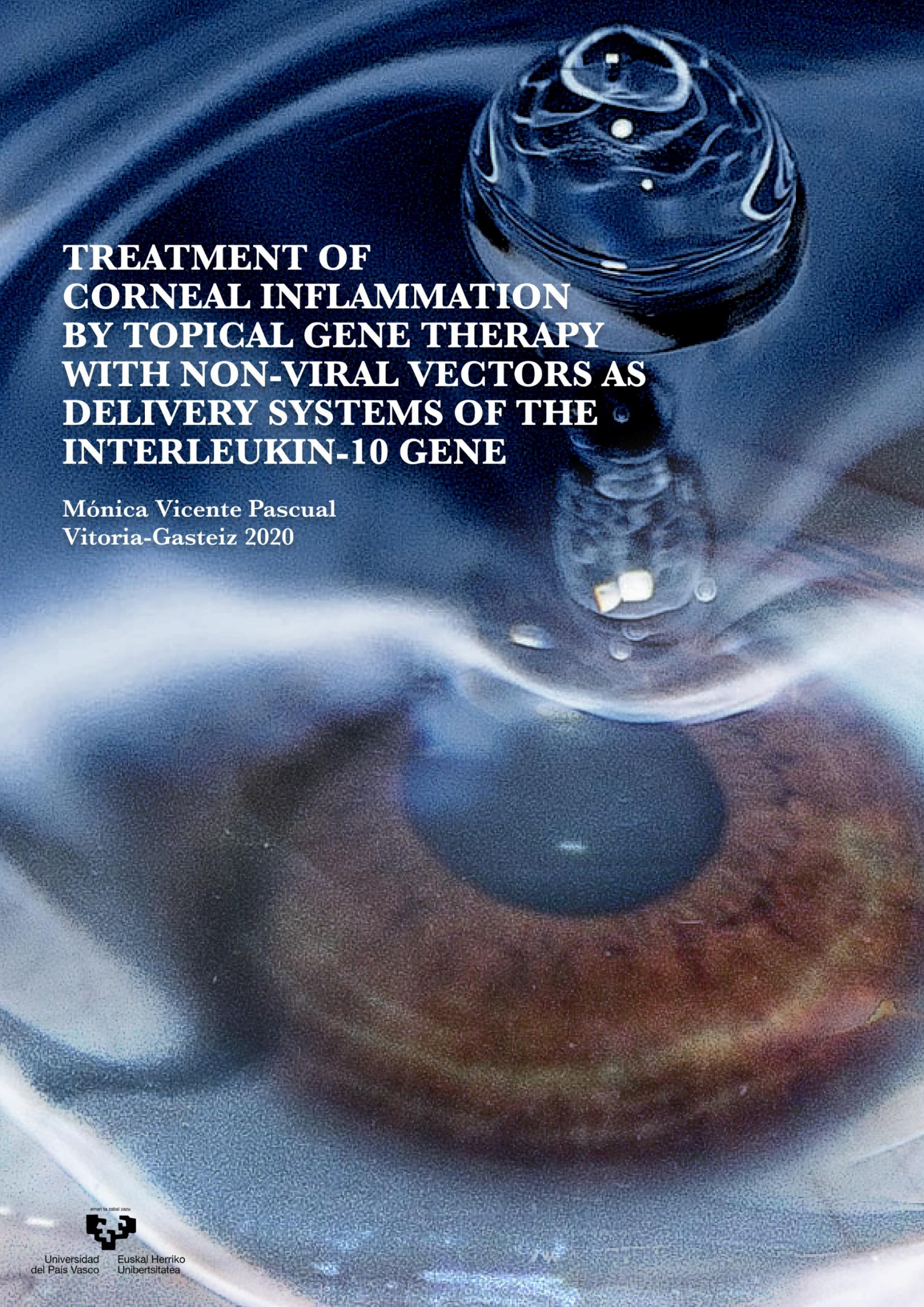

TREATMENT OF CORNEAL INFLAMMATION BY TOPICAL GENE THERAPY WITH NON-VIRAL VECTORS AS DELIVERY SYSTEMS OF THE INTERLEUKIN-10 GENE

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Vitoria-Gasteiz 2020

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“Hay momentos en los que la vida te coloca en una situación compleja: escoger entre lo que puede hacerte feliz y lo que quieres que te haga feliz. La primera opción es sencilla, no supone un esfuerzo importante ni te obliga a renunciar a nada. Se trata de oportunidades que la vida pone frente a tus ojos, oportunidades que normalmente aplauden los que te rodean. […] Entonces surgen los segundos caminos, esos en los que no se vislumbra el final, ni siquiera el recorrido. Están plagados de miedo y vértigo, de una indecisión que a veces parece que no termina, de dedos acusadores que llaman imprudencia y osadía a tu valor. Entrañan riesgos y, casi siempre, falta de seguridad, de la certeza de que aquello vaya a salir bien. Sin embargo, en esos lugares se encuentran los sueños, las verdaderas aspiraciones de cada uno, ya sean trabajos, relaciones o ganas de abrir nuevas puertas. En esos trayectos aparece de pronto la libertad más pura: la del alma. […]

Quiero decirte algo que he aprendido con el tiempo: la felicidad no es la meta, sino el camino.”

Días sin ti

Elvira Sastre

GLOSSARY

AAV: adeno-associated viruses

AD: adenovirus

ADA: adenosine deaminase

ADA-SCID: severe combined immunodeficiency due to adenosine deaminase

AIDS: acquired immune deficiency syndrome

ALL: acute lymphoblastic leukaemia

AMD: age-related macular degeneration

ASO: antisense oligonucleotides

ATP: adenosine triphosphate

BE: bemiparin

CAR T cell: chimeric antigen receptor T cell

CAR: Coxackie Adenovirus Receptor

CCK-8: cell counting kit-8

CCR5: C-C motif chemokine receptor type 5

CH: chitosan

CMC: chemistry, manufacturing and controls

CMV: cytomegalovirus

CNV: corneal neovascularization

CRISPR/Cas9: clustered regularly interspaced short palindromic repeats (CRISPR)/associated nuclease Cas9 (Cas9)

DAPI: 4’,6-diamidine-2’-phenylindole dihydrochloride

DC-cholesterol: 3ß-[N- (N', N'-dimethylaminoethane)-carbamoyl]-cholesterol

DLBCL: diffuse large B-cell lymphoma

DMD: Duchenne muscular dystrophy

DMEM/F-12: Dulbecco´s Modified Eagle´s Medium/Nutrient Mixture F-12

DMRIE: 1,2-dimyristyloxypropyl-3-dimethyldhydroxyethylammonium bromide

DNA: deoxyribonucleic acid

DNase I: Deoxyribonuclease I

DOPE: phospholipid 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine

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

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

DSB: double strand break

DX: dextran

ECM: extracellular matrix

ELISA: enzyme-linked immunosorbent assay

EMA: European Medicinal Agency

FBS: fetal bovine serum

FDA: Food and Drug Administration

FGF: fibroblast growth factor

GABP: GA binding protein

GFP: green fluorescent protein

GM-CSF: granulocyte-macrophage colony-stimulating factor

gRNA: guide RNA

GTMP: gene therapy medicinal product

HA: hyaluronic acid

Haplo-HSCT: haploidentical haematopoietic stem cell transplantation

hATTR: hereditary transthyretin amyloidosis

HCE-2: human corneal epithelial cells

HDL: high-density lipoprotein

HDR: homology-dependent repair

HIF: hypoxia-inducible factor

HIF-1α: subunit of a heterodimeric transcription factor hypoxia-inducible factor 1

