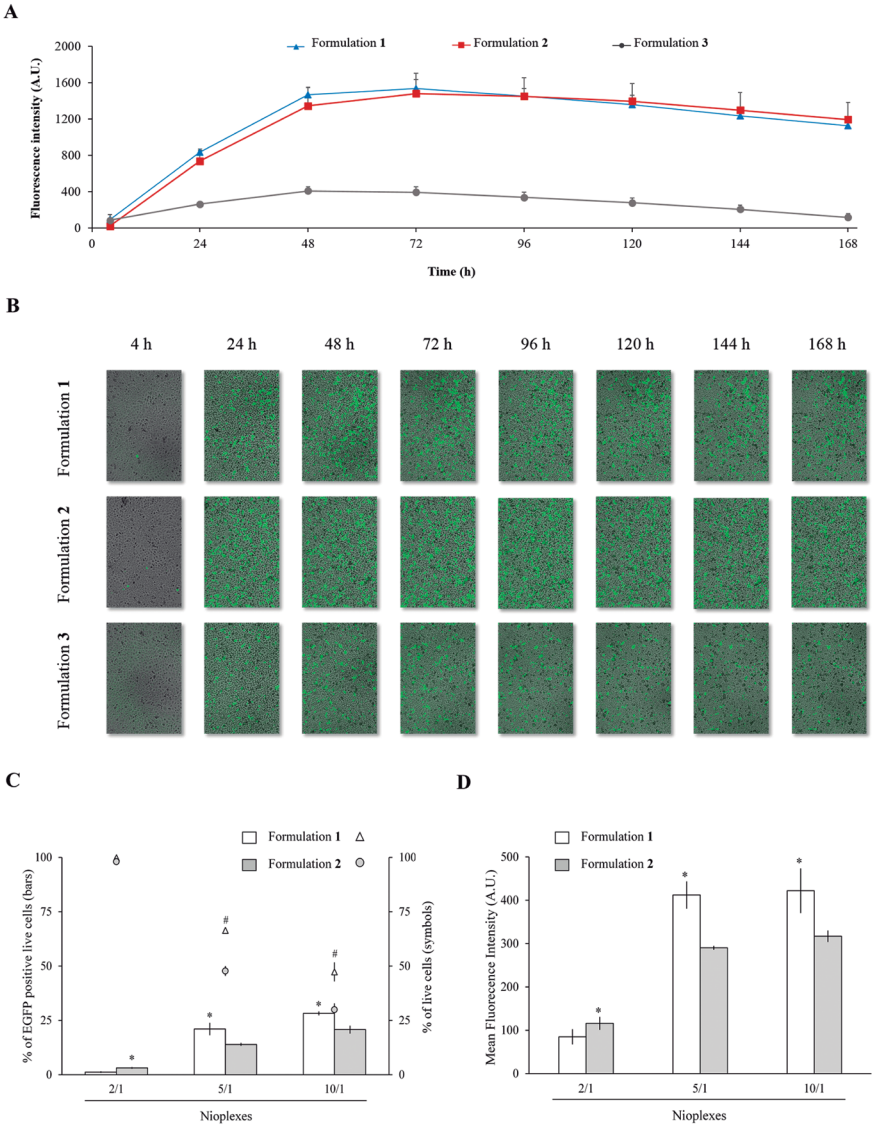


Figure 8. Transfection capacity and cell viability assays. (**A**) Fluorescence intensity over time in CuFi-1 cells transfected with nioplexes based on formulation **1**, **2** and **3** at cationic lipid/DNA mass ratio 5/1. Each value represents the mean ± SD of three measurements. (**B**) Fluorescence microscope images showing EGFP positive CuFi-1 cells transfected with nioplexes based on formulation **1**, **2** and **3** at cationic lipid/DNA mass ratio 5/1 and cellular appearance over time. (**C**) Percentages of EGFP positive live cells (bars) and cell viability (symbols) measured by flow cytometry in CuFi-1 cells 48 h after transfection with nioplexes **1** and **2** at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1. Each value represents the mean ± SD of three measurements. (**D**) Mean fluorescence intensity values. Each value represents the mean ± SD of three measurements. Statistical significance: $*p \leq 0.05$ in transfection and MFI; $\#p \leq 0.05$ in cell viability.

as a transfection positive control and showed 34.07 ± 2.52 % of EGFP expressing live cells, 65.36 ± 1.29 % of cell viability and a MFI value of 626.34 ± 26.30 (data not shown).

4. Discussion

In this work we determined, on the one hand, the influence of incorporating chloroquine and, on the other hand, the effect of varying non-ionic surfactant components and their phase of addition -aqueous or organic- on the biophysical performance of niosome formulations **1**, **2** and **3**. As it is well known by scientific community, chloroquine is a chemical compound that can promote the endosomal escape and enhance the transfection efficiency [42]. However, as far as we are concerned, how chloroquine affects the biophysical performance and correlate with their transfection efficiency of niosome formulations has not been assessed until now. It has also been well described that variations on chemical structure and composition, biophysical properties and preparation methods of lipid-based non-viral vectors can affect to their gene delivery efficiency and cytotoxicity both *in vitro* and *in vivo* [43]. In this respect, some works have described how liposomal formulations with different manufacturing process carrying amphotericin B resulted in significant differences in terms of physicochemical properties, efficiency and toxicity, despite the fact of presenting the same components in their formulations [44-46]. Additionally, the manufacturing processes required for the scale-up production of such formulations in order to reach clinical practice could also affect to their biological performance. To avoid this scenario, the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) specified the requirements required to obtain the bioequivalence of liposomal formulations [47] and compiled precise guidance documents for the complete characterization of nanosystems in order to guarantee the safety and efficacy of the new drugs based on nanomaterials [48,49]. All these requirements could also be applied to niosome formulations in order to track the manufacturing process as well as to evaluate their properties and activity, considering that they could represent an attractive alternative to liposomes with better properties for gene therapy purposes.

Regarding the chemical structure and composition of the niosome formulations **1**, **2** and **3** developed in the present work, the compounds used have been previously reported for nucleic acid delivery. In particular, the cationic lipid DTPA, which contains two saturated hydrocarbonated chains, a glycerol backbone and an amino as cationic head [18], has shown suitable properties for gene therapy [18,41,50,51]. As non-ionic surfactants polysorbate 80 and poloxamer 188 and 407 were included. Polysorbate 80 is one of the most employed non-ionic surfactant in niosomes because avoids nanoparticle aggregation, decreases the toxicity frequently associated to cationic lipids and improves the transfection efficiency due to its polyethylene glycol (PEG) chains [7,50,52]. Poloxamer 188 can prevent and repair membrane disruption and is useful to stabilize the lipid bilayer of niosomes [53-55], thereby improving the gene transfer efficiency [56]. Poloxamer 407 has been used in micro- and nanoparticles to stabilize and prolong the half-life of formulations as well as to prevent from aggregation [57,58] and to enhance the transfection process [59]. Regarding the HLB values of these non-ionic surfactants, poloxamer 188 (HLB = 28) has the highest value, followed by poloxamer 407 (HLB = 18-22) and, lastly, by polysorbate 80 (HLB = 15). Nevertheless, because of their amphiphilic nature, all of them could be incorporated either in the aqueous or in the organic phase during the preparation of nanoemulsions [39]. As "helper" component, formulations **1** and **2** included chloroquine due to its capacity to protect and interact with the DNA [60,61]. Moreover, chloroquine improves the endosomal escape impairing the fusion of endosomes and lysosomes because of its protonation inside the vesicles, causing a higher pH value that avoids the enzymatic lysosomal activity [30,62-65]. Therefore, in the present work, we hypothesized that chloroquine would not only act as a "helper" component, but also as a biophysical performance enhancer agent inside the niosomes. For this purpose, an exhaustive biophysical characterization is described in order to stablish its potential correlation with their biological activity.

First, niosome formulations **1**, **2** and **3** were prepared by the o/w emulsion technique and analyzed by DSC in order to determine their thermal stability and associated structural transitions. The Tm analysis, which corresponds to the maximum peak of endothermal events [66], showed relevant differences

among the three formulations. Such differences are commonly found in lipid nanoparticle dispersions with various components affecting molecular packing, which is reflected in the different melting points and enthalpies [67]. The "fine structure" of the thermogram in the region between 48°C and 70°C of formulations **1** and **2** is clearly associated with the effect caused by the chloroquine component on the disposition of lipids within the niosome. This effect could be related, on the one hand, to the higher packing shown by the lipids in both formulations and, on the other hand, to their higher transfection efficiency. The removal of chloroquine from formulation **3** caused disappearance of the well-defined transitions observed in that region and, instead, a possible transition between 40°C and 50°C was observed. On the other hand, the shift towards higher temperatures observed in formulation **1** could be due not only to the chloroquine content (formulation **1** vs. formulation **3**), but also to the substitution of poloxamer (188 in formulation **2**, by 407 in formulation **1**), and the incorporation of such surfactants in the organic phase (formulation **1**) instead of in the aqueous phase (formulation **2**). This could be related to its ability to stabilize the formulations, meaning that higher temperatures are needed in order to induce structural alterations in formulation **1** compared to its counterparts as indicated by the noticeable shift to the right of its thermogram [68]. In addition, the transition centered at 21.76°C (formulation **3**) or 24.47°C (formulation **1**) is possibly associated with the presence of poloxamer 407, since in formulation **2** a wide and low intensity transition is observed instead, which was centered around 15-16ºC and which could be due to the presence of poloxamer 188.

Next, rheological studies were conducted in order to evaluate the flow behavior of the niosome formulations. Formulation **2** appeared to be the most viscous among the three formulations, with values that clearly declined when increasing the shear rate, as it is common in solutions with a pseudoplastic rheological behavior [69]. Formulation **3** showed a similar pseudoplastic behavior, with lower viscosity values. Interestingly, formulation **1** showed the lowest and more constant viscosity values, which would indicate a Newtonian rheological behavior [69]. In this regard, the major viscosity values of formulation **2** could be in part attributed to its non-ionic surfactant poloxamer 188,

which has higher viscosity values than poloxamer 407 used in the preparation of formulations **1** and **3**. Therefore, these results suggested that the substitution of poloxamer 407 by poloxamer 188 and, probably, also its incorporation in the aqueous phase instead of the organic phase, increased the viscosity and affected to the rheological behavior of the formulation. On the other hand, the differences found between formulations **1** and **3** could only be due to the presence of chloroquine, as it is the only difference between both formulations. In this sense, the incorporation of chloroquine in formulation **1** significantly affects the arrangement of the lipid membrane, as evidenced by DSC studies and GP values (see below), modifying its rheological behavior. Taken together, rheological analysis suggests that the low viscosity value of formulation **1** could contribute, among many other physicochemical factors, to the highest transfection efficiency reported by this formulation. Such viscosity values would maintain constant at different shear stress due to the Newtonian rheological behavior [70].

Subsequently, in order to further understand the differences between the three formulations and their applicability for gene delivery purposes, niosomes **1**, **2** and **3** were complexed to the reporter pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1 to obtain their corresponding nioplexes. Their morphology, size, dispersity and zeta potential were studied, parameters that provide insights about the transfection capacity of the formulations. The size of all niosomes and nioplexes ranged between 100 and 230 nm, which it is appropriate to enhance the cellular uptake of the nanoparticles [71]. The positive zeta potential values of the three niosomes enhance electrostatic interactions with the plasmid DNA and, therefore, the nioplexes formation [72]. Additionally, these high positive values prevent particle aggregation and improve cellular internalization [73]. As predicted, surface charge data decreased after the addition of the genetic material due to the partial neutralization of positive charges of the cationic lipid amine groups by the negatively charged phosphate groups of the DNA [74]. Regarding dispersity, formulations **1** and **2** revealed narrow size distributions, as indicated by their low values, while formulation **3** showed higher values, indicating a more heterogeneous particle size distribution than its counterparts [7], which could be due to the lack of chloroquine.

The biophysical properties studied are closely related to another relevant parameter, specifically, to the niosome membrane packing. Therefore, we studied the membrane environment in niosome formulations **1**, **2** and **3** and their corresponding nioplexes vectoring pEGFP at cationic lipid/DNA mass ratios of 2/1, 5/1 and 10/1, by measuring the GP values. In niosomes measurements, the highest value was obtained by formulation **1** and the lowest by formulation **2**. The nioplexes values showed clear differences of formulations **1** and **2** respect to formulation **3**, especially at cationic lipid/DNA mass ratios of 2/1 and 5/1. Results revealed a more disordered membrane lipid packing profile in formulation **3** compared to formulations **1** and **2** after complexing with pEGFP, which showed GP values similar to the lipid packing values found in model membrane [75]. The highest membrane lipid packing profile was obtained by nioplexes based on formulations **1** and **2** with chloroquine content, yielding more condensed complexes than formulation **3**, devoid of chloroquine. In addition, the increase obtained in GP values from niosomes to nioplexes based in formulation **1** and **2**, could indicate that some ordered packing was formed via interaction of pDNA with the niosomes components, suggesting a relevant role of chloroquine in the packing of nioplexes possibly due to its ability to interact with the DNA [60,61]. Moreover, slight variations between formulations **1** and **2** could be attributed to the different poloxamer non-ionic surfactants content, since poloxamer 407 -used to prepare formulation **1**-, presents longer alkyl chains than poloxamer 188 -used to prepare formulation **2**-, which is often related to higher rigidity and, therefore, could contribute to increase the membrane packing of nioplexes based on formulation **1**. As indicated above, membrane packing is also related with other biophysical parameters studied in this work, such as thermal stability, viscosity and the rheological behavior of the formulations which, all together, could affect to the gene delivery process. In fact, it has been described that the presence of the drug astaxanthin in liposomes affects to the thermodynamic, viscoelastic and electrical properties of lipid membranes [76]. In our case, it could be assumed that the incorporation of chloroquine in the niosome formulation increases the membrane lipid packing of nioplexes as well as the thermal stability of formulations and, ultimately, enhances the transfection efficiency of these niosome formulations.