HIV: human immunodeficiency virus

HLA: human leukocyte antigen

hRPE65: human retinal pigment epithelium 65kDa protein

HSCT: haematopoietic stem cell transplantation

HSK: herpes simplex virus-induced stromal keratitis

HSV-1: herpes simplex virus type 1

HSV-TK: herpes simplex virus thymidine kinase

IFN-γ: interferon-gamma

IL: interleukin

ITR: inverted terminal repeats

k: consistency coefficient

KO: Knock Out

LDL-C: low-density lipoprotein-cholesterol

LEC: lymphatic endothelial cells

LTR: long terminal repeats

mcDNA: DNA minicircles

MHC: major histocompatibility complex

miRNA: microRNA

MLV: murine leukemia virus

MMPs: matrix metalloproteinases

MNPs: magnetic nanoparticles

MPS: mucopolysaccharidosis

mRNA: messenger RNA

n: flow behaviour index

NF-κB: nuclear factor-κB

NHEJ: non-homologous end joining

NLS: nuclear localization signals

NP2: neuropilin-2

NPC: nuclear pore complex

NSF: negative splicing factor

OTC: ornithine transcarbamylase

OV: oncolytic viruses

P: protamine sulfate salt

pbs: first binding site

PBS: phosphate buffered saline

PCSK9: pro-protein convertase subtilisin/kexin 9

PDGF: platelet-derived growth factor

PDI: polydispersity index

PEDF: pigment epithelium-derived factor

PEG: polyethylene glycol

PEI: polyethyleneimine

PFA: paraformaldehyde

p-IL10: plasmids that encode for IL-10

PMBCL: primary mediastinal large B-cell lymphoma

PPARγ: peroxisome proliferator-activated receptor gamma

ppt: poly-purine tract sequence

pre-miRNA: precursor miRNA

pri-miRNA: primary miRNA

PVA: polyvinyl alcohol

PVA9000: partially hydrolyzed polyvinyl alcohol 9000-10000 Da Mw

QbD: quality by design

R^2: high coefficient of determination

RFU: relative fluorescent units

RGD: arginine-glycine-aspartic acid

RISC: RNA induced silencing complex

RNA: ribonucleic acid

RNAi: interference RNA

S/MAR: scaffold matrix attachment regions

SCID: severe combined immunodeficiency

SDFA: China State Food & Drug Administration

SDS: sodium dodecyl sulphate

shRNA: short hairpin RNAs

siRNA: short interference RNA

SLN: Solid Lipid Nanoparticles

SMA: spinal muscular atrophy

SMN: survival of motor neuron

TALEN: transcription activator-like effector nucleases

TEM: transmission electronic microscopy

TGF-β: transforming growth factor beta

TNF: tumor necrosis factor

TTR: transthyretin

VEGF: vascular endothelial growth factor

ZFNs: zinc finger nucleases

ΔLNGFR: human low affinity nerve growth factor receptor

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RESUMEN

La terapia génica es una prometedora herramienta terapéutica para tratar, curar e incluso prevenir enfermedades, dado que permite reparar, desactivar o reemplazar los genes disfuncionales que las causan y así restablecer su función normal. Según la Agencia Europea del Medicamento (EMA), un producto terapéutico basado en terapia génica consiste en un vector o sistema de administración que contiene un constructo genético para expresar una secuencia terapéutica o proteína específica responsable de la regulación, reparación, adición o deleción de una secuencia genética determinada. Supone una revolución en cuanto a la manera de afrontar el tratamiento de algunas enfermedades, ya que abre la posibilidad de curar patologías para las que las terapias convencionales solo llegan a paliar algunos de los síntomas, aunque por el momento es un área principalmente experimental.

Los ácidos nucleicos administrados en terapia génica presentan propiedades físico-químicas muy diferentes a los fármacos convencionales. Por un lado, tienen una estabilidad limitada, ya que son degradados muy rápidamente en el organismo, y por otro, deben atravesar diferentes membranas para acceder a compartimentos intracelulares, como el núcleo o el citoplasma de las células, donde ejercerán su efecto modificando de manera artificial la expresión de un gen. Este proceso se conoce como transfección.

Teniendo todo ello en cuenta, es necesario un sistema de administración eficaz y seguro, que sea capaz de proteger el material genético, de internalizarlo en las células diana y una vez allí, que permita su liberación y favorezca su acceso al compartimento intracelular adecuado. El sistema de administración ideal va a depender del tipo de célula, del ácido nucleico que se quiere emplear y de la duración de la expresión. Se clasifican en dos grupos: vectores virales y no virales.

Los vectores virales se preparan a partir de virus genéticamente modificados, de manera que no puedan replicarse en las células diana, pero puedan expresar el gen terapéutico que transportan. Estos vectores son eficaces y permiten conseguir altos porcentajes de transfección. Además, es posible obtener una expresión del material genético a largo plazo. Sin embargo, presentan limitaciones relacionadas con su potencial oncogénico e inmunogénico debido a las proteínas virales. Otra de las desventajas, es que no pueden transportar ácidos nucleicos de gran tamaño. Los vectores virales más estudiados son los retrovirus, lentivirus, adenovirus y virus adenoasociados.

Los vectores no virales, en cambio, son más seguros, simples y baratos, además de ser sistemas más reproducibles. No presentan limitaciones en cuanto al tamaño del material genético que incorporan, pero la eficacia de transfección es menor comparada con los vectores virales, y, en

general, la duración de la expresión es menor, aunque en los últimos años se han desarrollado materiales y técnicas con los que se han mejorado los resultados. De hecho, el número de ensayos clínicos empleando este tipo de sistemas de administración ha aumentado en los últimos diez años. Entre los sistemas no virales, encontramos métodos físicos, como la electroporación, sonoporación o hidroporación, y sistemas químicos.

Los sistemas químicos se basan en el uso de diferentes tipos de compuestos, biocompatibles y biodegradables, capaces de encapsular o unir el material genético, tanto de manera electrostática como covalente. Dentro de este tipo de sistemas encontramos varias categorías: partículas inorgánicas, vectores poliméricos, lipídicos o peptídicos. Los sistemas lipídicos son los vectores no virales más estudiados a nivel clínico. Se han documentado hasta 119 ensayos clínicos, la mayoría de ellos en fases I y II, en los que se han utilizado vectores basados en lípidos como sistemas de liberación del material genético.

Dentro de los vectores lipídicos se encuentran las nanopartículas sólidas lipídicas (SLN). Son partículas esféricas de tamaño nanométrico formadas por un núcleo compuesto por lípidos sólidos a temperatura ambiente, el cual está rodeado por una capa de tensioactivos. En las SLN diseñadas para su aplicación en terapia génica, parte del efecto tensioactivo viene dado por un lípido catiónico, que además proporciona carga positiva a la superficie de las partículas, permitiendo su unión al material genético y a las superficies celulares, que poseen carga negativa. Debido a su carácter lipídico están compuestas por componentes bien tolerados, que han sido aprobados para preparados farmacéuticos de uso humano. Además, se han desarrollado diversos métodos para su producción, que se han aplicado con éxito en la industria farmacéutica y cosmética, pudiendo incluso esterilizarse y liofilizarse para su almacenaje.

Desde los primeros ensayos clínicos con productos terapéuticos basados en terapia génica, 9 de ellos se han aprobado en todo el mundo y casi 2.700 ensayos clínicos se han completado, están en curso o se han autorizado para un amplio rango de aplicaciones. La indicación más frecuente a la que se dirigen es al cáncer con un 66,8% de ensayos clínicos realizados, seguido de las enfermedades monogénicas, infecciosas y cardiovasculares, con porcentajes por debajo del 12%. Los ensayos clínicos con terapia génica dirigidos a enfermedades oculares suponen un 1,2%.

El ojo es un órgano muy especializado, con estructuras individuales implicadas en el proceso de captura y procesamiento de la información visual, que se encuentra dividido en compartimento posterior y anterior. En el segmento anterior del ojo se localiza la córnea, tejido transparente que realiza una función indispensable para la refracción de la luz y el control del enfoque. Entre

las patologías que afectan a este tejido la inflamación corneal o queratitis es relativamente frecuente y puede desembocar en problemas graves como la ceguera. Existe una gran variedad de factores que desencadenan inflamación corneal, incluyendo infecciones, ojo seco, patologías de los párpados, daños físicos y químicos y un amplio rango de enfermedades subyacentes. La queratitis, independientemente de su origen, presenta como síntomas dolor, visión borrosa, fotofobia, lagrimeo y enrojecimiento del ojo. Si la inflamación persiste y se vuelve crónica, puede alterar la visión y acabar en destrucción del tejido, llegando a producirse ulceración de la córnea, cicatrices e incluso perforación, causando discapacidad visual y ceguera. Las opciones terapéuticas actuales para el tratamiento de la inflamación corneal están restringidas por la eficacia limitada, los efectos adversos y la corta duración de acción.