The effect of chloroquine incorporation in the interaction of niosome formulations with DNA was further supported by ITC results. Titration of niosome formulations into DNA showed that formulations **1** and **2** reached to a plateau or saturation point at DTPA/pEGFP mass ratios around 2/1-3/1, whereas saturation with formulation **3** required a mass ratio of 9/1, which suggests a less binding affinity for DNA molecules. Therefore, it can be concluded that the addition of chloroquine increased the DNA binding affinity of niosome formulations, which is in accordance with previous reports [4] and would also in part explain the higher transfection efficiency of these formulations compared to formulation **3**. Additionally, ITC results suggested that variations reported in the structural and functional properties of nioplexes based in formulations **1** and **2** at mass ratios above 2/1 likely reflect a redistribution of DNA leading to nioplexes with a decreasing fraction of plasmid molecules bound as niosome concentration was further increased. Besides, the titration results were in accordance with the results obtained from nioplexes packing since the presence of chloroquine promoted greater order of niosome membrane packing. Once characterized the capacity to bind DNA, agarose gel electrophoresis assays were performed to evaluate the ability to protect and release the genetic material from enzymatic degradation (Figure S2). Results showed that the three formulations were able to protect the genetic material against enzymatic degradation at cationic lipid/DNA mass ratios 5/1 and 10/1, but not at the lower mass ratio 2/1, suggesting that this ratio would not be suitable for gene delivery purposes. For subsequent cell transfection assays, we selected the cationic lipid/DNA mass ratio 5/1 in all formulations, because it reported enough capacity to protect the genetic material and contributed more than the higher ratio 10/1 to mitigate the cytotoxic effect sometimes associated to high amounts of cationic lipids [77].

The last parameters that were evaluated before moving on to biological assays were the buffer capacity and the endosomal escape ability from artificial PS micelles (mimicking cellular endosomes) of the niosome formulations. Considering that DNA quantity loaded in all wells was the same, the differences observed in the percentages of released DNA from nioplexes could be explained by the different chemical composition of the formulations. Among

the three niosomes, formulation **3** showed the lowest buffering capacity and the lowest ability to escape from artificial endosomes. This suggests that the incorporation of chloroquine in formulations **1** and **2** might enhance the endosomal escape via the proton sponge effect, which is a widely used strategy in formulations with a high buffering capacity, as is the case of these two formulations [14,30]. However, other possible endosomal escape mechanisms such as pore formation in the endosomal membrane, flip-flop mechanisms or fusion in the endosomal membrane mechanisms, among others, could also contribute to the endosomal escape behavior observed [78-80]. The ability to release the DNA once the formulations contact the lipid membrane of the endosomal compartment is essential for an efficient transfection process [41].

Finally, the biological performance of nioplexes **1**, **2** and **3** at cationic lipid/DNA mass ratio 5/1 was studied in CuFi-1 cells, because of the autosomal monogenic recessive condition of cystic fibrosis disease, which makes it particularly attractive for future gene therapy applications using niosomes as non-viral vectors [81]. In particular, the cell viability after exposure to nioplexes, the transfection efficiency of the formulations and the duration of gene expression were evaluated. Results clearly showed that the presence of chloroquine was necessary to achieve high transfection levels and, in addition, formulations with this component were well tolerated by the cells as indicated by the healthy cellular appearance along 7 days in cells exposed to formulations **1** and **2**. Generally, chloroquine is considered to be cytotoxic at concentrations superior to 100 μM, while it has been reported to enhance endosomal escape of polymeric nanoparticles at a concentration of 75 μM [82]. However, in this work cells were exposed to a concentration of chloroquine of 24.23 μM, which was enough to enhance endosomal escape and far away from the cytotoxic concentration. Considering that most niosome formulations enter the cells via endocytic pathways, which usually end in late endosomes, the ability to promote endosomal escape is essential in order to reach high transfection efficiencies [7]. Therefore, the endosomal escape observed in formulations **1** and **2** would in part explain the higher transfection efficiency of chloroquine-containing niosomes compared to formulation **3**, devoid of chloroquine.

Regarding the duration of gene expression, the fluorescence intensity values reached their maximum 48-72 h after transfection and then were maintained quite stable or declined slightly over time. Interestingly, the gene expression was maintained over seven days, which affects to the design of specific dosage regimens for gene therapy applications. Hence, the kinetics evaluation of transgene expression employing formulations **1**, **2** and **3**, corroborated the key role of chloroquine in this process. In order to further analyze the influence of non-ionic surfactants and the inversion of the addition phase on the biological performance of formulations **1** and **2**, additional more accurate flow cytometry studies were carried out at 48 h post-transfection [7]. Results showed significant better cell tolerance as well as higher EGFP positive cell percentages and MFI values in cells treated with formulation **1**, revealing this formulation as the best of the three evaluated for gene delivery purposes. This could be attributed to the specific biophysical advantages shown by formulation **1**, including its superior thermal stability and a Newtonian rheological behavior, as well as to other non-considered parameters such as the cell entry pathways or the cellular uptake capacity. Therefore, it could be deduced that not only the chloroquine content, but also the non-ionic surfactant chemical composition and protocol of incorporation also affects to the transfection efficiency of niosome formulations, although further research is needed in order to elucidate the exact mechanism of such effect. Hence, formulation **1**, which contains chloroquine in the aqueous phase and surfactants incorporated in the organic phase, might be an encouraging non-viral strategy for gene therapy aimed at cystic fibrosis disease. In this regard, previous *in vivo* studies carried out by our group showed a successful gene delivery capacity of formulation **1** in central nervous system [62], but an in depth biophysical study and implementation in congenital disease model, as cystic fibrosis, was a missing issue.

5. Conclusions

In conclusion, the main findings of the present study are obtained from the comparative evaluation, on the one hand, of formulations **1** and **2**, which differed on the composition of non-ionic surfactant and on the phase of addition of those components and, on the other hand, of formulations **1** and

3, which only differed in the presence or absence of the helper component chloroquine. Differences observed on formulations **1** and **3** clearly revealed the importance of chloroquine content in niosome vesicles since affected not only to physicochemical parameters but also to the biological performance of niosomes. Overall, results revealed that chloroquine improved thermal stability, lowered viscosity and reduced particle size and dispersity values. In addition, chloroquine content improved DNA binding affinity and membrane packing organization in corresponding nioplexes. Such relevant parameters along with the enhanced buffering capacity could explain, at least in part, the highest transfection efficiency values reported when chloroquine was incorporated into the niosome formulations. The increased capacity of such niosomes that contain chloroquine to escape from artificial endosomes could also contribute to improve gene delivery efficiency. Thus, chloroquine emerges as an interesting material able to improve the biophysical properties and the transfection efficiency of niosomes for non-viral gene therapy applications. On the other hand, the effect of both chemical composition and protocol of incorporation of non-ionic surfactants (formulation **1** vs. formulation **2**), resulted in subtle biophysical variations, although formulation **1** showed better thermal stability, lower viscosity values with Newtonian rheological behavior and higher transfection efficiency. Taken together, these results support the requirements of the regulatory agencies for the complete characterization of nanoparticles aimed for biomedical applications and scaling-up. Of note, the biophysical parameters evaluated for full physicochemical and biological characterization of niosomes and their corresponding nioplexes, could be used as gold standard for further gene therapy nanosystems evaluation. Hence, the present work provides an in depth analysis of different biophysical parameters and characterization strategies that are relevant for nanosystems in gene delivery purposes and that might be interesting to include in the specifications of regulatory agencies for the evaluation of new drugs based on nanomaterials.

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Supplementary materials

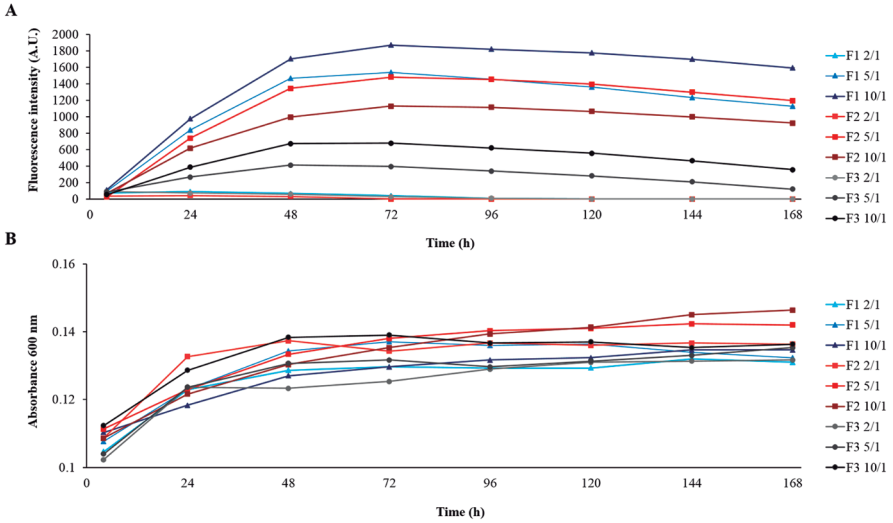

Figure S1. Transfection efficiency and cell viability assays in CuFi-1 cells transfected with nioplexes based on formulation **1** (F1), **2** (F2) and **3** (F3) at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1. (**A**) Fluorescence intensity over time. Each value represents the mean ± SD of three measurements. (**B**) Mean absorbance curves over time. Each value represents the mean ± SD of three measurements.

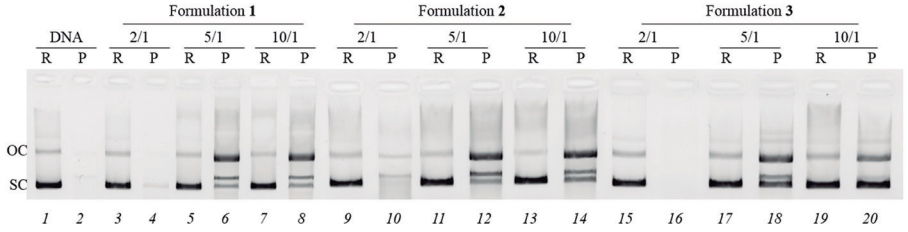

Figure S2. Protection and SDS-induced release of DNA in niosome formulations **1**, **2** and **3** complexed to pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1 visualized by agarose gel electrophoresis. Lanes 1-2 correspond to naked DNA; lanes 3-4 to formulation **1** at ratio 2/1; lanes 5-6 to formulation **1** at ratio 5/1; lanes 7-8 to formulation **1** at ratio 10/1; lanes 9-10 to formulation **2** at ratio 2/1; lanes 11-12 to formulation **2** at ratio 5/1; lanes 13-14 to formulation **2** at ratio 10/1; lanes 15-16 to formulation **3** at ratio 2/1; lanes 17-18 to formulation **3** at ratio 5/1; lanes 19-20 to formulation **3** at ratio 10/1. Nioplexes were treated with SDS (lanes 3, 5, 7, 9, 11, 13, 15, 17 and 19) and DNase I + SDS (lanes 4, 6, 8, 10, 12, 14, 16, 18 and 20). Upon the addition of SDS, naked DNA (lane 1) completely migrated in the gel, and so did the DNA complexed to the formulations at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1 as indicated by the intense supercoiled (SC) signal (lanes 3, 5, 7, 9, 11, 13, 15, 17 and 19). After the addition of the enzyme DNase I, no DNA signal was observed in the case of naked DNA (lane 2) and very faint or no signal was neither observed in the case of DNA complexed to formulations **1**, **2** and **3** at cationic lipid/DNA mass ratio 2/1 (lanes 4, 10 and 16, respectively). However, the intense DNA bands observed with DNA complexed to formulations **1**, **2** and **3** at cationic lipid/DNA mass ratio 5/1 (lanes 6, 12 and 18, respectively) as well as with DNA complexed to formulations **1**, **2** and **3** at cationic lipid/DNA mass ratio 10/1 (lanes 8, 14 and 20, respectively), indicated that the genetic material was protected and released by the formulations at those higher cationic lipid/DNA mass ratios. R: DNA release. P: DNA protection. OC: open circular. SC: supercoiled.

Appendix 4

Non-viral mediated gene therapy in human cystic fibrosis airway epithelial cells recovers chloride channel functionality

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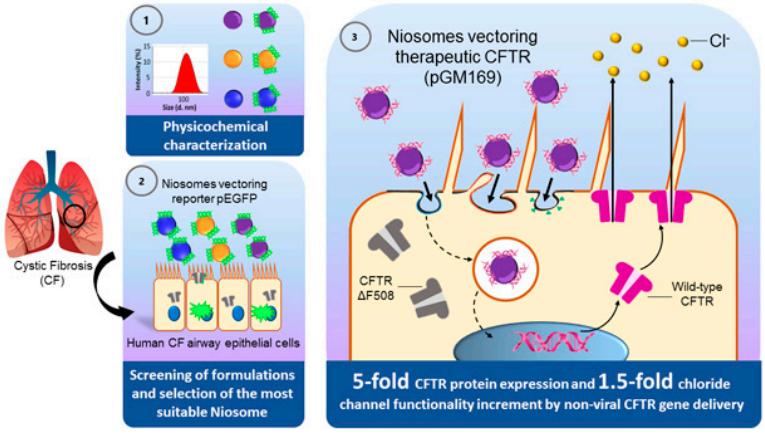
Graphical abstract



Non-viral mediated gene therapy in human cystic fibrosis airway epithelial cells recovers chloride channel functionality

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ABSTRACT

Gene therapy strategies based on non-viral vectors are currently considered as a promising therapeutic option for the treatment of cystic fibrosis (CF), being liposomes the most commonly used gene carriers. Niosomes offer a powerful alternative to liposomes due to their higher stability and lower cytotoxicity, provided by their non-ionic surfactant and helper components. In this work, a three-formulation screening is performed, in terms of physicochemical and biological behavior, in CF patient derived airway epithelial cells. The most efficient niosome formulation reaches 28 % of EGFP expressing live cells and follows caveolae-mediated endocytosis. Transfection with therapeutic cystic fibrosis transmembrane conductance regulator (CFTR) gene results in 5-fold increase of CFTR protein expression in transfected versus non-transfected cells, which leads to 1.5-fold increment of the chloride channel functionality. These findings highlight the relevance of niosome-based systems as an encouraging non-viral gene therapy platform with potential therapeutic benefits for CF.

Keywords: gene therapy • non-viral • niosomes • CFTR • cystic fibrosis

1. Introduction

Nowadays, some gene therapy products have already reached approval for human use and are commercially available (Dunbar et al., 2018). Therefore, this kind of strategy is recognized as a realistic medical option for the treatment of both inherited and acquired human diseases. However, in order to make gene therapy evolve into a leading therapeutic option in clinical practice, still some relevant issues need to be addressed, especially those related to the design and development of safe and effective gene carriers.

Gene therapy results particularly attractive for the treatment of inherited and monogenic diseases such as cystic fibrosis (CF), an autosomal recessive condition, caused by different mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Although the gene was identified more than 30 years ago, currently, there is no curative treatment (Cooney et al., 2018). New pharmaceuticals such as Kalydeco® (ivacaftor), Orkambi® (lumacaftor/ivacaftor), and Symdeko® (tezacaftor/ivacaftor) have given substantial benefit to some people with CF, contributing to improve both quality and expectancy of life (Donnelley and Parsons, 2018). Although these approaches alleviate or minimize the consequences of CF, they are not able to repair the underlying genetic defect. In this sense, gene therapy is aimed at transferring correct copies of the CFTR gene into airway epithelial cells, targeting the genetic origin of the disease. Earliest clinical trials in CF patients have established proof-of-principle for transfer of the wild-type CFTR gene to human airway epithelial cells using liposome based non-viral vectors (Alton et al., 2015). Therefore, non-viral gene therapy for CF could become a real medical option as an alternative to viral vectors, due to relevant advantages including, among others, high nucleic acid packing capacity required to deliver CFTR gene, low immunogenicity which allows repeated dose administration and low costs that allow the access of gene therapy products to the general population (Pezzoli et al., 2012).

Among the wide variety of nanosized materials that can be employed to develop non-viral vectors, cationic lipid based niosomes are considered promising candidates for gene delivery purposes due to their high biocompatibility

and long-term stability (Ojeda et al., 2016). The preparation of these formulations requires three main components: a cationic lipid – to allow electrostatic interactions with negatively charged biomolecules such as DNA (Karmali and Chaudhuri, 2007), a helper component – to improve the biological performance of the formulation (Dabkowska et al., 2012) and non-ionic surfactants – to prevent particle aggregation and enhance the stability of the formulation (Liu et al., 1996). Different combinations of those components, along with the variety in their chemical composition, affect to the physicochemical parameters of niosome formulations, including size, surface charge and morphology. In turn, these characteristics determine the biological properties of niosomes, such as their cellular uptake, intracellular trafficking and the ability to deliver the DNA cargo in suitable subcellular compartments (Dabkowska et al., 2012). Along with the design of proper non-viral vectors for gene therapy purposes, the size and composition of the genetic material to deliver have to be considered. In this sense, several efforts have been conducted in order to enhance the performance of conventional plasmids, including the removal of bacterial backbones, the optimization of the promoters or the elimination of CpG islands, which confer not only lower immunogenicity but also higher transfection efficiencies and sustained transgene expression (Gallego et al., 2019; Hyde et al., 2008). The therapeutic pGM169 plasmid, which encodes for a correct copy of the CFTR gene, is currently used in CF clinical trials as an optimized gene construct vectored by liposome based non-viral vectors (Alton et al., 2015; Hyde et al., 2008). In this regard, niosome based formulations offer a promising alternative to liposomes due to their enhanced stability and lower cytotoxicity, provided by the non-ionic surfactant and the helper component (Bartelds et al., 2018; Choi et al., 2004).

Taking all those factors into account, our goal was to develop a suitable non-viral formulation based on niosomes able to transfer efficiently correct copies of the CFTR gene into human CF airway epithelial cells. For that purpose, in this work, we designed and prepared niosome formulations **1**, **2** and **3** -named as N**1**, N**2** and N**3**- based on different cationic lipids, helper components and non-ionic surfactants. The resulting niosomes were bound to different DNA constructs of interest, depending on the assay, to form the

corresponding nioplexes. Niosomes and nioplexes were physicochemically characterized in terms of size, dispersity, zeta potential and morphology. *In vitro* experiments were carried out in human CF airway epithelial cells to evaluate the transfection efficiency and cell viability, as well as the cellular uptake and intracellular trafficking of nioplexes. Additionally, transfection efficiency was further evaluated through both, the CFTR transgene and protein quantification and the biological activity of the CFTR channel.

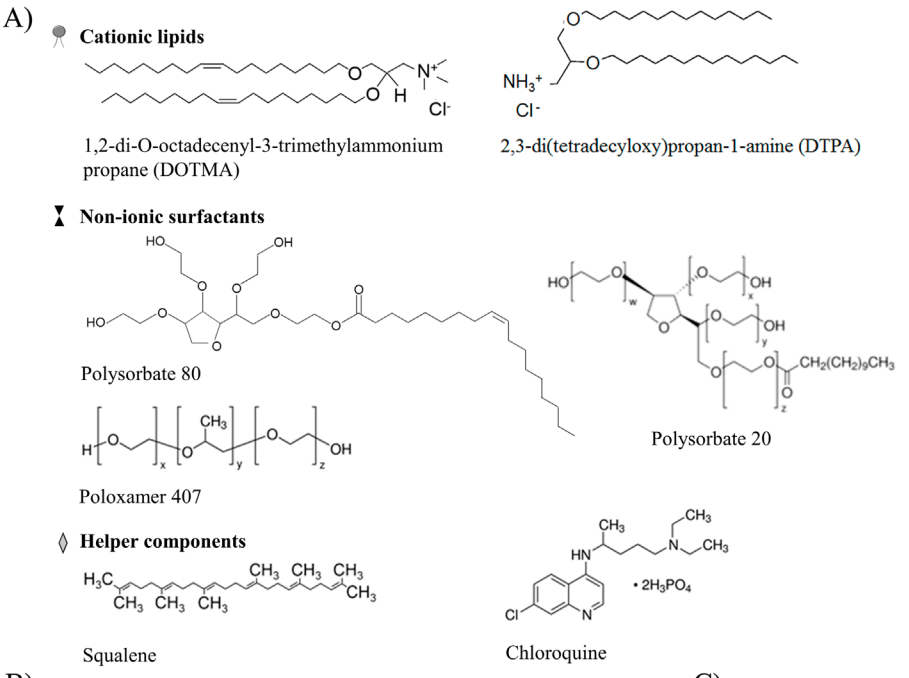
2. Materials and methods

2.1. Preparation of niosomes and nioplexes

Niosome formulations were prepared by the oil-in-water emulsion technique as previously described (Ojeda et al., 2016). Fig. 1 indicates the chemical structure (Fig. 1A), the components used for the elaboration of N**1**, N**2** and N**3** formulations (Fig. 1B) and a general scheme showing the distribution of the components in niosomes (Fig. 1C). The organic phase of N**1** and N**2** contained the cationic lipid 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) (6.7 mg) (Avanti Polar Lipids, USA) and helper lipid squalene (19 µL) (Sigma-Aldrich, USA) dissolved in dichloromethane (DCM) (1 mL) (Panreac, Spain). In contrast, the organic phase of N**3** contained the cationic lipid 2,3-di(tetradecyloxy)propan-1-amine (DTPA) (5 mg) tailor-synthesized (Ojeda et al., 2016), non-ionic surfactants polysorbate 80 (12.5 mg) (Panreac, Spain) and poloxamer 407 (12.5 mg) (Sigma-Aldrich, USA), all dissolved in DCM (1 mL). The water phase of N**1** and N**2** contained, respectively, polysorbate 20 (0.49 %) (Sigma-Aldrich, USA) and polysorbate 80 (0.52 %) dissolved in distilled water (5 mL). The water phase of N**3** contained the helper component chloroquine diphosphate salt (2.5 mg) (Sigma-Aldrich, USA) dissolved in distilled water (5 mL). In all formulations, the organic phase and the water phase were emulsified by sonication (Branson Sonifier 250, Danbury) for 30 s at 50 W. The organic solvent was removed from the emulsion by evaporation under magnetic agitation for 1 h at room temperature.

The nioplexes were formed by mixing an appropriate volume of a stock solution of DNA with different volumes of the niosome formulation to obtain,

the following cationic lipid/DNA mass ratios: 2/1, 5/1 and 10/1. Niosomes and DNA were incubated for 30 min at room temperature to enhance electrostatic interactions and allow the formation of nioplexes.



		Cationic lipids		Non-ionic surfactant			Helper components	
		DOTMA	DTPA	Polysorbate 20	Polysorbate 80	Poloxamer 407	Squalene	Chloroquine
Niosomes	N1	✓		✓			✓	
	N2	✓			✓		✓	
	N3		✓		✓	✓		✓

Fig. 1. Overview of niosomes N**1**, N**2** and N**3**. **A**. Chemical structures of the components. **B**. Description of the components that constitute each niosome. **C**. General scheme of the disposition of components in a niosome.

2.2. Genetic material

Four plasmid constructs DNA were used in this work: pCMS-EGFP (5.5 kb, PlasmidFactory, Germany), fluorescence isothiocyanate (FITC) labelled pCMS-EGFP (DareBio, Spain), pEGFP-CFTR (9.1 kb, kindly provided by Unit of Viral Infections and Comparative Pathology at University of Lyon 1) and

pGM169 (6.5 kb, CpG free CFTR plasmid kindly gifted from Professor Deborah Gill and co-workers at the University of Oxford from UK Cystic Fibrosis Consortium).

pCMS-EGFP, named as pEGFP, was used for the initial formulation screening assays. FITC labelled pCMS-EGFP, named as FITC-pEGFP, was used for cellular uptake and intracellular trafficking studies. pEGFP-CFTR and pGM169 were used for gene transfection efficiency and CFTR channel functional assessment.

The pEGFP and pEGFP-CFTR plasmids used in this work were propagated in *Escherichia coli* DH5α and One Shot™ Stbl3™ chemically competent *Escherichia coli*, respectively, and purified using the Qiagen endotoxin-free plasmid purification Maxi-prep kit (Qiagen, Santa Clarita, CA, USA) according to manufacturer's instructions. The concentration of pDNA was quantified by measuring the absorbance at 260 nm with a SimpliNano™ device (GE Healthcare, Buckinghamshire, UK).

2.3. Size, dispersity, zeta potential and morphology

The hydrodynamic diameter, which includes the particle size and dispersity (Đ), and the zeta potential of both niosomes and their corresponding nioplexes were determined by Dynamic Light Scattering (DLS) and by Lasser Doppler Velocimetry (LDV), respectively, using a Zetasizer Nano ZS (Malvern Instrument, UK) and the morphology of niosomes was determined by transmission electron microscopy (TEM), all as previously described (Puras et al., 2014).

2.4. Cell culture and transfection assays

Human CF airway epithelium cells (CuFi-1) (ATCC® CRL-4013™), a specific CF patient derived cell model homozygous for the ΔF508 mutation (Buchanan et al., 2013) were grown in bronchial epithelial growth medium -2 bulletkit- (BEGM) (Lonza, Basel, Switzerland) previous flask/plate pretreatment with collagen type IV (Sigma-Aldrich, USA). Cells were incubated at 37°C and 5 % CO_2 atmosphere, and were split every 3-4 days to maintain monolayer coverage.

For transfection efficiency assays, cells were seeded in 24-well plates at a density of 1.6×10^5 cells per well and incubated overnight to achieve 70 % of confluence at the time of transfection with nioplexes at cationic lipid/DNA mass ratio 2/1, 5/1 and 10/1. For that purpose, electrostatic interactions between the cationic lipid and the corresponding plasmid were left to occur for 30 min at room temperature in Opti-MEM (Gibco, San Diego, CA, USA) transfection medium. Once removing the growth medium from the plate, cells were exposed for 4 h to nioplexes containing DNA (1.25 µg per well). After the incubation, nioplexes were removed and fresh medium was added to the cells. Transfection efficiency was analyzed 48 h after the exposure to nioplexes. Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was used as positive control. Transfection negative control cells were not exposed to nioplexes, but were incubated in Opti-MEM for 4 h. Each condition was performed in triplicate.

2.5. Analysis of EGFP expression and cell viability

Qualitative analysis of EGFP signal was conducted using an inverted microscope equipped with an attachment for fluorescence observation (EclipseTE2000-S, Nikon). For quantitative determination of EGFP expression and cell viability, flow cytometry analysis was conducted using a FACSCalibur system flow cytometer (Becton Dickinson Bioscience, San Jose, USA). For that purpose, N**1**, N**2** and N**3** nioplexes-transfected cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) with calcium and magnesium (Lonza, Basel, Switzerland) and detached with trypsin/EDTA (200 µL per well, 0.25 %) (Gibco, San Diego, CA, USA). Trypsin inhibitor (500 µL DPBS with 1 % of fetal bovine serum) (Gibco, San Diego, CA, USA) were added and cells were centrifuged at 1,100 rpm for 5 min. The resulting pellet was resuspended in BEGM (500 µL) and transferred to specific flow cytometer tubes. Cell viability was evaluated using propidium iodide (Sigma-Aldrich, USA) at 1:300 dilution in each sample. The fluorescent signals corresponding to dead cells and to EGFP positive cells were measured at 650 nm (FL3) and 525 nm (FL1), respectively. Non-transfected cells, used as control samples, were displayed on a forward scatter (FSC) versus side scatter (SSC) dot plot to establish a collection gate and exclude cells debris. Positive transfection control samples transfected with

Lipofectamine™ 2000 were used to establish cytometer settings and channel compensations. Cell viability data were normalized in relation to the value of non-transfected control cells. The experiments were carried out in triplicate for each condition. For each sample 10,000 events were collected.

2.6. Cellular uptake

The cellular internalization of nioplexes bound to FITC-pEGFP was analysed both qualitatively and quantitatively by fluorescence microscopy and flow cytometry, respectively. For qualitative assays, human CF cells were seeded on coverslips in 24 well plates and transfected with nioplexes for 4 h. After that, cells were washed with phosphate buffered saline (PBS, Gibco, San Diego, CA, USA) and fixed with 4 % formaldehyde (Panreac, Spain). Then, cells were washed twice with PBS and incubated for 40 min with phalloidin (5 µL) diluted in a 1 % bovine serum albumin (BSA) in PBS solution to stain cells cytoskeleton. Once washing with PBS, cells were mounted with Fluoroshield™ with DAPI (Sigma-Aldrich, USA) and observed under Zeiss Axio Observer fluorescence microscopy with Apotome 2 illumination system (Zeiss Axiobserver, Jena, Germany). Captured images were analysed with ImageJ software.

For quantitative analyses, a FACSCalibur system flow cytometer was used to quantify the percentage of cells that contained FITC-labelled EGFP coding plasmid DNA. Cells were detached and processed for flow cytometry measurements as previously mentioned. Cellular uptake was expressed as the percentage of FITC-labelled plasmid positive cells after excluding dead cells and analysed as described above.