La terapia convencional contra la inflamación ocular consiste en la administración sistémica o la instilación tópica de corticosteroides. Sin embargo, su uso prolongado frecuentemente da lugar a cataratas y aumento de la presión intraocular como efectos adversos. Por lo tanto, es necesario el desarrollo de nuevas estrategias para el tratamiento de la inflamación corneal. Un posible enfoque terapéutico es la administración de interleuquina-10 (IL-10), una potente citoquina inmunomoduladora, producida por varios tipos de células, que interactúa con las células presentadoras de antígenos inhibiendo la producción de citoquinas proinflamatorias como la IL-1, IL-6, IL-8 y el factor de necrosis tumoral (TNF)-alfa. Varios estudios han confirmado el papel esencial que desempeña la IL-10 en la respuesta inmunológica asociada a las patologías de la superficie ocular. Sin embargo, la baja biodisponibilidad de la IL-10 tras su administración tópica, debido a la barrera corneal, y su corta semivida de eliminación, dificultan el efecto antiinflamatorio, incluso administrando dosis altas de manera frecuente. La suplementación genética, en la que se administra en la córnea el plásmido que codifica la IL-10, es una alternativa que puede resultar útil para superar estos inconvenientes, ya que permite la expresión *de novo* de IL-10 en células corneales.

La terapia génica ha emergido como una alternativa eficaz para las enfermedades corneales, gracias, en parte, a las ventajas que presenta la córnea en cuanto a transparencia, accesibilidad, facilidad de administración y monitorización de manera no invasiva. Además, la circulación sanguínea y el sistema inmune en este tejido son limitados, confiriéndole “inmunoprivilegio”. Por otro lado, la posibilidad de mantener la córnea *ex vivo* durante varias semanas facilita la evaluación y eficacia de los productos de terapia génica y la optimización de la transfección. Aun así, los ensayos clínicos de terapia génica aplicada a la córnea en humanos son escasos y la mayoría de los estudios se encuentran a nivel preclínico en modelos animales. Una de las principales limitaciones para la aplicación de la terapia génica en la córnea está asociada a las

dificultades para diseñar sistemas de administración seguros y eficaces. Es necesario desarrollar sistemas que puedan ser autoadministrados mediante instilación ocular y que sean capaces de superar las barreras corneales que limitan la penetración del material genético en la córnea, y proporcionar una disponibilidad adecuada en las células diana.

Teniendo todo esto en cuenta, el objetivo principal de esta tesis fue el desarrollo de un sistema de administración para terapia génica basado en SLN, para su administración ocular tópica con el fin de abordar la inflamación corneal mediante la producción *de novo* de IL-10.

Para ello, en primer lugar, se diseñaron, optimizaron y caracterizaron diferentes vectores con dos tipos de SLN: SLN_{EE}, preparadas mediante el método de emulsificación/evaporación del solvente y SLN_C, preparadas mediante coacervación, que evita el uso de solventes. Para la formación de los vectores se emplearon diferentes componentes: SLN, ADN plasmídico (que codifica la proteína verde fluorescente (GFP) o la IL-10), un péptido catiónico (protamina) y un polisacárido (bemiparina, dextrano o ácido hialurónico). Estos componentes se unieron mediante interacciones electrostáticas. El hecho de ser un vector con varios componentes confiere gran versatilidad a este sistema por varias razones: en primer lugar, el polisacárido va a determinar la interacción con las células diana, y como consecuencia, el proceso de internalización y la disposición intracelular del material genético, y, en segundo lugar, la protamina favorece el proceso de transcripción y la internalización nuclear, además de contribuir a la unión y protección del ácido nucleico.

El tamaño de estos vectores se encontró en el rango nanométrico y su carga superficial fue positiva. Ambos factores favorecen la entrada celular y la penetración en la barrera corneal, y evitan la irritación y posibles molestias tras su administración tópica. Se estudió también su morfología mediante microscopía electrónica de transmisión y se comprobó su capacidad para unir, liberar y proteger el ADN plasmídico.

Tras estos estudios se evaluó la viabilidad celular y la eficacia de transfección *in vitro* en células humanas del epitelio corneal (HCE-2). Primero se emplearon vectores que contenían el plásmido reportero pcDNA3-GFP para estudiar la expresión de GFP y el porcentaje de células transfectadas, y más tarde la producción de IL-10 con los sistemas conteniendo el plásmido que codifica para esta proteína. Se obtuvieron niveles de IL-10 secretada que previamente habían demostrado ser suficientes para ejercer el efecto antiinflamatorio deseado. Además, se empleó un ADN marcado con el fluoróforo monoazida de etidio para poder estudiar la distribución intracelular y la condensación del mismo, observando diferencias en función del vector utilizado.

En la siguiente etapa se evaluaron *ex vivo* los vectores basados en SLN en explantes de córnea de conejo, un modelo estático que se asemeja más a las condiciones fisiológicas que se dan *in vivo*. Los diferentes vectores fueron capaces de transfectar los explantes corneales sin afectar a la estructura del tejido, aunque la eficacia de transfección y el tipo de célula corneal transfectada dependieron del polisacárido empleado, siendo los vectores formulados con dextrano o ácido hialurónico los más efectivos. Todos los vectores transfectaron las células epiteliales, pero los que contenían ácido hialurónico también transfectaron el estroma, y con los vectores preparados con dextrano, la proteína se produjo extensamente en el epitelio, en el estroma y también en el endotelio. Por lo tanto, el diseño adecuado de los vectores no virales basados en SLN permite modular la biodistribución y, por lo tanto, la transfección dentro de las diferentes capas celulares de la córnea.

Teniendo en cuenta estos resultados, para los siguientes estudios se emplearon los vectores con SLN_{EE} que contenían dextrano y ácido hialurónico, y se reformularon las SLN_C; el compuesto catiónico glicol quitosano se reemplazó por el DEAE-dextrano, que es un polímero muy utilizado para administración de ácidos nucleicos. De acuerdo con los estudios de caracterización y eficacia de transfección *in vitro* se seleccionó el vector compuesto por las nuevas SLN_C combinadas con protamina y ácido hialurónico para los siguientes ensayos.

Para incrementar el tiempo de residencia de la formulación en la superficie de la córnea y reducir su eliminación debido al lagrimeo natural del ojo, se decidió incorporar alcohol polivinílico (PVA), un polímero sintético utilizado comúnmente en preparaciones oftálmicas y que ha demostrado su capacidad para aumentar la absorción ocular de medicamentos. En primer lugar, se estudiaron los factores tecnológicos de las formulaciones relacionados con su administración por vía tópica ocular. Se observó que el PVA aumentaba la viscosidad e incrementaba la adhesión de las formulaciones *in vitro*, y que todas las formulaciones tenían un pH bien tolerado por el ojo. De hecho, la tinción tricrómica de Masson de las muestras *in vivo* no mostró cambios en la histología de las córneas en comparación con la córnea sin tratar tras 3 días de tratamiento.

En el estudio de las propiedades reológicas se observó que la mayoría de las formulaciones presentaban un comportamiento pseudoplástico, de manera que durante el parpadeo la baja viscosidad permite que la formulación se extienda sobre la superficie corneal, mientras que en reposo la viscosidad aumenta, mejorando la retención y evitando el drenaje.

A continuación, se evaluó la capacidad de los vectores formulados como gotas oculares, para superar las barreras corneales tras su administración tópica en ratones wild type. El vector formado por las SLN_{EE} y el ácido hialurónico como polisacárido fue el más eficaz para penetrar

en la córnea. El receptor CD44 parece estar involucrado en el proceso de internalización de las formulaciones que contienen ácido hialurónico. Además, la combinación de PVA y ácido hialurónico en la formulación final, mejoró la capacidad de los vectores para penetrar en la córnea, lo que podría estar relacionado con la mayor adhesividad y la disminución de la viscosidad observada para esa combinación en los estudios reológicos.

Inicialmente se realizaron estudios preliminares de transfección *in vivo* en ratones wild type con el plásmido que codifica la proteína GFP, ya que al ser una proteína intracelular permite identificar las células corneales transfectadas. La distribución del GFP coincidió con la distribución de los vectores observados previamente en el estudio de localización, confirmando que sólo se transfectaron las células epiteliales, y la combinación de ácido hialurónico y PVA en las formulaciones finales dio lugar a mayores niveles de expresión de la proteína.

Para los estudios de transfección con el plásmido que codifica la IL-10 se emplearon solo las formulaciones con PVA al ser las más eficaces. En estos estudios se administraron los vectores a ratones wild type y ratones knock out para IL-10, y se detectó la IL-10 en todas las secciones analizadas. La IL-10 es una proteína que se secreta, por lo que su presencia en una determinada capa no significa necesariamente que se haya expresado allí. De hecho, la IL-10 se pudo observar incluso en las células endoteliales. En este caso también las formulaciones con ácido hialurónico resultaron ser las más eficaces, siendo la intensidad de fluorescencia en el endotelio mayor en el caso del vector preparado con las SLN_{EE}. Tras confirmar la capacidad de los sistemas no virales desarrollados para producir la IL-10 tras su administración tópica, el siguiente paso a seguir será la realización de estudios a largo plazo que permitan valorar a nivel histológico el impacto de la expresión de la IL-10 sobre la inflamación corneal.