2.7. Intracellular trafficking

For cellular internalization assays, human CF cells were transfected with the FITC-labelled pEGFP for 3 h over coverslips, as aforementioned. Then, the following endocytic vesicle markers were added and incubated for 1 h with either Transferrin-Alexa Fluor™ 594 (50 µg mL^{-1}), Cholera toxin B-Alexa Fluor™ 594 (10 µg mL^{-1}) or Dextran-Alexa Fluor™ 594 (1 µg $µL^{-1}$), which are markers for clathrin mediated endocytosis (CME), caveolae mediated endocytosis (CvME) and macropinocytosis, respectively. After that, cells were washed with

PBS, and then, they were fixed, mounted with and observed under fluorescence microscopy as described above. Co-localization of the green and red signal was analyzed by ImageJ software and quantified by a cross-correlation analysis, as previously described (van Steensel et al., 1996; Villate-Beitia et al., 2018). Incubation with the endocytic vesicle markers without previous transfection with FITC-pEGFP was carried out to determine the quantity of clathrins, caveolae or macropinocytosis vesicles in human CF cells by flow cytometry analyzing 10,000 events per sample.

2.8. CFTR protein quantification

The protein expression of CFTR (150-170 kDa) from cell cultures was analyzed by Western blot. For that, cells were seeded in 6-well plates at a cell density of 8×10^5 per well and transfected with nioplexes vectoring pGM169. After trypsinization and centrifugation, total protein was extracted from the resulting pellet with lysis buffer (TRIS 1 mM, NaCl 15 mM, EDTA 0.2 mM, 2 % Triton X-100) and protease inhibitor EDTA-free cocktail 1 % (Roche, Germany), which was then placed on ice for 30 min. After that, the contents were centrifuged for 10 min (10,000 g) at 4°C and the supernatant was employed for protein quantification by a Pierce™ BCA protein assay kit (Thermo Fisher, USA), according to the manufacturer instructions. Protein containing Laemmli buffer (30 µg) (Bio-Rad, USA) was loaded into a SDS-PAGE, 7 % acrylamide, midi-PROTEAN® TGX™ electrophoresis gel (Bio-Rad, USA) and separated at 130 V for 75 min. Proteins were transferred in a semi-dry system (Trans-Blot® Turbo™ Transfer System, Bio-Rad, USA) to a PVDF membrane which was then blocked with 5 % non-fat milk in TBST 0.1 % for 1 h at room temperature.

A monoclonal mouse anti-human/-mouse against CFTR (CF3; Thermo Fisher, USA) at a dilution of 1:500 in blocking solution, was incubated with the membrane overnight at 4°C. Bands were detected using peroxidase-conjugated goat anti-mouse IgG (1:10,000; Bio-Rad, USA) secondary antibody diluted in blocking solution. Wash steps were carried out with TBST 0.1 %. Specific protein expression was detected by chemiluminescence with ECL Plus (Bio-Rad, USA) and observed in a ChemiDoc System (Bio-Rad, USA). β-actin

was used as a loading control, employing a rabbit polyclonal anti β-actin antibody (1:400, Abcam) followed by peroxidase-conjugated secondary antibody (1:20,000, GE Healthcare, UK). Densitometric evaluation of bands was performed using the Image Lab™ 4.0.1 software. Data were expressed as arbitrary densitometric units (ADU) relative to β-actin expression.

2.9. CFTR channel functionality

For CFTR channel functionality, human CF cells were seeded in a 96-well plate at a density of 3×10^4 cells and incubated overnight. Cells were transfected with nioplexes vectoring pGM169. After 48 h, CFTR chloride-channel activity was measured by monitoring iodide efflux evolution along time, which is commonly used as surrogate for chloride (Mitomo et al., 2010). For that purpose, cells were washed twice with Hanks' Balanced Salt Solution (HBSS) and incubated for 45 min with 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) (10 mM) fluorescent molecule (ThermoFisher Scientific, USA) diluted in 1:1 NaI buffer:Milli-Q® water hypotonic solution. Then, cells were washed with the NaI buffer [NaI (130 mM), KNO_3 (4 mM), $Mg(NO_3)_2$ (1 mM), $Ca(NO_3)_2$ (1 mM), glucose (10 mM) (Sigma-Aldrich, USA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (20 mM) (Lonza, Basel, Switzerland) in MilliQ® water (200 mL)] and incubated for 30 min in fresh NaI buffer. After that, cells were washed again twice with the NaI buffer and incubated for 20 min in nitrate buffer, which consisted of NaI buffer but replacing NaI by $NaNO_3$ (130 mM) and supplemented with the CFTR channel agonists forskolin (FSK) (10 μM) and 3-isobutyl-1-methylxanthine (IBMX) (200 μM) (Sigma-Aldrich, USA). In order to revert the basal state, nitrate buffer was replaced by NaI buffer. The fluorescent signal emitted by SPQ ($n \geq 5$) was read at 443 nm in an Infinite M200 microplate reader (TECAN Trading AG. Männedorf, Switzerland) employing the Tecan i-control 1.7 software.

2.10. Statistical analysis

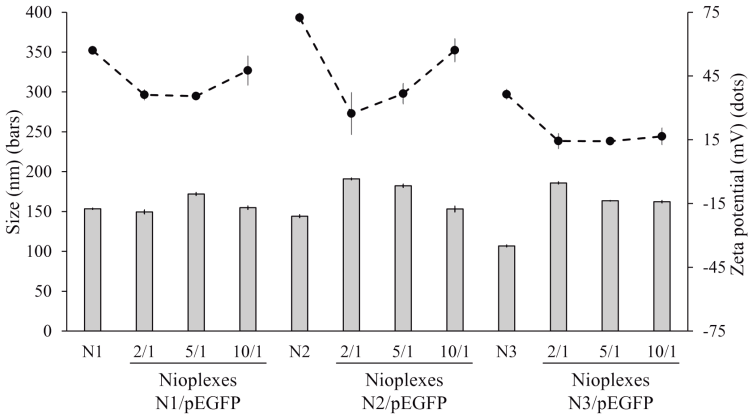
Statistical analysis was carried out using IBM® SPSS® Statistics 25 software. Normal distribution of samples was assessed by the Kolmogorov-Smirnov test, and the homogeneity of the variance by the Levene test. Under parametric conditions, one-way ANOVA followed by post-hoc HSD Tukey test

was performed. Otherwise, the non-parametric Kruskal-Wallis test followed by Mann-Whitney *U* test for 2-2 comparisons was employed. In all cases, *p* values ≤ 0.05 were regarded as significant. Data were presented as mean ± standard deviation (SD).

3. Results

3.1. Screening of formulations in terms of physicochemical characterization

A)



B)

	N1	Nioplexes N1/pEGFP			N2	Nioplexes N2/pEGFP			N3	Nioplexes N3/pEGFP		
		2/1	5/1	10/1		2/1	5/1	10/1		2/1	5/1	10/1
Mean Đ	0.14	0.21	0.26	0.18	0.21	0.34	0.39	0.32	0.16	0.36	0.37	0.32
SD	0.01	0.03	0.01	0.01	0.01	0.01	0.03	0.02	0.01	0.02	0.01	0.01

Fig. 2. Physicochemical characterization, transfection efficiency and cell viability of N**1**, N**2** and N**3** niosomes and nioplexes vectoring pEGFP at different cationic lipid/DNA mass ratios. **A**. Particle size (bars) and zeta potential (dots) of niosomes and their corresponding nioplexes. Each value represents the mean ± SD of three measurements. **B**. Dispersity (Đ) values of niosomes and their corresponding nioplexes. Each value represents the mean ± SD of three measurements. **C**. Percentage of EGFP positive live cells (bars) and cell viability (dots) evaluated by flow cytometry. Data are presented as mean ± SD, n = 3.

Based on previous experiments (Mashal et al., 2017; Ojeda et al., 2016; Puras et al., 2014), we selected and prepared three niosomes N**1**, N**2** and N**3** as starting formulations for the preparation of nioplexes. N**1** and N**2** contained DOTMA as cationic lipid and squalene as helper lipid (Fig. 1). They differ in

the use of Polysorbate 20 (N**1**) or Polysorbate 80 (N**2**) as non-ionic surfactant. Niosome N**3** was prepared using DTPA as cationic lipid, chloroquine as helper component and Polysorbate 80 and Poloxamer 407 as non-ionic surfactants.

The physicochemical analysis of formulations revealed that the mean particle size of N**1**, N**2** and N**3** niosomes were 153.47 ± 0.76 nm, 144.17 ± 1.89 nm and 106.93 ± 0.31 nm, respectively (Fig. 2A, bars). Upon the addition of pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1, the mean particle size values remained similar in N**1**, meanwhile slightly increased in N**2** and N**3** at 2/1 ratio and steadily decreased when augmenting the cationic lipid/DNA ratio (Fig. 2A, bars). Zeta potential values of N**1**, N**2** and N**3** niosomes were +57.03 ± 0.51 mV, +72.43 ± 2.16 mV and +36.40 ± 3.00 mV, respectively (Fig. 2A, dots). After complexing to pEGFP, zeta potential values of the three nioplexes at cationic lipid/DNA mass ratio 2/1 decreased moderately, but increased again with augmenting cationic lipid/DNA mass ratios. As represented in Fig. 2B, all formulations showed low dispersity (Đ) values, below 0.4.

3.2. Screening of formulations in terms of transfection efficiency and cell viability

Cell viability and transfection assays were performed with N**1**, N**2** and N**3** nioplexes vectoring pEGFP at cationic lipid/DNA mass ratios 2/1, 5/1 and 10/1 in human CF airway epithelial cells (Fig. 3). For nioplexes N**1** and N**2**, the percentage of EGFP expressing cells was lower than 5 % in all cases and no significant differences were found when comparing the aforementioned ratios (Fig. 3A, bars). In the case of N**3** nioplexes, transfection with cationic lipid/DNA mass ratio 5/1 achieved 28.14 % of EGFP expressing cells, which was significantly higher ($p \leq 0.05$) than the values obtained with 2/1 and 10/1 ratios, as well as than values obtained with the rest of formulations (Fig. 3A, bars). Regarding cell viability, formulations containing N**1** and N**2** niosomes induced high cellular death of more than 50 %, while the formulation based on N**3** niosomes at the cationic lipid/DNA mass ratio 5/1 presented high cell viability values near 90 % (Fig. 3A, dots). In all cases, the 10/1 ratio caused higher cell death rates than their counterparts ratios. The transfection positive control Lipofectamine™ 2000 showed 38.02 ± 4.16 % of EGFP expressing live

cells and a cellular viability of 50.77 ± 5.01 % (data not shown). The qualitative analysis of the transfection efficiency by the examination of cells under the microscope 48 h after transfection can be observed in Fig. 3B.

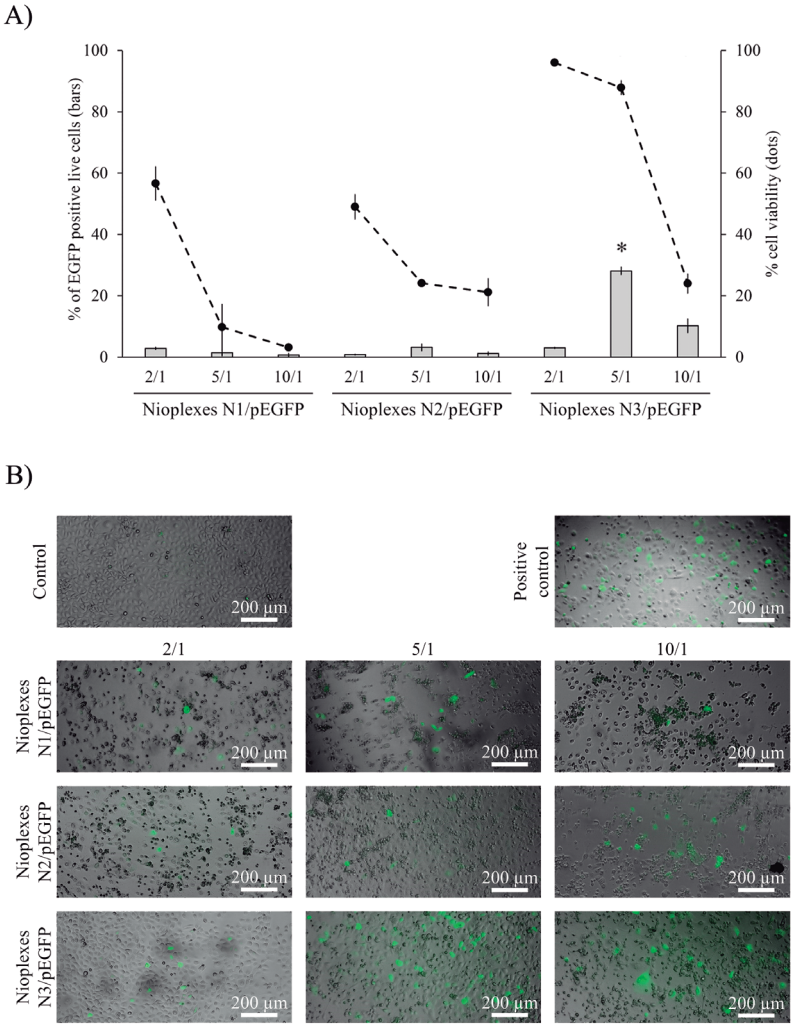


Fig. 3. Transfection efficiency and cell viability of N**1**, N**2** and N**3** niosomes and nioplexes vectoring pEGFP at different cationic lipid/DNA mass ratios in human CF airway epithelial cells. **A**. Percentage of EGFP positive live cells (bars) and cell viability (dots) evaluated by flow cytometry. Data are presented as mean ± SD, n = 3. Statistical significance $*p \leq 0.05$ in terms of transfection efficiency. **B**. Representative fluorescence microscope images of EGFP signal in human CF airway epithelial cells transfected with N**1**, N**2** and N**3** niosomes complexed with pEGFP at the indicated cationic lipid/DNA mass ratios. Control: non-transfected cells. Positive control: cells transfected with Lipofectamine™ 2000 complexed with pEGFP at cationic lipid/DNA mass ratio 2/1. Scale bar: 200 µm.

*3.3. Cellular uptake of N**3** nioplexes*

Cellular uptake in terms of percentages of FITC positive live cells for N**3** nioplexes vectoring FITC labelled pEGFP at the cationic lipid/DNA mass ratio 5/1 was 75.11 % at 2 h and 80.23 % at 4 h post-exposure (Fig. 4A). Representative images of control and cells exposed to nioplexes for 4 h are shown in Fig. 4B. The transfection positive control Lipofectamine™ 2000 showed 86.53 ± 0.63 % and 84.60 ± 2.70 % of FITC positive live cells 2 h and 4 h after exposure to lipoplexes, respectively.