Los resultados obtenidos en este trabajo permiten concluir que la terapia génica es una estrategia viable para abordar la inflamación corneal mediante la expresión de IL-10 *de novo* en las células de la córnea, a través de la administración tópica de sistemas de administración de genes basados en SLN formulados como gotas oculares.

SUMMARY

Gene therapy is a novel approach to treat, cure, or ultimately prevent disease by changing the expression of a person's gene. According to the European Medicinal Agency (EMA), a gene therapy medicinal product (GTMP) generally consists of a vector or delivery formulation/system containing a genetic construct engineered to express a specific transgene ('therapeutic sequence') for the regulation, repair, replacement, addition or deletion of a genetic sequence. Gene therapy works by repairing, deactivating, or replacing dysfunctional genes that cause disease with the aim of (re)establishing normal function. Thanks to the advances of genetics and bioengineering, gene therapy becomes possible, although at present, it is an area predominantly experimental.

Nucleic acids possess physical-chemical properties very different to that of conventional drugs. They present limited stability in the biological medium, and they must overcome several barriers to access to the appropriate intracellular compartment (the cytoplasm or the nucleus), a process known as transfection. These two characteristics are mainly responsible for the difficulties to develop a medicinal product.

A fundamental aspect for the success of gene therapy is the availability of delivery systems capable of protecting the genetic material from degradation, facilitating its internalization in target cells and releasing them inside. The ideal delivery system depends on the target cells, the kind of nucleic acid to be delivered and the duration of expression. Delivery systems can be classified into two large groups: viral and non-viral vectors.

Viral vectors are prepared from genetically modified viruses so that they are not able to replicate in the target cells, but they express the therapeutic gene they transport. Viral vectors allow high long-term transfection efficiencies; however, they present important safety limitations due to the oncogenic and immunogenic potential (due to viral proteins). Another problem associated to viral vectors is the inability to transport large nucleic acids. The most studied viral vectors are retrovirus, lentivirus, adenovirus and adeno-associated virus.

Non-viral vectors are safer, simpler, cheaper and more reproducible systems. In addition, they do not present limitations regarding the size of the genetic material they can incorporate. Nevertheless, a disadvantage of non-viral systems is that their transfection efficiency is lower compared to viral vectors, although in recent years new non-viral systems have been developed with materials that exhibit higher transfection efficiencies. In fact, the number of clinical trials with products based on non-viral vectors has increased in the last decade. Non-viral vectors are divided in physical methods, such as electroporation, sonoporation or hydrofection, and chemical carriers.

Chemical vectors are based on the use of different types of biocompatible and biodegradable compounds, capable of encapsulating or binding, electrostatically or covalently, the genetic material. This type of systems can be classified in different groups: inorganic, polymeric, lipidic or peptidic particles. Among them, lipid-based systems are the most studied non-viral vectors at the clinical level. Up to 119 clinical trials have been documented, most of them in phases I and II, in which lipid vectors have been used as delivery systems for the genetic material.

Lipidic vectors include Solid Lipid Nanoparticles (SLNs), which are spherical particles in the range of nanometres, formed by a core composed of a solid lipid at room temperature surrounded by a layer of surfactants. In the case of SLNs designed to be applied in gene therapy, cationic lipids exert part of the surfactant effect and, in turn, confer positive charge to the surface of the particles, making possible their union with genetic material and cell surfaces that have negative charge. SLNs are usually composed of well-tolerated physiological lipids that have been approved for pharmaceutical preparations for human use. Furthermore, a variety of production methods, which have been successfully implemented in the pharmaceutical and cosmetic industries, have been developed to manufacture SLNs and have furnished stable delivery systems that can undergo long-term storage.

Since first gene therapy clinical trials in the early 1990s, only nine gene therapy medicinal products have received approval worldwide; nevertheless, almost 2,700 gene therapy-based clinical trials have been completed, are ongoing or have been approved for a broad range of applications. The main indications these clinical trials address are cancer diseases (66,8%), monogenic, infectious and cardiovascular diseases (less than 12%), and ocular diseases (1,2%) among others.

The eye is an extremely specialized organ, with individual structures that work together to capture and process visual information. It is broadly divided into two compartments, the anterior and the posterior segment. The cornea, a transparent window at the anterior segment of the eye, performs a significant function in eyesight by refracting the light to focus a visual image. Among the pathologies that affect this tissue, corneal inflammation or keratitis is relatively frequent and can lead to serious problems such as blindness. A wide range of factors may be responsible of corneal inflammation, including infections, dry eye, disorders of the eyelids, physical and chemical injury, and a large variety of underlying diseases. Keratitis, regardless of its origin, presents common symptoms and signs, including eye pain, blurred vision, photophobia, tearing, and eye redness. Furthermore, chronic inflammation of the cornea induces visual disturbance, and often results in tissue destruction that leads to corneal ulceration, scarring and, even, perforation, causing visual impairment and blindness. Current

treatment options for corneal inflammation are restricted by limited efficacy, adverse effects and short duration of action.

The conventional therapy against ocular inflammation is the systemic administration or topical instillation of corticosteroids. However, cataracts and increased intraocular pressure are adverse effects that are frequently caused by the long-term use of corticosteroids. Therefore, the development of new therapeutic strategies for the treatment of corneal inflammation becomes necessary. One possible approach is the administration of interleukin-10 (IL-10). It is a potent immunomodulatory cytokine that interacts with antigen presenting cells inhibiting the production of proinflammatory cytokines such as IL-1, IL-6, IL-8 and tumor necrosis factor (TNF)-alpha. Several studies have confirmed the essential role that IL-10 plays in the regulation of the immune response associated with ocular surface pathologies. However, the low bioavailability of this protein after topical administration, which is caused by the corneal barrier, and its short half-life, hamper the anti-inflammatory effect, even after frequent topical administration at high doses. Gene supplementation, in which the plasmids that encode for IL-10 are administered in the cornea, is an alternative that may overcome these drawbacks.

Gene therapy has emerged as a new approach for corneal diseases, thanks to the advantages of the cornea in terms of accessibility, transparency, ease of vector administration and visual monitoring, and ability to perform frequent non-invasive corneal assessment. In addition, the general circulation and the systemic immune system in the cornea are limited, a concept termed “immune privilege”. Other important favouring circumstance is that the cornea can be maintained as an *ex vivo* organ cultured for several weeks, which allows to evaluate the efficacy and safety of gene therapy products, and to optimise gene transfer. Nevertheless, clinical trials of gene therapy applied to the cornea in humans are scarce and most studies are at the pre-clinical level in animal models. One of the main limitations for the application of gene therapy to the cornea is associated with the difficulties in designing safe and effective delivery systems. It is necessary to develop systems that can be self-administered by ocular instillation and that are able to overcome the barriers that limit the penetration of genetic material into the cornea, and provide adequate bioavailability in the target cells.

Accordingly, the main objective of this thesis was to develop a gene therapy system, particularly, a non-viral vector based on SLNs, for topical instillation to address corneal inflammation by *de novo* IL-10 production. In order to achieve this aim, the following steps were carried out.

Firstly, the design, characterization and optimization of different nanovectors based on two types of SLNs: either SLN_{EE}, prepared by emulsification/solvent evaporation method or SLN_{C}, by coacervation technique, which avoids the use of solvents. The vectors were formed by

electrostatic interaction between the SLNs with ligands of different nature, including the cationic peptide protamine and polysaccharides (bemiparin, dextran or hyaluronic acid). A plasmid encoding the green fluorescent protein (GFP) or the IL-10 was employed. The final vector containing various ligands confers the system a high versatility for different reasons. Firstly, polysaccharides determine the interaction with targeted cells, and as a consequence, the internalization process and the intracellular behaviour of the genetic material. Secondly, protamine, apart from favouring the transcription process and nuclear entry, also contributes binding and protecting the genetic material at intra and extracellular level, thanks to its cationic nature.

The final vectors presented particle size in the nanometre range and positive superficial charge. These properties facilitate corneal penetration and cellular uptake and avoid ocular irritation or discomfort. Their morphology was also studied using Transmission Electron Microscopy and their ability to bind, release and protect the plasmid DNA.