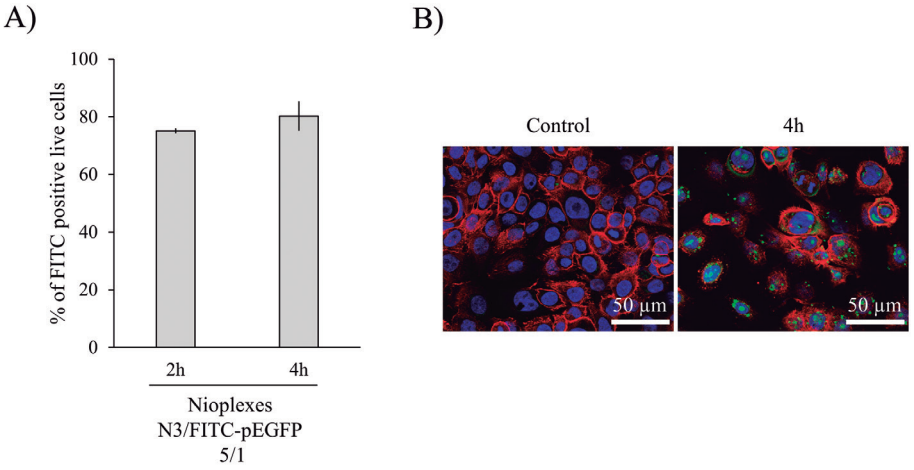


Fig. 4. Cellular uptake in human CF airway epithelial cells co-incubated with N**3** nioplexes vectoring FITC labelled EGFP plasmid at 5/1 cationic lipid/DNA mass ratio. **A**. Percentage of FITC positive live cells after 2 and 4 h of incubation with N**3** nioplexes, analysed by flow cytometry. Each value represents the mean ± SD, n = 3. **B**. Representative merged confocal microscopy images of cellular uptake. Cell nuclei were coloured in blue (DAPI); F-actin in red (Phalloidin); and N**3** nioplexes in green (FITC). Scale bar: 50 µm.

*3.4. Intracellular trafficking of N**3** nioplexes*

The cellular internalization pathways studied in this work include clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME) and macropinocytosis. The fluorescent signal emitted by the specific endocytic vesicle markers in human CF airway epithelial cells was higher for CvME and macropinocytosis than for CME (Fig. 5A). Co-localization analysis of these N**3** nioplexes with the intracellular trafficking pathways showed the following mean CCF peak values: of 0.39 ± 0.06 with macropinocytosis markers, 0.13

± 0.02 with CME markers and 0.36 ± 0.01 with CvME markers (Fig. 5B). Representative images of these conditions are shown in Fig. 5C.

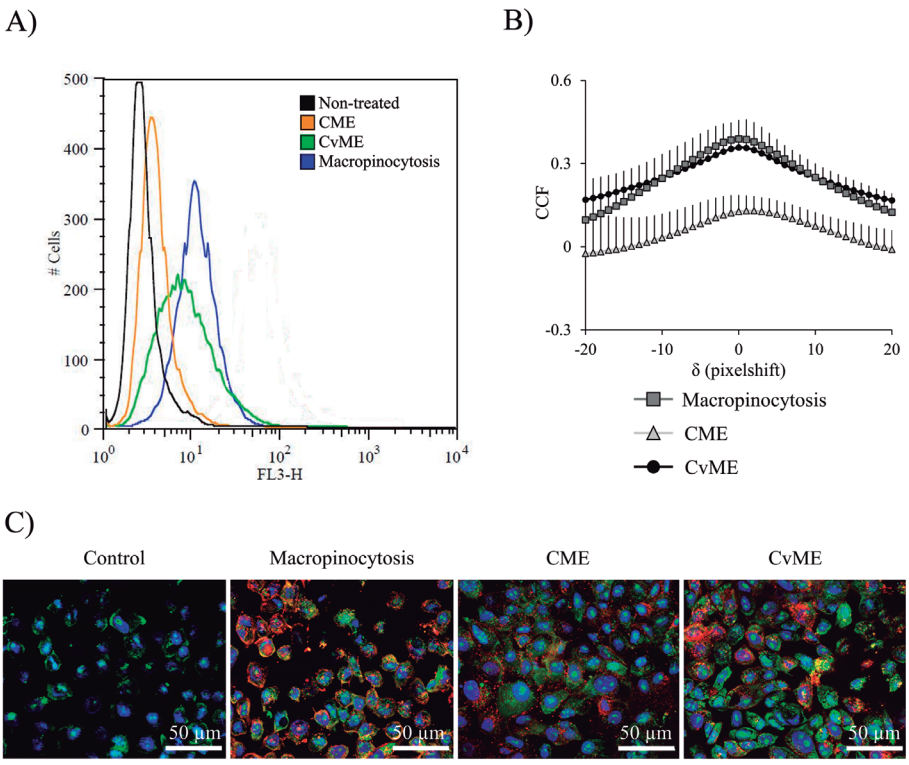
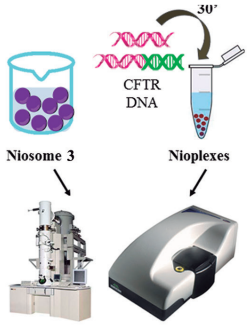


Fig. 5. Intracellular trafficking assays in human CF airway epithelial cells co-incubated with N**3** nioplexes vectoring FITC labelled EGFP plasmid at 5/1 cationic lipid/DNA mass ratio. **A**. Fluorescent signal of the endocytic vesicle markers by number of cells quantified by flow cytometry. **B**. Co-localization values of FITC labelled pEGFP and the endocytic pathways signals determined by cross-correlation analysis in each case. Each value represents the mean ± SD, n = 3. **C**. Representative merged confocal microscopy images of intracellular trafficking. Green colouring shows the FITC labelled EGFP plasmid, blue colouring shows cells nuclei stained with DAPI and red colouring shows one of the following endocytic vesicles: Dextran-Alexa Fluor™ 594 for macropinocytosis, Transferrin-Alexa Fluor™ 594 for clathrin mediated endocytosis (CME) and Cholera toxin B-Alexa Fluor™ 594 for caveolae mediated endocytosis (CvME). Scale bar: 50 µm.

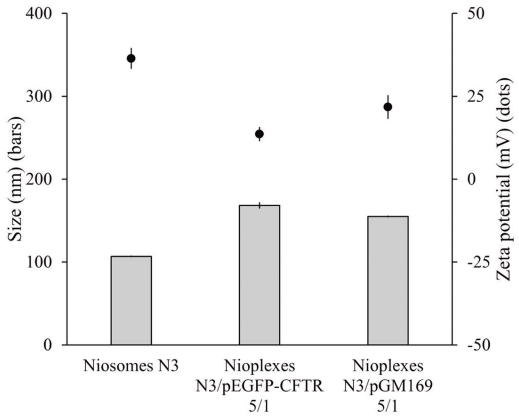
*3.5. Physicochemical characterization of N**3** nioplexes vectoring CFTR gene*

The physicochemical characterization of N**3** niosome formulations vectoring the CFTR gene at the established 5/1 cationic lipid/DNA mass ratio (Fig. 6A) showed similar patterns to those observed in N**3** vectoring pEGFP. Precisely, N**3**/pEGFP-CFTR and N**3**/pGM169 nioplexes showed mean size values of 168.47 nm and 155.1 nm and mean zeta potential values of +13.6 mV and +21.77 mV, respectively (Fig. 6B). Mean Đ values were below 0.4 in all cases (Fig. 6C). When observed under TEM, N**3** niosomes showed a clear spherical morphology (Fig. 6D).

A)



B)



C)

	Mean Đ	SD
Niosomes N3	0.16	0.01
Nioplexes N3/pEGFP-CFTR 5/1	0.35	0.01
Nioplexes N3/pGM169 5/1	0.25	0.01

D)

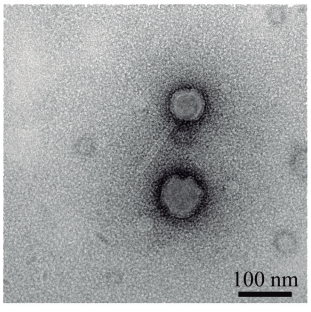


Fig. 6. Physicochemical characterization of N**3** niosomes and their corresponding nioplexes vectoring pEGFP-CFTR and pGM169 at 5/1 of cationic lipid/DNA mass ratio. **A**. Overview of the physicochemical characterization process. **B**. Particle size (bars) and zeta potential (dots). Each value represents the mean ± SD of three measurements. **C**. Dispersity (Đ) values. Each value represents the mean ± SD of three measurements. **D**. TEM image of N**3** niosomes. Scale bar: 100 nm.

3.6. CFTR expression and chloride channel biological activity

Transfection efficiency assays in human CF airway epithelial cells exposed to N**3** nioplexes vectoring pEGFP-CFTR at 5/1 cationic lipid/DNA mass ratio (Fig. 7A) revealed 16.34 ± 0.41 % of EGFP expressing positive live cells, with a cellular viability of 99.48 ± 3.24 % (data not shown). The immunoblot upon transfection of human CF airway epithelial cells with N**3** vectoring pGM169 at cationic lipid/DNA mass ratio 5/1, showed an intense band at approximately 170 KDa that corresponded to the glycosylated CFTR protein (Fig. 7B). Band intensity normalization with β-actin confirmed 5-fold higher ($p \leq 0.05$) CFTR protein levels in transfected cells compared with non-transfected cells (Fig. 7C).

Regarding CFTR channel biological activity, mean fluorescence signal resulting from the iodide efflux raised remarkably after the addition of cAMP agonists FSK and IBMX (Fig. 7D). Fluorescence signal continued to increase progressively, achieving 1.5-fold higher mean fluorescence peak values ($p \leq 0.01$) in N**3**/pGM169 transfected versus non-transfected CF cells, until the addition of NaI buffer, when fluorescent signal decreased to baseline values.

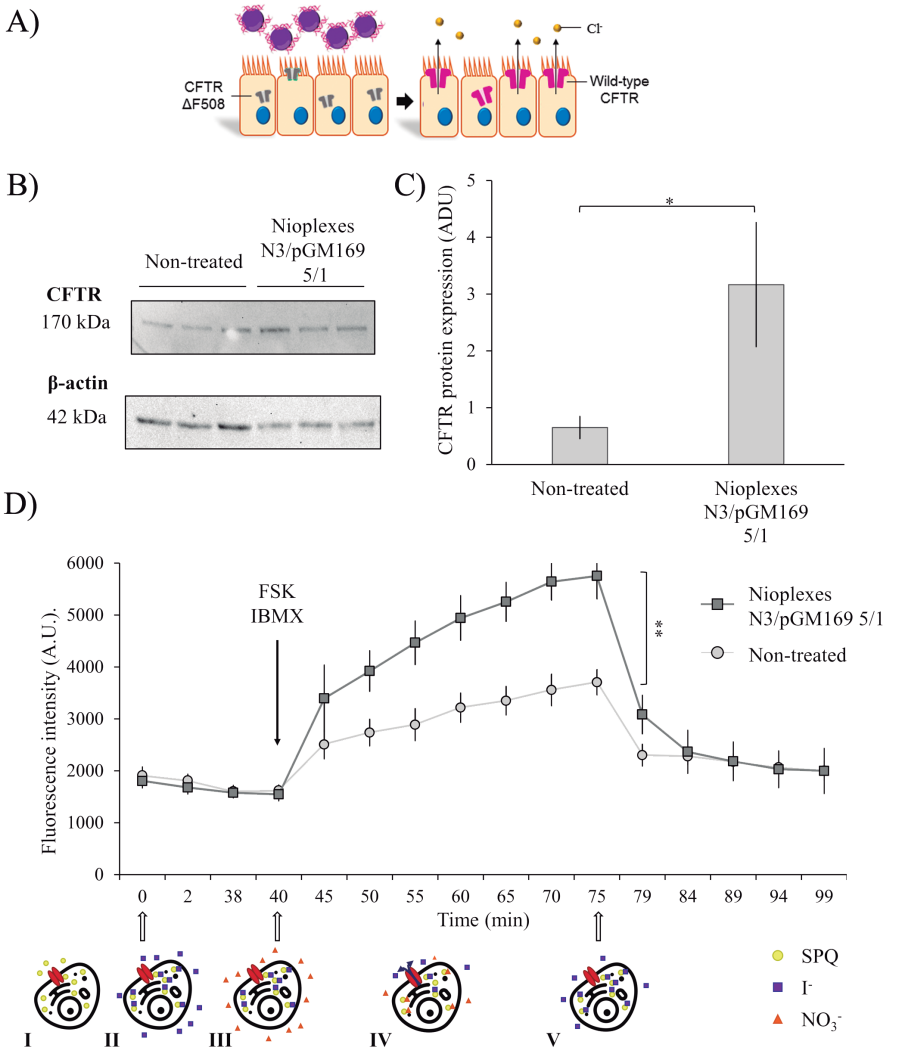


Fig. 7. Evaluation of the expression and the biological activity of the CFTR protein produced by human CF airway epithelial cells transfected with N**3** nioplexes vectoring pGM169 at 5/1 cationic lipid/DNA mass ratio, compared with non-transfected ones. **A**. General scheme of the transfection process. **B**. CFTR and β-actin (loading control) bands, analysed by Western Blot. **C**. CFTR/β-actin protein expression ratio. Each value represents the mean ± SD, n = 3. Statistical significance $*p \leq 0.05$. **D**. CFTR chloride channel activity determined by the rate of iodide efflux monitored with the fluorescent SPQ molecule. General scheme of the performed functional assay: (D-I) SPQ loading into human CF cells; (D-II) quenching of SPQ by I^- of the NaI buffer; (D-III) addition of nitrate buffer containing NO_3^- and CFTR channel agonists forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX), indicated by a black arrow; (D-IV) exit of I^- from human CF cells across the CFTR channel and dequenching of SPQ; and (D-V) addition of I^- from NaI buffer and secondary quenching of SPQ. Each value represents the mean ± SD, n > 3. Statistical significance $**p \leq 0.01$.

4. Discussion

The basic concept of gene therapy involves the addition of a healthy copy of a gene into target cells in order to cure, prevent or slow down the progression of a disease. Unlike most conventional therapeutic approaches, gene therapy aims to correcting the underlying cause of the disease instead of treating its symptoms. Due to its monogenic nature, CF disease is a good candidate for this technology, since the transfer of a healthy copy of the CFTR gene would override the wide variety of mutations present in the genome of CF patients. The delivery of large genetic sequences, such as the CFTR gene, requires efficient vector systems able to transport the DNA into target cells. To date, most of the published clinical trials for CF that use non-viral approaches are based on liposomes (Griesenbach et al., 2015). However, these studies usually involve either, general transfection cell lines in the *in vitro* stages, or healthy animal models in the preclinical stages, both with little relevance for CF disease. In this concern, our study aims to provide exhaustive data regarding niosome mediated CFTR gene delivery efficiency, protein expression and chloride channel functionality in specific CF patient derived airway epithelial cells homozygous for the ΔF508 mutation.