After these studies, cell viability and transfection efficacy were assessed *in vitro* in Human Corneal Epithelial (HCE-2) cells. Transfection studies were carried out first with the vectors containing the reporter plasmid pcDNA3-EGFP, encoding the intracellular protein GFP, in order to quantify the percentage of transfected cells, and then, with the vectors bearing the therapeutic plasmid pUNO1-IL10, obtaining enough secreted levels to exert anti-inflammatory effect. Moreover, the plasmids labelled with the fluorophore ethidum monoazide were employed in order to follow its intracellular disposition and condensation; differences regarding the composition of the vector were observed.

For the next step, rabbit corneas were used to perform the *ex vivo* studies, with the aim of studying cellular uptake and transfection efficacy of the formulations. All the vectors were able to transfect the corneal explants without affecting the tissue structure, although the transfection efficiency and the type of corneal cell transfected depended on the polysaccharide used, with vectors formulated with dextran or hyaluronic acid being the most effective. All the vectors transfected the epithelial cells, but those containing hyaluronic acid also transfected the stroma, and with vectors prepared with dextran, the protein was extensively produced in the epithelium, stroma and also in the endothelium. Therefore, the appropriate design of SLN-based non-viral vectors allows modulating the biodistribution and, therefore, the transfection within the different cell layers of the cornea.

Based on these results, HA-SLN_{EE} and DX-SLN_{EE} vectors were selected for the next assays. In addition, SLN_C were reformulated: the cationic agent glycol chitosan was replaced by DEAE-dextran, which is a polymer extensively used in nucleic acid delivery. According to

characterization and *in vitro* studies the SLN_C-based vectors containing hyaluronic acid were selected for further studies.

In order to enhance the residence time of the formulation in contact with the cornea and reduce drainage from lachrymal fluid, polyvinyl alcohol (PVA), widely used as viscosity modifier in ophthalmic preparations because of its ability to increase ocular drug absorption, was included in the final formulation. First, technological aspects of the formulations related to the ocular administration route were considered. PVA increased the viscosity and the adhesion of the formulations *in vitro*, and all the formulations showed a pH well tolerated by the eye. In fact, Masson's trichrome staining of the *in vivo* samples showed no change in the histology of the corneas compared to the untreated cornea after 3 days of treatment.

Regarding rheological properties, most of the formulations had pseudoplastic behaviour, so that during blinking the low viscosity allows the formulation to spread over the corneal surface, while at rest the viscosity increases, improving retention and preventing drainage.

Thereafter, the ability of the vectors formulated as eye drops to overpass the corneal barriers after topical administration *in vivo* in wild type mice was evaluated. The vector composed by SLN_{EE} and hyaluronic acid was the most effective to penetrate into the cornea. In addition, the combination of PVA and hyaluronic acid in the final formulation improved the ability of the vectors to penetrate to the cornea, which may be related to the increased adhesiveness and decreased viscosity observed for that combination in rheological studies.

Preliminary *in vivo* transfection studies were conducted in wild type mice with the plasmid encoding the intracellular reporter GFP to identify the transfected corneal cells. The distribution of GFP matched with the distribution of the vectors previously observed in the localization study, confirming that only the epithelial cells were transfected. The combination of hyaluronic acid and PVA resulted in higher protein expression.

For the following studies with the therapeutic plasmid pUNO1-hIL10, PVA-containing formulations were selected. In these studies, vectors were administered to wild type and IL-10 knock out mice. IL-10 was detected in all the sections analysed, not only in the epithelium but also in the endothelial layer with all the vectors. However, IL-10 is a secreted protein and its presence in a certain layer does not necessarily mean that it was expressed there. Hyaluronic acid formulations proved to be the most effective, with the intensity of fluorescence in the endothelium being higher in the case of the SLN_{EE}-based vector. After confirming the ability of the developed non-viral systems to produce IL-10 after topical administration, the next step will be to carry out long-term studies to assess the impact of IL-10 expression on corneal inflammation at the histological level.

These promising results highlight the potential contribution of gene therapy deto address corneal inflammation through the expression of IL-10 *de novo* in corneal cells, after topical administration of SLN-based gene delivery systems formulated as eye drops.

INTRODUCTION

The content of this section is based on the following publications, in which I am co-author:

del Pozo-Rodríguez A, Rodríguez-Gascón A, Rodríguez-Castejón J, **Vicente-Pascual M**, Gómez-Aguado I, Battaglia LS, Solinís MÁ. Gene Therapy. In: Current Applications of Pharmaceutical Biotechnology. Advances in Biochemical Engineering/Biotechnology. Silva A, Moreira J, Lobo J, Almeida H, (Ed.), Springer International Publishing, Switzerland. 2020; Volume 171:321–368. https://doi.org/10.1007/10_2019_109. *Adapted by permission from Springer Nature. Current Applications of Pharmaceutical Biotechnology. Advances in Biochemical Engineering/Biotechnology by Silva A, Moreira J, Lobo J, Almeida H, (Ed.). COPYRIGHT 2020.*

Torrecilla J, del Pozo-Rodríguez A, **Vicente-Pascual M**, Solinís MÁ, Rodríguez-Gascón A. Targeting corneal inflammation by gene therapy: Emerging strategies for keratitis. Exp. Eye Res. 2018; 176:130-40. https://doi.org/10.1016/j.exer.2018.07.006.

Rodríguez-Castejón J, Gómez-Aguado I, **Vicente-Pascual M**, Rodríguez-Gascón A, Isla A, Solinís MÁ, del Pozo-Rodríguez A. Gene-terapia: Ikuspegi terapeutiko berria begietako gaitzen tratamenduan. (Gene therapy: Novel therapeutic approach for treating ocular disorders). Ekaia. 2019; 36:31-48. https://doi.org/10.1387/ekaia.20754.

1. Gene therapy

Gene therapy is a novel approach to treat, cure, or ultimately prevent disease by changing the expression of a person's gene[1]. According to the European Medicinal Agency (EMA), a gene therapy medicinal product (GTMP) generally consists of a vector or delivery formulation/system containing a genetic construct engineered to express a specific transgene ('therapeutic sequence') for the regulation, repair, replacement, addition or deletion of a genetic sequence[2]. However, from a scientific point of view, it is accepted a broader concept of gene therapy, including the therapeutic application of products containing any nucleic acid. Gene therapy works by repairing, deactivating, or replacing dysfunctional genes that cause disease with the aim of (re)establishing normal function.

Depending on the cell type to be modified, gene therapy can be classified in two categories: somatic, and germline gene therapy[3]. Somatic gene therapy targets to body somatic cells such as bone marrow or blood cells. This type of gene therapy cannot be passed on to descendents. In germline gene therapy, egg and sperm cells (germ cells) are the objective of therapy, and the inserted gene passes on to future generations. The idea of germline gene therapy is controversial due to ethical concerns.

Two fundamental strategies have evolved to restore or modify target cell function: *ex vivo* or *in vivo* gene delivery[4]. In *ex vivo* therapy, cells from the patient or a donor are harvested, and the therapeutic gene is then transduced in a cell therapy manufacturing setting. The modified cells are later re-infused into the patient. *In vivo* gene therapy consists on functional modification of targets by direct transgene injection into the patient. Figure I features a scheme with the *ex vivo* and *in vivo* approaches to gene therapy.

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1 American Medical Association. Gene Therapy. 2015. https://www.immortalitymedicine.tv/gene-medicine/gene-therapy-american-medical-association. Accessed 30 March 2020.

2 EMA (European Medicine Agency). Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal product. 2018a. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-quality-non-clinical-clinical-aspects-gene-therapy-medicinal-products_en.pdf. Accessed 30 Mar 2020.

3 Genetics Home Reference. Gene Therapy. Lister Hill National Center for Biomedical Communications U.S. National Library of Medicine. National Institutes of Health. Department of Health & Human Services. 2017. https://ghr.nlm.nih.gov/primer. Accessed 30 Mar 2020.

4 Thorne B, Takeya R, Vitelli F, Swanson X. Gene Therapy. Adv. Biochem. Eng. Biotechnol. 2018; 165:351-99. https://doi.org/10.1007/10_2016_53.

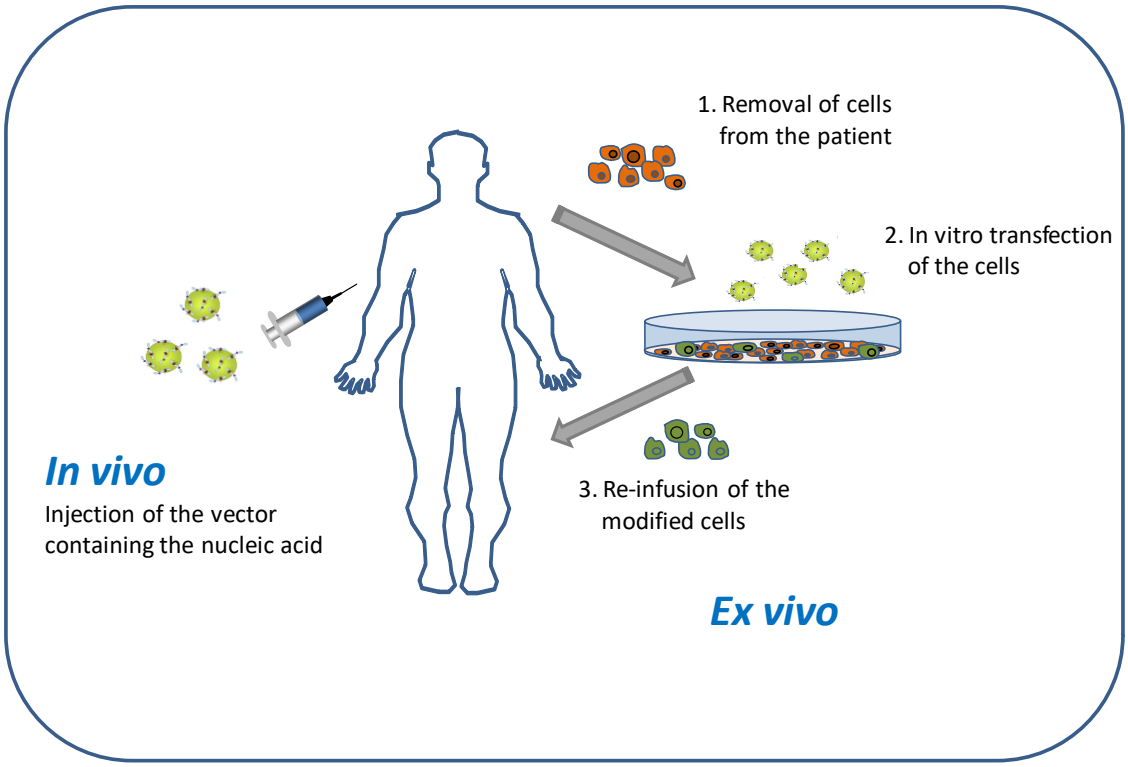

Figure I. Ex vivo and in vivo approaches to gene therapy.

Thanks to the advances of genetics and bioengineering, gene therapy becomes possible, although at present, it is an area predominantly experimental. However, in the last five years, enormous advances have occurred, with the approval of a few drug products by the Food and Drug Administration (FDA) and the EMA, and others that are expected to be marketed in the near future.

1.1. Nucleic acids for gene therapy

Historically many gene therapy approaches have been based on expression of a transgene encoding a functional protein (i.e. the transgene product). However, newer tools including directly acting nucleic acid sequences such as microRNA (miRNA), interference RNA (RNAi) via short hairpin RNAs (shRNA) or short interference RNA (siRNA), molecular scissor and gene editing approaches such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)/associated nuclease Cas9 (Cas9) (CRISPR/Cas9), are being extensively applied in research and for developing new medicinal products.

Nucleic acids are negatively charged and high molecular weight molecules, with physical-chemical properties very different to that of conventional drugs. They present limited stability in the biological medium, and must access to an intracellular compartment (the cytoplasm or the nucleus); these two characteristics are responsible for the difficulties to develop a medicinal product.

Depending on the application, the objective of the therapy can be gene augmentation, gene suppression, or gene editing[5].

1.1.1. Gene augmentation

The objective of gene augmentation is to restore normal cellular function by delivering a functional copy of a gene (DNA) or messenger RNA (mRNA).

1.1.1.1. DNA

Typically, the gene of interest is inserted into a plasmid or expression cassette, which are high molecular weight, double-stranded DNA constructs containing transgenes, which encode specific proteins[6]. Plasmids also contain a promoter and a terminator signal to drive and end gene transcription, respectively[7]. Transfection with DNA leads to much higher protein and persistent expression than those obtained with mRNA, but starts at a later time point[8], since it has to reach the nucleus of the target cell, being this process one of the most limiting steps for transfection. At present, the EMA and/or the FDA have approved six gene therapy products based on DNA.

Apart from plasmid DNA, DNA minicircles (mcDNA) are emerging due to their safety and persistent transgene expression in both quiescent or actively dividing cells[9]. mcDNA are episomal, covalently closed circular gene expression systems, generally biosynthesized in recombinant bacteria, that consist in minimalistic backbones with potential to meet the clinical requirements for safe and long-lasting expression[10]. An alternative approach to sustain prolonged gene expression is the inclusion of scaffold matrix attachment regions (S/MAR)

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5 Anguela XM, High KA. Entering the Modern Era of Gene Therapy. Annu. Rev. Med. 2019; 70:273-88. https://doi.org/10.1146/annurev-med-012017-043332.

6 Pushpendra S, Arvind P, Anil B. Nucleic acids as therapeutics. In V.A. Erdmann and J. Barciszewski (eds.), From Nucleic Acids Sequences to Molecular Medicine, RNA Technologies, Springer-Verlag Berlin Heidelberg. 2012.

7 Papadakis ED, Nicklin SA, Baker AH, White SJ. Promoters and control elements: designing expression cassettes for gene therapy. Curr. Gene Ther. 2004; 4: 89-113. https://doi.org/10.2174/1566523044578077.

8 Guan S, Rosenecker J. Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems. Gene Ther. 2017; 24:133-43. https://doi.org/10.1038/gt.2017.5

9 Gaspar V, de Melo-Diogo D, Costa E, Moreira A, Queiroz J, Pichon C et al. Minicircle DNA vectors for gene therapy: advances and applications. Expert Opin Biol Ther. 2015; 15:353-79. https://doi.org/10.1517/14712598.2015.996544.

10 Maniar LEG, Maniar JM, Chen Z-Y, Lu J, Fire AZ, Kay MA. Minicircle DNA vectors achieve sustained expression reflected by active chromatin and transcriptional level. Mol. Ther. 2013; 21:131-8. https://doi.org/10.1038/mt.2012.244.

moieties in mcDNA constructs. Amon others, mcDNA has been proposed as a potential therapeutic strategy for cancer[11].

1.1.1.2. Messenger RNA

Messenger RNA (mRNA) is the template for the synthesis of proteins. The use of synthetic mRNA to produce a desired protein in cells is a very promising technology to apply in clinic. Contrary to plasmid DNA, mRNA based therapeutics are still in their infancy, in spite of the important advantages. mRNA must only be delivered to the cytoplasm where cellular translation machinery is located. From a therapeutic perspective, protein expression arising from mRNA is more transient than that from DNA. From a safety point of view, no risk of genomic integration associated with DNA insertional mutagenesis is involved[12]. Moreover, mRNA gene therapy circumvents the need for selecting a specific promoter, and thus the transfection process is relatively efficient and facile[13,14]. Another advantage of mRNA refers to the production process, raw material synthesis and the quality product, which are more easily standardized than that for DNA, leading to a higher reproducibility[15].

Foreign RNA possesses inherent immune-activating adjuvant properties, and this effect has been studied for the intracellular delivery of mRNAs coding for specific antigens, with potential application in cancer immunotherapy[16], prophylactic vaccines[17], and allergy tolerance[18]. Actually, this application of mRNA is the most clinically advanced.

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11 Zuo Y, Wu J, Xu Z, Yang S, Yan H, Tan L et al. Minicircle-oriP-IFNg: a novel targeted gene therapeutic system for EBV positive human nasopharyngeal carcinoma. Oncol. Rep. 2014; 32:2564-70. https://doi.org/10.1371/journal.pone.0019407.

12 Kauffman KJ, Webber MJ, Anderson DG. Materials for non-viral intracellular delivery of messenger RNA therapeutics. J. Control. Release. 2016; 240:227-234. https://doi.org/10.1016/j.jconrel.2015.12.032.

13 Meng Z, O'Keeffe-Ahern J, Lyu J, Pierucci L, Zhou D, Wang W. A new developing class of gene delivery: messenger RNA-based therapeutics. Biomater. Sci. 2017; 5:2381-92. https://doi.org/10.1039/c7bm00712d.