In this work, three different cationic lipid based niosome formulations were prepared and evaluated, and the most suitable one according to physicochemical and biological properties to transfer correct copies of the CFTR gene into human CF cells was selected. Different combinations and quantities of the niosome components affect to the gene transfer ability and cell tolerance of the formulations. In this sense, the components used in this work to prepare N**1**, N**2** and N**3** niosome formulations presented suitable characteristics for gene therapy purposes according to previous studies. In fact, the cationic lipids DOTMA and tailor-synthesized DTPA have previously reported high efficiency for gene delivery *in vitro* and *in vivo* (Mashal et al., 2017; Puras et al., 2014). As a helper component, squalene based niosomes have proven efficiency to transfect retinal cells (Ojeda et al., 2016), while chloroquine has the ability to prevent endosomal acidification as well as to inhibit lysosomal enzymes that could damage the genetic material (Cheng et al., 2006; Sanz et al., 2012). In addition, it is well known the suitability of non-ionic surfactants polysorbate 20

(Villate-Beitia et al., 2018), polysorbate 80 (Puras et al., 2014) and poloxamer 407 (Monti et al., 2010) for gene delivery purposes.

Physicochemical characterization of niosomes in terms of size, zeta potential, dispersity and morphology is useful to predict the potential applicability of these formulations for gene therapy purposes. In this regard, N**1**, N**2** and N**3** niosomes and nioplexes revealed mean particle sizes between 100 nm and 200 nm, appropriate for nucleic acid delivery (Andar et al., 2014). In fact, mean particle size can determine cellular uptake, being bigger size particles (>500 nm) more difficult and slowly taken, contrary to smaller particles (<40 nm), which are easily and rapidly captured (dos Santos et al., 2011). Regarding zeta potential, the three niosome formulations showed positive values which promote electrostatic interactions with negatively charged DNA to form nioplexes (Hosseinkhani and Tabata, 2006). In addition, positive surface charges above +20 mV presented by N**1**, N**2** and N**3** formulations prevent particle aggregation and enhance cellular uptake (Caracciolo and Amenitsch, 2012). As expected, zeta potential values declined after the addition of DNA at the low cationic lipid/DNA mass ratio 2/1, demonstrating a partial neutralization of surface charges due to the electrostatic interactions between the amine groups of the cationic lipids and the phosphate groups of the DNA (Paecharoenchai et al., 2012).

Once physicochemically characterized, we evaluated the biological performance of N**1**, N**2** and N**3** formulations vectoring the reporter pEGFP in human CF airway epithelial cells. Regarding cell viability, N**3** nioplexes showed excellent values above 90 %, while N**1** and N**2** were worse tolerated. Considering that N**1** and N**2** share the same cationic lipid DOTMA and helper lipid squalene, these results suggested that the components used to elaborate N**3** niosomes were more suitable for gene therapy applied to human CF cells. Concerning transfection efficiency in terms of EGFP expression, we found that N**3** nioplexes, mainly at the cationic lipid/DNA mass ratio 5/1, presented significantly higher values than its counterparts. In fact, the chloroquine component present in N**3** may enhance its endosomal escape and, therefore, improve the intracellular delivery of the genetic cargo (Xie et al., 2018). Hence, based on those cell viability and transfection efficiency results, we selected the N**3**

formulation for the following studies in human CF airway epithelial cells.

The evaluation of cellular uptake is a primary assay that helps to understand part of the transfection process and shows the capacity of the nioplexes to be internalized when they are in contact with the cells. The 80 % of cellular internalization obtained after 4 h incubation with N**3** nioplexes at 5/1 mass ratio supported the high transfection values obtained. In order to further understand the transfection process of N**3** nioplexes, we studied the intracellular trafficking pathways followed by this formulation. In general, most non-viral vectors enter the cells via endocytosis, being CME and CvME the best studied endocytic pathways (Rejman et al., 2004). Although there is no a clear consensus, the CvME has been generally described as a non-acidic and non-digestive pathway (Nichols, 2003), while it is considered that the CME pathway integrates the endocyted vesicles into late endosomes (Luzio et al., 2009). On the other hand, it has been described that the acid pH in lysosomes causes the degradation of non-viral vector/DNA complexes (Khalil et al., 2006). Here, we found that the N**3** formulation followed predominantly both the CvME and the macropinocytic pathways, pointing out to the non-acidic and non-digestive hypothesis of the CvME pathway. This is consistent with the superior fluorescent signal observed with CvME and macropinocytosis markers compared to CME markers in human CF cells, which would suggest a higher presence of these endocytic vesicles in this cell type. These results, together with the ability of the chloroquine component of N**3** to enhance endosomal escape, would contribute to explain the high transfection efficiency of this formulation in human CF cells. In this regard, it is also known that chloroquine, by itself, can enhance transfection efficiency whenever included to the cell culture medium or incorporated into cationic-peptide-DNA complexes (Yang et al., 2009) in a dose-dependent matter. However, the pre-treatment with chloroquine, has shown high toxicity levels that limit further clinical applications (Zhang et al., 2003). For this reason, chloroquine was incorporated within the niosome formulation in order to avoid such deleterious effect. Such inclusion of chloroquine into a niosome formulation, rather than as a co-/pre-treatment of cells, would be a more logical approach for *in vivo* settings.

Once fully characterized and corroborated the suitability of N**3** formulation for gene delivery purposes in CF cells, this niosome was complexed to the therapeutic CFTR gene constructs at the cationic lipid/DNA mass ratio 5/1. The physicochemical analysis corroborated suitable particle size, Đ, surface charge and morphology. As expected, N**3**/pGM169 nioplexes showed a slight increase in zeta potential values, probably due to the lack of CpG islets of that plasmid. We evaluated the transfection efficiency of the N**3**/pGM169 formulation in terms of CFTR protein expression, which in consequence would mean a correct translation of the delivered CFTR gene into protein. We obtained glycosylated CFTR protein bands in accordance with previous studies (Garbuzenko et al., 2019; Hyde et al., 2008). The increment of CFTR protein expression was of 5-fold respect to non-transfected cells, which would correspond to an equal or superior increase at the mRNA level. According to previous reports, 5 % of wild CFTR mRNA is enough to correct the chloride ion transport defect in mice (Dorin et al., 1996; Harvey et al., 1999). Importantly, the high CFTR protein expression levels obtained in this work lead to 55 % increment (corresponding to approximately 1.5-fold) of the chloride channel functionality, which, according to the literature, would largely exceed the values needed to provide therapeutic benefits (Dorin et al., 1996; Ramalho et al., 2002). Of note, previous studies in this field obtained lower rates of CFTR functionality measured by iodide efflux monitoring. For instance, the use of the same therapeutic pGM169 genetic construct employed in this work but complexed to the commercial liposome Lipofectamine™ 2000, achieved an increment of around 25 % in CFTR channel activity in HEK-293 transfection model cells (Hyde et al., 2008). Other authors reported an increment of around 36 % in CF cells transfected with lipid nanoparticles vectoring a modified CFTR mRNA (Robinson et al., 2018). Although in an initial stage, our thorough research holds great relevance, mainly considering that we reached high levels of CFTR protein and chloride channel functionality in CF patient derived airway epithelial cells, employing for the first time niosomes as a non-viral gene therapy strategy for this disease.

5. Conclusions

In conclusion, our data demonstrate that: (i) N**3** niosomes present suitable physicochemical properties for gene delivery purposes and are able to efficiently transfect human cystic fibrosis airway epithelial cells, (ii) N**3** based nioplexes followed the caveolae-mediated endocytic route, (iii) transfection with N**3** complexed with the therapeutic CFTR gene construct results in a 5-fold increase at protein level and (iv) this transfection leads to a 55 % increment of the chloride channel functionality in human cystic fibrosis airway epithelial cells, pointing out to a potential therapeutic benefit.

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Appendix 5

3D bioprinting technology application to evaluate gene delivery efficiency of niosomes in cystic fibrosis disease

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Sociedad Española de Farmacia Industrial y Galénica

Applying 3D constructs for gene therapy

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1. Introduction

The main objective of three-dimensional (3D) bioprinting technology is the development of 3D constructs that combine biomaterials and cells to replicate tissues and organs. Using this technique, different kinds of epithelia can be produced in order to mimic the *in vivo* environment more accurately than cell cultures [1]. Such scaffolds are often characterized before assessing cellular response upon the addition of different drugs and genetic materials [2].

The aim of this study was to make use of 3D bioprinting technology to develop scaffolds that mimic the *in vivo* lung environment to evaluate the ability of niosomes (non-viral vectors) to deliver genetic material for the treatment of cystic fibrosis (CF) genetic disorder [3] (Fig. 1).

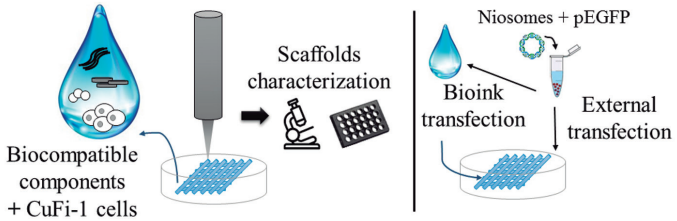

Fig. 1. General scheme of the 3D bioprinting process, scaffold characterization and transfection approaches.

2. Material and methods

2.1. 3D bioprinting

The scaffolds were obtained in an extrusion-based 3D Cellink® BioX bioprinter equipment using bioink with biocompatible components such as hyaluronic acid, alginate, gelatin, fibrin and A-proteinin, combined with human airway epithelial cells (CuFi-1) being homozygous for F508del (mutation responsible for 70 % of CF patients).

2.2. Scaffolds characterization

Assays were carried out on days 1, 3, 7, 14 and 21 after bioink printing. Live/dead™ staining was used for tracking cell status. Cell Counting Kit 8 (CCK-8) and lactate dehydrogenase (LDH) activity assays were performed to monitor metabolic activity and cell cytotoxicity, respectively.

2.3. Cellular transfections

Two different transfection approaches were evaluated. The first one consists of the addition of nioplexes into the scaffolds medium (2.5 µg DNA/scaffold) every 72 h (4 times). In the second one, nioplexes were added inside the bioink, using 3 different doses (7.5; 15 y 22.5 µg DNA/scaffold). The genetic material used in both approaches was the EGFP reporter plasmid at a cationic lipid/DNA mass ratio of 5/1. Transfection efficiency was evaluated in a Cytation™ 1 equipment for 13 days.

3. Results and discussion

Fluorescence images (Fig. 2) show live cells in green, which contain a high number of cells inside each scaffold, and dead cells in red, which scarcely appear in all pictures. The number of live cells was higher as the days go by and the number of dead ones was highest on days 7 and 14.

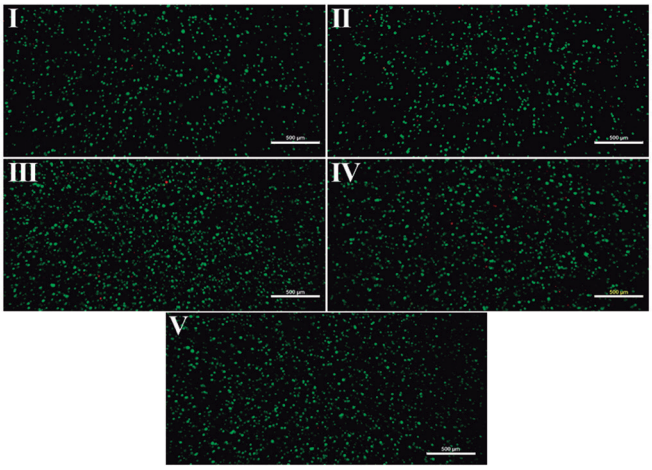

Fig. 2. Representative fluorescence images of Live/dead™ stained scaffolds on I) day 1; II) day 3; III) day 7; IV) day 14 and V) day 21. Scale bar: 500 µm.

Metabolic activity (Fig. 3A) was similar on all days, except on day 7 when it was higher, without statistical significance. As for the cytotoxicity effect (Fig. 3B), the first day reported the highest toxic value, showing stable and minimum values during next days.

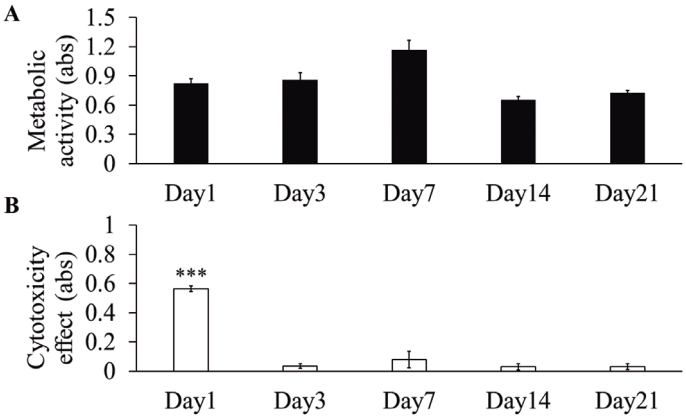

Fig. 3. Evolution of (**A**) metabolic activity and (**B**) cell cytotoxicity of scaffolds. Statistical significance ***$p < 0.001$.

External transfection (Fig. 4) obtained the lowest values until day 7 when fluorescence began to increase. This approach has 4 doses because nioplexes have more barriers to overcome and need more time to reach the cells. Bioink transfection (Fig. 4) has a unique dose being 3 dose-dependent groups (7.5, 15 and 22.5 μg DNA/scaffold). The same pattern was followed for each dose: fluorescence increased until day 4 and the following days remained stable; 7.5 was the dose with the highest fluorescence increase, followed by 15 and ending by 22.5 (the higher the dose, the lower the fluorescence). At the end point, external transfection showed more fluorescence value than 7.5.