14 Tavernier G, Andries O, Demeester J, Sanders NN, De Smedt SC, Rejman J. mRNA as gene therapeutic: how to control protein expression. J. Control. Release. 2011; 150:238-47. https://doi.org/10.1016/j.jconrel.2010.10.020.

15 Sahin U, Karikó K, Türeci Ö. mRNA-based therapeutics--developing a new class of drugs. Nat. Rev. Drug Discov. 2014; 13:759-80. https://doi.org/10.1038/nrd4278.

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17 Pollard C, De Koker S, Saelens X, Vanham G, Grooten J. Challenges and advances towards the rational design of mRNA vaccines. Trends Mol. Med. 2013; 19:705-713. https://doi.org/10.1016/j.molmed.2013.09.002.

18 Weiss R, Scheiblhofer S, Roesler E, Weinberger E, Thalhamer J. mRNA vaccination as a safe approach for specific protection from type I allergy. Expert Rev. Vaccines 2012; 11:55-67. https://doi.org/10.1586/erv.11.168.

Since the protein expression profile of mRNA and DNA is completely different, the codelivery of these two nucleic acids has been proposed to take advantage of both[19].

1.1.2. Gene silencing

1.1.2.1. Antisense oligonucleotides

Antisense oligonucleotides (ASO) are short sequences of modified DNA or RNA that can be used as therapeutic tools through 1) activation of RNase H to achieve specific knockdown of the target transcript or 2) modulation of pre-mRNA splicing to enable the restoration of a (partially) functional protein or alternatively a protein with reduced toxicity[20]. The ASO's unprecedented specificity for transcripts make them unique as a therapeutic entity as it allows very specific targeting and provides the opportunity to, for example, correct genetic defects for rare genetic diseases with a current unmet medical need, modulate splice defects in autoimmune or neurodegenerative diseases or target transcripts expressed by tumors or viruses.

The utilization of synthetic ASOs is increasing in areas ranging from clinical diagnostics to novel biopharmaceutical therapeutics and the efficacy and safety of ASOs is being investigated for the treatment of various genetic disorders where no treatment is currently available. Recently, several first-in-class ASO drugs have been approved by the FDA or EMA, including Mipomersen for the treatment of familial hypercholesterolemia, Eteplirsen for the treatment of Duchenne muscular dystrophy, and Nusinersen for spinal muscular atrophy.

1.1.2.2. Aptamers

Aptamers are short single-stranded RNA or DNA oligonucleotides, normally 15-80 nucleotides, with the capacity to fold in stable three-dimensional structures. These molecules present very high affinity with nucleic acids through structural recognition and bind to them through electrostatic interactions, hydrogen bonding, Van der Waals forces, base stacking or a combination of them[21].

Aptamers recognize and bind targets of interest just like antibodies, and have important advantages over conventional antibodies: 1) easy to synthesize by automated methods, 2) easy to modify to improve the stability, binding strength, and specificity to the target nucleic acid, 3)

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19 Thorne B, Takeya R, Vitelli F, Swanson X. Gene Therapy. Adv. Biochem. Eng. Biotechnol. 2018; 165:351-99. https://doi.org/10.1007/10_2016_53.

20 Sridharan K, Gogtay JN. Therapeutic nucleic acids: current clinical status. Br J Clin Pharmacol 2016; 82:659-672. https://doi.org/10.1111/bcp.12987.

21 Rozenblum GT, Lopez VG, Vitullo AD, Radrizzani M. Aptamers: current challenges and future prospects. Expert Opin. Drug Discov. 2016; 11:127-35. https://doi.org/10.1517/17460441.2016.1126244.

structure very flexible, 4) display low to no immunogenicity when administered in preclinical doses 1000-fold greater than doses used in animal and human therapeutic applications[22].

Due to the molecular recognition of their targets, aptamers have a variety of diagnostic and therapeutic applications, such as biosensors and target inhibitors. Due to simple preparation, easy modification, and stability, aptamers have been used in the diverse areas within molecular biology, biotechnology, and biomedicine[23]. However, up to know, the introduction of aptamers into the market has not been very successful, and only one aptamer based product have been approved for clinical use Macugen® (Pegaptanib) for the treatment of age-related macular degeneration.

1.1.2.3. RNA Interference

RNA interference (RNAi) is a posttranscriptional mechanism of gene silencing through chromatin remodeling, inhibition of protein translation, or direct mRNA degradation. RNAi is a naturally occurring process of gene regulation present in plants and mammalian cells, and it can be used to downregulate disease-causing genes. Typically, there are three different types of commonly use RNAi molecules[24]:

- microRNA (miRNA)
- Short interfering RNA (siRNA)
- Short-hairpin RNA (shRNA), also called expressed RNAi activators

1.1.2.3.1. miRNA

miRNA and their role in regulating normal physiological processes was discovered in the last decade, as well as their involvement in pathological disorders such as cancer[25]. They are non-coding RNA molecules of 18-25 nucleotide in length that regulate at post-transcriptional level the expression of genes by binding to the 3'-UTR of target genes[26]. A miRNA can regulate

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22 Eyetech Study Group. Preclinical and phase 1A clinical evaluation of an anti-VEGF pegylated aptamer (EYE001) for the treatment of exudative age-related macular degeneration. Retina. 2002; 22:143-152. https://doi.org/10.1097/00006982-200204000-00002.

23 Zhang Y, Lai BS, Juhas M. Recent Advances in Aptamer Discovery and Applications. Molecules. 2019; 24. pii: E941. https://doi.org/10.3390/molecules24050941.

24 Torrecilla J, Rodríguez-Gascón A, Solinís MÁ, del Pozo-Rodríguez A. Lipid nanoparticles as carriers for RNAi against viral infections: Current status and future perspectives. Biomed. Res. Int. 2014; 2014:161794. https://doi.org/10.1155/2014/161794.

25 Iorio MV, Croce CM: MicroRNAs in cancer: Small molecules with a huge impact. J. Clin. Oncol. 2009; 27: 5848-56. https://doi.org/10.1200/JCO.2009.24.0317.

26 Ha M, Kim VN. Regulation of microRNA biogenesis. Nat. Rev. Mol. Cell Biol. 2014; 15: 509-24. https://doi.org/10.1038/nrm3838.

different mRNAs, because they are not specific to a single mRNA[27]. miRNA are transcribed from DNA as primary miRNA (pri-miRNA), which is later processed into a precursor miRNA (pre-miRNA) by two proteins: Pasha and Drosha. The pre-miRNA is transported to the cytoplasm, where it is processed by Dicer to obtain the miRNA, which is incorporated into the RNA induced silencing complex (RISC), where a helicase unwinds the miRNA. The resulting antisense strand guides the RISC to its complementary mRNA, which is cleaved (Figure II).

miRNAs have emerged as key players in a wide array of biological processes, and changes in their expression and/or function have been associated with plethora of human diseases, such as myocardial infarction and stroke [28], Parkinson disease[29], or cancer. The application of miRNA in cancer therapy is based on the finding that miRNA expression is deregulated in cancer tissues and also due to the ability of miRNA to target multiple genes and alter cancer phenotypes[30]. In fact, in neoplastic diseases, miRNA can be downregulated when they function as tumor suppressors or overexpressed when they function as oncogenes[31].

27 Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009; 10: 704-14. https://doi.org/10.1038/nrg2634.

28 Laffont B, Rayner KJ. MicroRNAs in the Pathobiology and Therapy of Atherosclerosis. Can. J. Cardiol. 2017; 33:313-324. https://doi.org/10.1016/j.cjca.2017.01.001.

29 Nakamori M, Junn E, Mochizuki H, Mouradian MM. Nucleic Acid-Based Therapeutics for Parkinson's Disease. Neurotherapeutics. 2019. https://doi.org/10.1007/s13311-019-00714-7.

30 Lam JK, Chow MY, Zhang Y, Leung SW. siRNA versus miRNA as therapeutics for gene silencing. Mol. Ther. Nucleic Acids 2015; 4, e252. https://doi.org/10.1038/mtna.2015.23.

31 Fernandez-Piñeiro I, Badiola I, Sanchez A. Nanocarriers for microRNA delivery in cancer medicine. Biotechnol Adv. 2017; 35:350-60. https://doi.org/10.1016/j.biotechadv.2017.03.002.