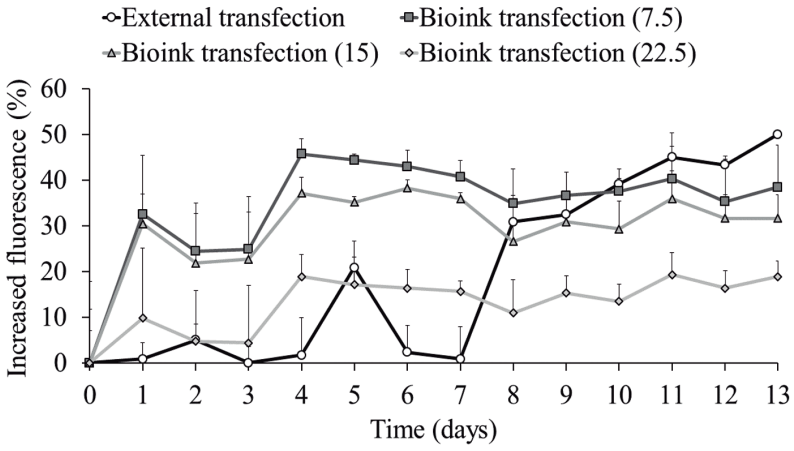

Fig. 4. Fluorescence increase of the scaffolds using different transfection approaches.

4. Conclusions

The main findings were as follows: after 21 days in culture, cells embedded in the scaffolds remain viable and metabolically active; cells were more affected the first day after bioprinting due to the technique employed, but then recovered their values; external multi-dose transfection has higher fluorescence values than bioink transfection.

5. Acknowledgements

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Chapter 4

RESUMEN

La terapia génica es el conjunto de técnicas que permiten modificar o manipular la expresión de un gen o alterar las propiedades biológicas de células vivas para su uso terapéutico. El objetivo de esta terapia es tratar o curar enfermedades mediante la adición, deleción, reemplazamiento, reparación o regulación de un gen en una persona. Según la Agencia Europea del Medicamento (EMA), un producto terapéutico basado en terapia génica presenta dos componentes principales: un constructo genético y un vector o sistema de liberación. Pueden diferenciarse tres grupos principales atendiendo a la composición y al mecanismo de acción terapéutico.

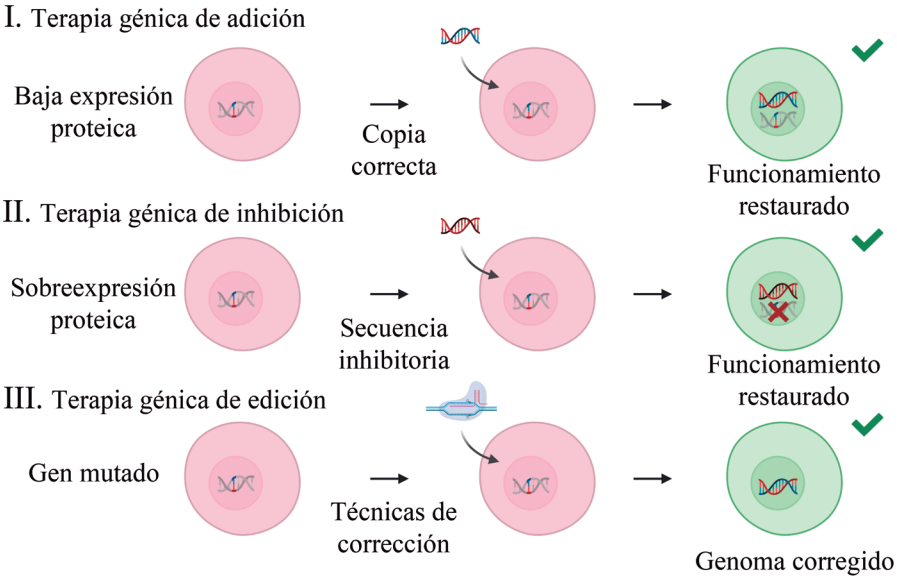


Figura 1. Representación esquemática de las diversas estrategias de la terapia génica dependiendo de su mecanismo de acción.

Dependiendo del tipo celular al que esté dirigida la terapia podemos diferenciar entre: terapia génica de células somáticas, en la que se corrige la mutación en un paciente en concreto pero no es transmisible a generaciones venideras; o terapia génica de células germinales, en la que la mutación es corregida en espermatozoides u óvulos y la versión correcta del gen se transfiere a todas las células del sujeto y a sus descendientes. Además, dependiendo del mecanismo de acción del tratamiento se puede diferenciar entre terapia

génica de adición, de inhibición y de edición, como se observa en la Figura 1. La terapia génica de adición suele emplearse en enfermedades que presentan una baja expresión proteica determinada, y consiste en la incorporación de una copia correcta del gen en las células. La terapia génica de inhibición consiste en la incorporación de una secuencia inhibitoria dentro de las células utilizada en el tratamiento de enfermedades que cursan con una sobreexpresión proteica determinada. La terapia génica de edición consiste en la incorporación de diferentes moléculas capaces de modificar ("editar") las mutaciones en el propio genoma celular.

Para conseguir el efecto deseado en cada terapia, el material genético ha de llegar a las células objetivo. La manera más simple y segura de poder alcanzarlo es a través de la administración del material genético "desnudo". Sin embargo, esta situación deriva en una baja eficiencia de transfección debido a la falta de estabilidad de este material genético en el medio extracelular y a su baja internalización celular. Para aumentar la protección y la internalización celular del material genético, se provee de un sistema de liberación o vector. El desarrollo de vectores eficientes y seguros conlleva un laborioso trabajo de diseño, elaboración, caracterización y evaluación. Los vectores se clasifican en dos grupos principales atendiendo a su naturaleza: vectores virales y no virales. Para poder elegir el vector más efectivo y seguro en cada caso, se ha de tener en cuenta la aplicación del tratamiento que engloba las células objetivo, la vía de administración y todas las barreras biológicas que han de atravesar. De esta manera, los vectores pueden ser diseñados específicamente para cada tratamiento.

Los vectores virales son virus genéticamente modificados que utilizan su capacidad intrínseca para la internalización celular e integración del material genético dentro del genoma celular. Estos vectores presentan altas eficiencias de transducción que son ensombrecidas por su alto potencial oncogénico, alta inmunogenicidad y alta citotoxicidad. Además, no son capaces de transportar ácidos nucleicos de gran tamaño y su producción es cara y compleja. Los vectores víricos más estudiados y utilizados son los retrovirus, lentivirus, virus adenoasociados y adenovirus.

Los vectores no virales son una alternativa con menos inmunogenicidad y mutagenicidad que sus homólogos virales. Además, su proceso de elaboración es más barato, más fácil y escalable, y obtiene mejor reproducibilidad. Asimismo, permiten transportar ácidos nucleicos de tamaño casi ilimitado y realizar administraciones con régimen multidosis. Sin embargo, uno de los principales obstáculos es su baja tasa de transfección, que se trata de solventar gracias a los continuos avances en el desarrollo de nuevos materiales y técnicas. Dentro de los vectores no virales nos encontramos con estrategias basadas en métodos físicos, como la electroporación, sonoporación, fotoporación, magnetofección o hidroporación; y en métodos químicos, incluyendo compuestos como polímeros, lípidos, péptidos, materiales inorgánicos o sistemas híbridos.

Hasta ahora, los vectores no virales más usados son aquellos basados en lípidos, más específicamente en lípidos catiónicos, donde pueden destacarse los liposomas. Los liposomas son sistemas vesiculares fosfolipídicos con forma de esfera y constituidos por una o más bicapas lipofílicas. Posteriormente, los niosomas surgieron como una alternativa a los liposomas debido a su mayor estabilidad y su baja toxicidad, presentando además procesos de elaboración más baratos y fáciles. Los niosomas son vesículas de surfactantes no iónicos formados por una o más bicapas y están constituidos, en el caso de su utilización en el campo de la terapia génica, por tres componentes principales: surfactantes no iónicos, lípidos catiónicos y componentes "auxiliares" (Figura 2). Tanto la composición química de los componentes de la formulación como el método de elaboración de los niosomas influyen en sus propiedades biofísicas.

Los componentes que conforman los niosomas desempeñan funciones específicas dentro de él. El principal componente de los niosomas son los surfactantes no iónicos que estabilizan las emulsiones, previenen las agregaciones entre partículas e integran correctamente el material genético. Además, no presentan toxicidad debido a la ausencia de cargas iónicas en su estructura química. Los parámetros más relevantes de estas moléculas para la elaboración de niosomas son: el balance hidrofílico-lipofílico (HLB), el parámetro crítico de empaquetamiento o la temperatura de transición entre el estado gel y líquido. Los lípidos catiónicos proveen a los niosomas de cargas positivas, que

les permiten interactuar electrostáticamente con las cargas negativas del material genético formando los complejos, además de promover la fusión con las membranas celulares cargadas negativamente. Dentro de su estructura química encontramos tres principales dominios (una cabeza catiónica, una cola hidrofóbica y un grupo enlace entre ambos) y las diferentes modificaciones químicas de cada uno de ellos influyen en las propiedades fisicoquímicas de los lípidos resultantes. Estos lípidos se pueden adquirir comercialmente, pero también pueden diseñarse y sintetizarse optimizándolos para aumentar su eficiencia de liberación, disminuir sus problemas de toxicidad y alcanzar un objetivo específico. Los componentes "auxiliares" aumentan la fluidez y la estabilidad de la membrana y promueven los procesos de transfección. Normalmente, esta función la han desempeñado lípidos neutros, aunque, recientemente, se han introducido nuevos compuestos con propiedades específicas.

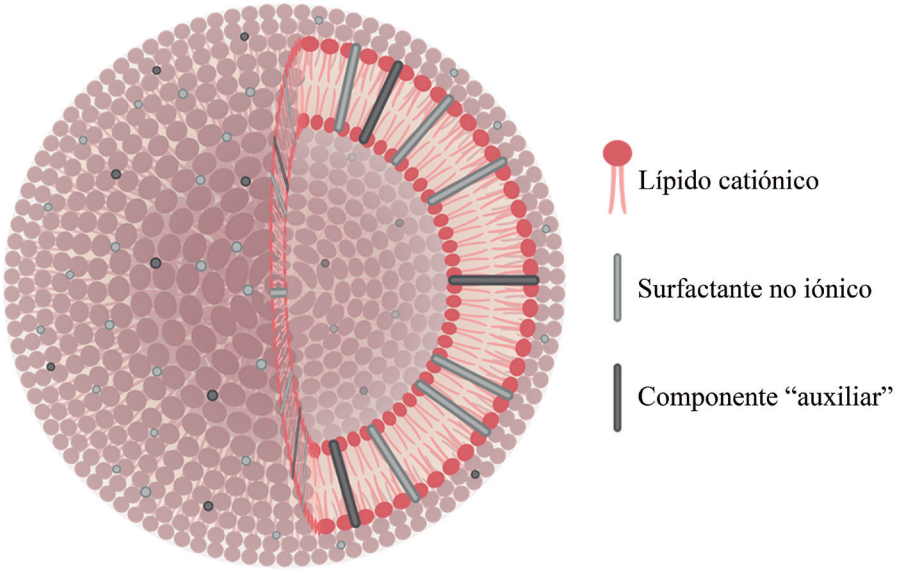


Figura 2. Esquema general de la estructura de los niosomas.

Para que los vectores consigan llegar a su objetivo desde la vía de administración, deberán solventar los problemas y las barreras biológicas que se encuentren en su camino. Las primeras barreras son las extracelulares como aclaramientos rápidos, interacciones no específicas con proteínas y células,

y degradación por parte de enzimas. Además de otras barreras más específicas como la hematoencefálica y la hematorretiniana. Una vez los vectores están cerca de las células objetivo, las barreras que se encuentran dependen de la captación e internalización celular. Los vectores pueden interaccionar inespecíficamente con las cargas negativas de las membranas celulares o específicamente tras su recubrimiento con ligandos. En la internalización celular caben destacar cuatro vías principales: endocitosis mediada por clatrinas (CME), endocitosis mediada por caveolas (CvME), macropinocitosis y fagocitosis. Después de este paso, es fundamental el escape endosomal, antes de la fusión de los endosomas con los lisosomas, para evitar el atrapamiento y la degradación de los vectores. Es posible promover este paso gracias a la inclusión de compuestos con capacidad de escape endosomal, como son los péptidos de penetración celular o la molécula cloroquina.

La terapia génica ha emergido como una alternativa eficaz para tratar enfermedades, destacando la fibrosis quística (FQ). La FQ es una enfermedad monogénica autosómica recesiva causada por diferentes mutaciones en el gen regulador de la conductancia transmembrana de la fibrosis quística (CFTR). La proteína CFTR es un canal transmembrana que se encuentra incluido en la membrana apical de las células epiteliales secretoras regulando el flujo de cloro y bicarbonato, principalmente. En los pacientes con FQ, estas mutaciones generan una proteína disfuncional, afectando primordialmente a tejidos como las vías respiratorias, el páncreas, el hígado y los tractos digestivo y reproductivo. Por tanto, los síntomas más comunes son derivados de la afectación de estos tejidos incluyendo la piel con sabor salado, tos, frecuentes infecciones de pulmón, infertilidad o problemas digestivos. Esta enfermedad está considerada una "enfermedad rara" debido a su baja prevalencia (uno de cada 2.000-3.000 nacimientos en población caucásica). La mutación más prevalente, presente en el 60-65 % de estos pacientes, radica en la deleción de un aminoácido fenilalanina en la posición 508 (F508del) en la proteína CFTR, provocando un problema de plegamiento proteico. A pesar de que la esperanza y calidad de vida de estos pacientes ha aumentado en los últimos años gracias a los avances en los tratamientos, todos ellos son sintomáticos sin abarcar la causa genética. Los más nuevos derivan de la combinación

entre fármacos potenciadores y correctores de la proteína CFTR. No obstante, desde que el gen CFTR fue identificado y clonado en 1989, la terapia génica ha tenido en su punto de mira a esta enfermedad. De hecho, numerosos ensayos clínicos, tanto con vectores virales como no virales, se han desarrollado desde entonces.

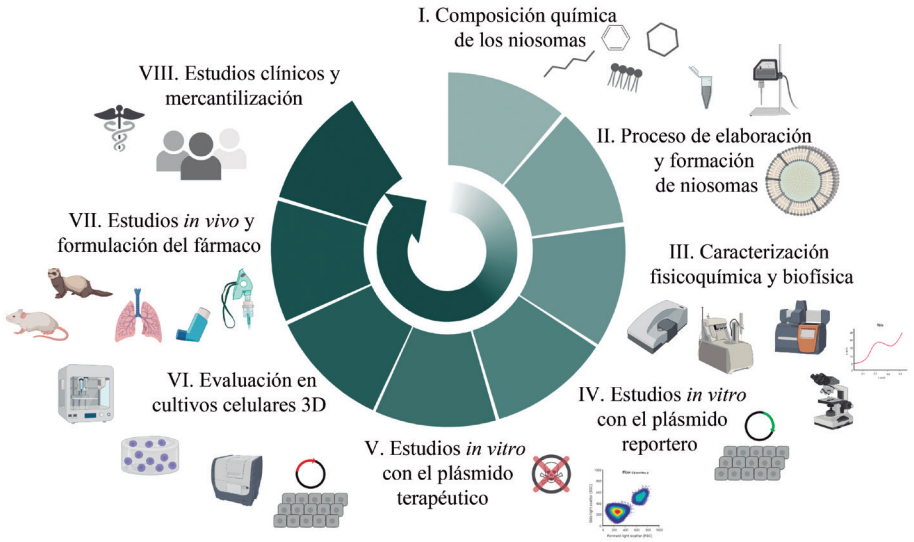


Figura 3. Esquema general de la estructura de los niosomas.

Con el fin de seguir avanzando en el campo de la terapia génica para el tratamiento de enfermedades como la FQ, podría ser interesante el desarrollo de vectores no virales, especialmente los niosomas. Para poder desarrollar niosomas adecuados, hay algunos aspectos clave que han de tenerse en cuenta (Figura 3). En primer lugar, según el objetivo principal del tratamiento, se deben determinar los compuestos que conforman las nanopartículas y su método de preparación. Después, estas nanopartículas se someten a una evaluación biofísica exhaustiva para corroborar su idoneidad antes de comprobar su comportamiento en ensayos *in vitro* con líneas células. Una vez completados todos los pasos, y tras pasar por los comités de ética apropiados, se utilizan modelos animales para los estudios *in vivo*. Actualmente, la correlación entre los ensayos *in vitro* e *in vivo* no es demasiado buena y, por ello, se están desarrollando nuevas estructuras que asemejan, más acertadamente, el entorno *in vivo* en las estructuras *in vitro*. En este caso, las

estructuras más destacadas son los cultivos celulares tridimensionales (3D) como los andamios, biorreactores, modelos de órganos-en-chip, organoides o esferoides. Para elaborar estas estructuras se utilizan numerosas técnicas, pero una de ellas destaca especialmente: la bioimpresión tridimensional. En ella se combinan materiales biocompatibles y células formando la biotinta con la que se crean constructos de tejidos funcionales para múltiples aplicaciones.

Teniendo en cuenta esta información, el objetivo principal de esta tesis doctoral es el desarrollo y la exhaustiva caracterización, biofísica y biológica, de vectores no virales basados en niosomas catiónicos para hacer frente a una enfermedad como la FQ.

Los primeros pasos del trabajo derivaron del desarrollo de formulaciones de niosomas para observar los cambios biofísicos derivados, por una parte, de la inclusión de la cloroquina en la formulación y, por otra parte, del cambio del surfactante no iónico y su fase de adición en el proceso de elaboración. Todas las formulaciones de niosomas se elaboraron a través de la técnica de emulsión de aceite en agua para, posteriormente, evaporar el solvente orgánico y obtener las nanopartículas en solución acuosa. Los compuestos utilizados para elaborarlas fueron: el polisorbato 80 y los poloxámeros 407 y 188 como surfactantes no iónicos, el lípido catiónico DTPA (sintetizado específicamente) y la cloroquina como componente "auxiliar". El material genético utilizado en el estudio para obtener los complejos fue el plásmido reportero codificante para la proteína verde fluorescente (pEGFP). Los niosomas fueron evaluados a través de ensayos de calorimetría diferencial de barrido y de caracterización en términos de morfología, tamaño, dispersión y carga superficial. También se realizaron estudios de afinidad de unión al ADN, como la calorimetría isoterma de titulación y el empaquetamiento de la membrana lipídica. Más adelante se llevaron a cabo ensayos de capacidad tamponante y de liberación del material genético desde endosomas artificiales. Por último, se comprobó su eficiencia de transfección y su toxicidad celular en células epiteliales humanas de vías respiratorias con mutación F508del para la FQ.

Para llevar a cabo el estudio, se prepararon dos formulaciones idénticas que diferían en el contenido del componente "auxiliar", ya que una de ellas

presentaba cloroquina en su composición (formulación **1**) y la otra no (formulación **3**). En este caso, todos los cambios observados en las propiedades biofísicas de estas formulaciones fueron debidas a la inclusión de la cloroquina en la formulación. Los resultados revelaron que la cloroquina aumentaba la estabilidad térmica y disminuía el tamaño y la dispersión de las partículas. Además, aumentaba la afinidad por las moléculas de ADN y mejoraba el empaquetamiento de la membrana en los complejos. Todos estos parámetros, junto con el aumento de la capacidad tamponante y su mayor capacidad de escape desde endosomas artificiales, podrían explicar, en parte, el aumento de la eficiencia de transfección en los niosomas que incluyen cloroquina en su composición.

En el caso de evaluar cuál es el papel que desarrollan los surfactantes no iónicos, se prepararon dos formulaciones: la formulación **1** presentaba los surfactantes no iónicos en la fase oleosa (poloxámero 407 y polisorbato 80) y la formulación **2** los presentaba en la fase acuosa (poloxámero 188 y polisorbato 80). Los resultados de los ensayos biofísicos obtenidos por ambas formulaciones revelaron diferencias sutiles entre ellas. No obstante, la formulación **1** presentó mayor estabilidad térmica, mayor eficiencia de transfección y mayor viabilidad celular que la formulación **2**, la cual obtuvo mayores valores de carga superficial positiva y peor viabilidad celular.

En el siguiente estudio, otros tres tipos de formulaciones de niosomas fueron evaluadas en células epiteliales humanas de vías respiratorias con mutación F508del para la FQ. Su finalidad radica en comprender cómo transcurren los procesos de transfección e intentar obtener suficiente eficiencia de transfección para recuperar la funcionalidad del canal CFTR. Para ello, se desarrollaron tres formulaciones de niosomas, la formulación **1**, ya utilizada en el primer estudio, y las formulaciones **4** y **5**, constituidas por el lípido comercial DOTMA y escualeno como componente "auxiliar" en la fase oleosa, y polisorbato 20 y 80, respectivamente, como surfactante no iónico en la fase acuosa. Todas las formulaciones fueron realizadas mediante la técnica de emulsión aceite en agua evaporando el solvente orgánico una vez obtenidos los niosomas. A su vez, se utilizaron diferentes constructos de ADN para poder llevar a cabo los diferentes ensayos. El plásmido reportero elegido fue

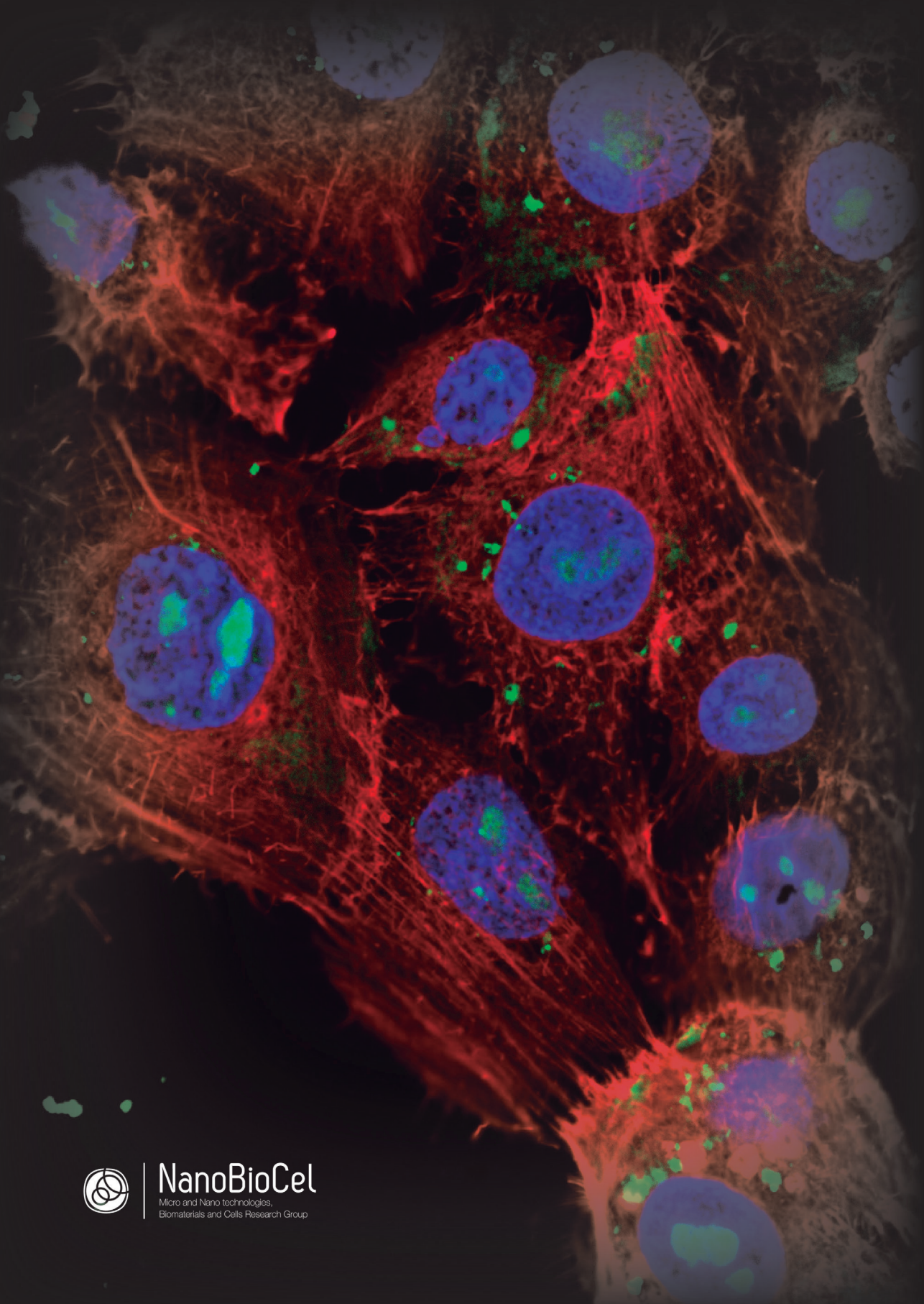
el codificante para la proteína verde fluorescente (pEGFP) utilizado en los ensayos de caracterización fisicoquímica, así como en los primeros ensayos de transfección. El plásmido reportero marcado con isotiocianato de fluoresceína (FITC) fue utilizado para seguir los complejos en los ensayos de captación e internalización celular. Y dos plásmidos terapéuticos codificantes para la proteína CFTR: uno de ellos codificante también para la proteína reportera (pEGFP-CFTR) utilizado en ensayos de transfección; y el otro es un constructo liberado de dinucleótidos de citosina y guanina (islas CpG) llamado pGM169, utilizado en ensayos de transfección para la cuantificación y evaluación de la funcionalidad de la proteína CFTR.

Los resultados obtenidos tras evaluar fisicoquímicamente los niosomas y sus correspondientes complejos concluyeron que las formulaciones presentaban valores de tamaño en escala nanométrica, carga superficial positiva y dispersión bastante homogénea, todos ellos adecuados para su utilización en terapia génica. Los ensayos *in vitro*, en células epiteliales humanas de vías respiratorias con mutación F508del para la FQ, demostraron que la formulación **1** presentaba mayor eficacia de transfección así como menor toxicidad celular que las otras dos formulaciones testadas. Posteriormente, se realizaron los ensayos de captación e internalización celular revelando que, la formulación **1**, se adentraba en las células predominantemente por la vía endocítica mediada por caveolas y macropinocitosis. En la siguiente etapa y tras la transfección con los plásmidos terapéuticos, se observó que la formulación **1** incrementaba en cinco veces los niveles de proteína CFTR, con respecto a las células no transfectadas, que se traducía en un aumento del 55 % de la funcionalidad de dicha proteína en las células epiteliales humanas de vías respiratorias con mutación F508del para la FQ, postulándose así para un potencial beneficio terapéutico.

Por último, se comenzó a desarrollar un constructo tridimensional para conseguir mejorar los modelos *in vitro* con el objetivo de asemejar, en mayor medida, el entorno *in vivo* y poder utilizarlo como modelo de evaluación de nuevos tratamientos. Para ello, se utilizó la tecnología de bioimpresión tridimensional. En una primera etapa, se optimizaron tanto la estructura del andamio como la biotinta utilizada, en la que se emplearon compuestos

biocompatibles y células epiteliales humanas de vías respiratorias con mutación F508del para la FQ. Seguidamente, se caracterizaron las células embebidas en el andamio en términos de viabilidad celular, actividad metabólica y efecto citotóxico, demostrando que las células se encuentran viables y metabólicamente activas tras 21 días en cultivo. Además, en estos constructos, se realizaron ensayos de transfección celular con los niosomas de la formulación **1** cargados con el plásmido reportero, pEGFP, comparando dos aproximaciones diferentes. En la primera de ellas se trató de administrar externamente los complejos, introduciéndolos en el medio de cultivo, con una pauta de administraciones repetidas. En la segunda de ellas se trató de administrar los complejos dentro de la biotinta, realizando 3 dosificaciones diferentes. En ambos casos se monitorizó el incremento de fluorescencia de las células embebidas dentro del andamio bioimpreso llegando a la conclusión de que la primera de las aproximaciones presentaba mejores valores. En cualquier caso, se demostró que los constructos tridimensionales son una herramienta muy útil para aproximarse al entorno *in vivo* permitiendo evaluar nuevos fármacos. Su importancia radica en que permiten simular diferentes pautas de administración utilizándolas para planificar y evaluar tanto los intervalos de dosificación como las concentraciones de tratamientos para implementarlas posteriormente en ensayos *in vivo*. Aplicados a la FQ, esta estrategia puede ser el inicio, con necesidad de optimización y mejora de sus características, para la obtención de un modelo de epitelio pulmonar realista *in vitro* que sirva para la evaluación de nuevos tratamientos y de sus pautas de administración.

Para concluir, la terapia génica ha surgido como una herramienta realista para tratar las enfermedades genéticas. El auge de la nanomedicina ha abierto la oportunidad de elaborar y optimizar vectores no virales, como los niosomas, para abordar una enfermedad como la fibrosis quística mediante un enfoque basado en terapia génica. Para ello, la inclusión de nuevos compuestos en las formulaciones y la elección del método de elaboración de niosomas más adecuado, así como una amplia caracterización biofísica y su evaluación *in vitro* en un modelo más realista, abren nuevas perspectivas y oportunidades para progresar en el tratamiento de esta enfermedad y poder ofrecer una solución basada en terapia génica.



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