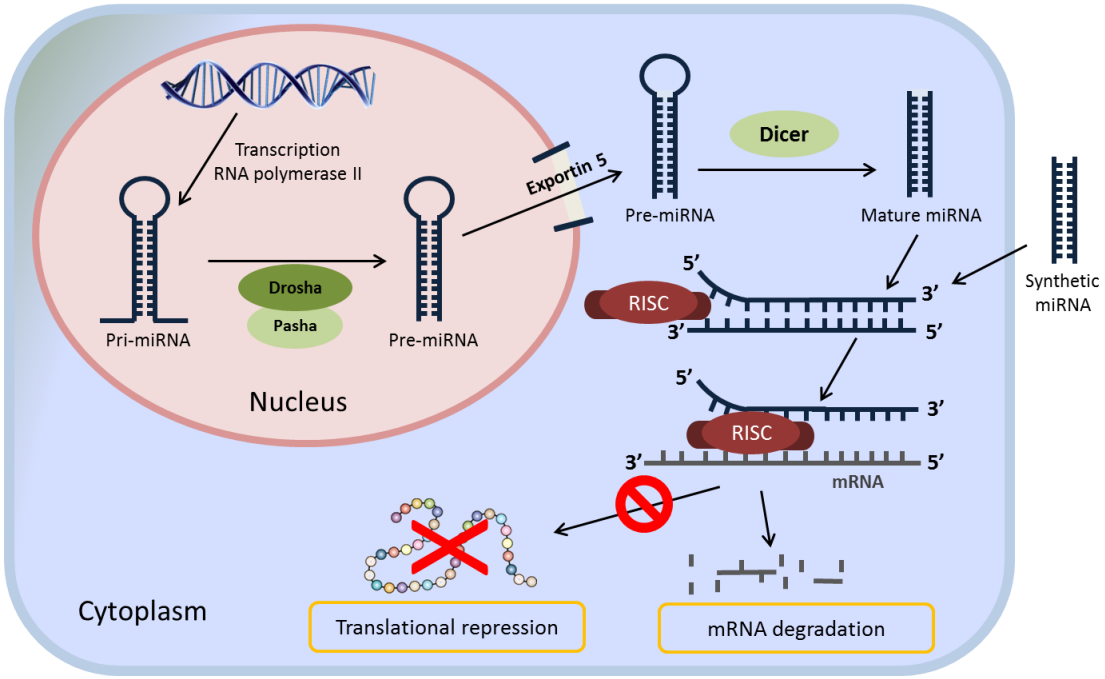

Figure II. Mechanism of miRNA. miRNA: microRNA.

1.1.2.3.2. siRNA

siRNA are short double-stranded RNA segments with 21-23 nucleotides and are complementary to the mRNA sequence of the protein whose transcription is to be blocked. siRNA molecules are incorporated into the RISC complex, which bind to the mRNA of interest and stimulate degradation of mRNA or the suppression of the translation process[32]. Figure III shows the mechanism of siRNA.

32 Rozenblum GT, Lopez VG, Vitullo AD, Radrizzani M. Aptamers: current challenges and future prospects. Expert Opin. Drug Discov. 2016; 11:127-35. https://doi.org/10.1517/17460441.2016.1126244.

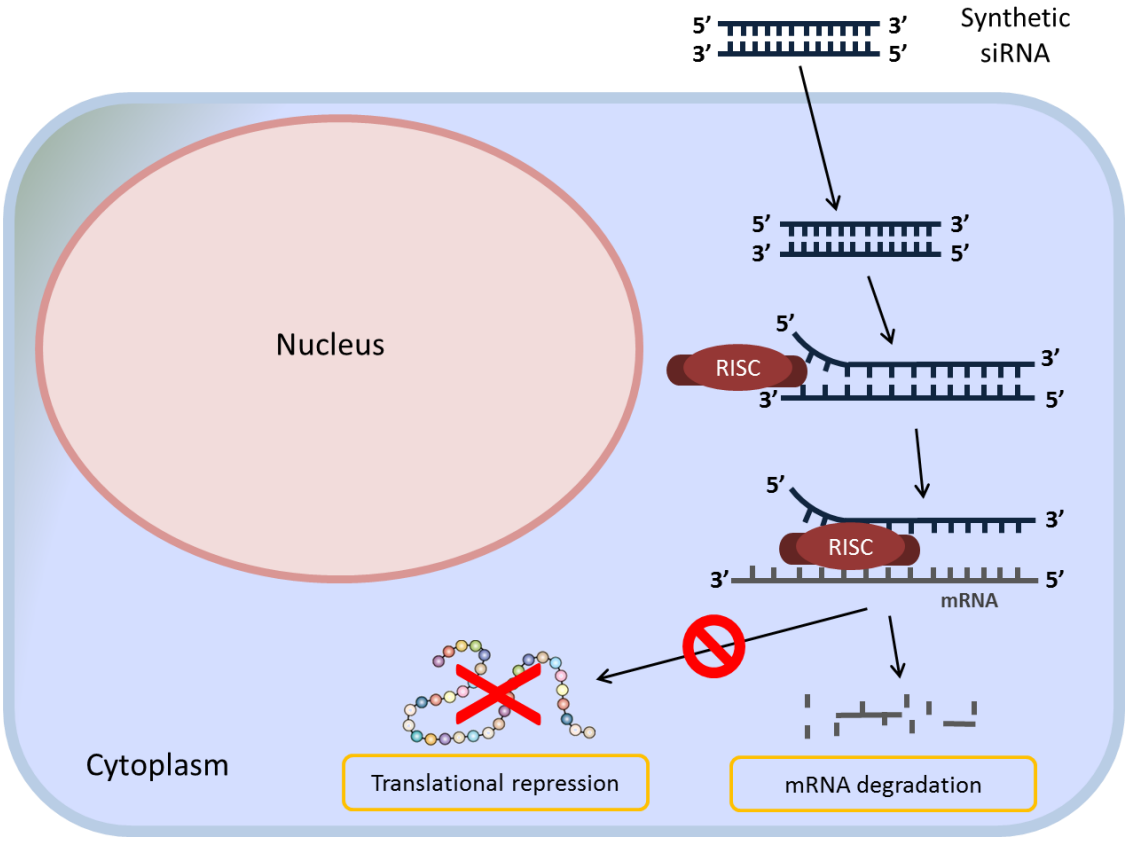

Figure III. Mechanism of siRNA. siRNA: short interfering RNA.

The main advantage of synthesized siRNA is that these molecules do not need to reach the nucleus to induce the therapeutic effect. As a drawback, their stability must be improved in order to optimize the efficacy[33].

Because of their small size and low potential to elicit adaptive immune responses, several anti-human immunodeficiency virus (HIV) RNAs have advanced to clinical trials. A potential advantage of anti-HIV-1 siRNAs over current therapies is that their sequences could be tailored to target a patient's particular viral strains and provide a personalized approach to therapy[34]. A major challenge for the development of anti-HIV-1 siRNAs is that lymphocytes, which represent the major cell-type for HIV-1 replication, are widely distributed in the body and extremely difficult to penetrate with existing siRNA delivery technologies[35]. Other viral infections with limited treatment options and more easily accessible target that could be treated with siRNA

33 Rozenblum GT, Lopez VG, Vitullo AD, Radrizzani M. Aptamers: current challenges and future prospects. Expert Opin. Drug Discov. 2016; 11:127-35. https://doi.org/10.1517/17460441.2016.1126244.

34 Scarborough RJ, Gatignol A. RNA Interference Therapies for an HIV-1 Functional Cure. Viruses. 2017; 10. pii: E8. https://doi.org/10.3390/v10010008.

35 Mizrahy S, Hazan-Halevy I, Dammes N, Landesman-Milo D, Peer D. Current progress in non-viral RNAi-based delivery strategies to lymphocytes. Mol. Ther. 2017; 25:1491-500. https://doi.org/10.1016/j.ymthe.2017.03.001.

include hepatitis B virus, Ebola, and respiratory syncytial virus[36]. Other indications of siRNA under clinical investigation are hepatocellular carcinoma, hepatic fibrosis, dry eye syndrome, melanoma and pancreatic ductal adenocarcinoma.

At present there is a siRNA based therapy (Patisiran) recently approved by the FDA and the EMA for the treatment of hereditary transthyretin-mediated amyloidosis. This is a rapidly progressive, heterogeneous disease caused by the accumulation of misfolded transthyretin protein as amyloid fibrils at multiple sites, and it is characterized by peripheral sensorimotor neuropathy, autonomic neuropathy and/or cardiomyopathy[37]. Another product, Inclisiran, is an experimental therapeutic agent for the treatment of hypercholesterolemia that is being tested in late-stage clinical trials.

1.1.2.3.3. Short-hairpin RNA

Short-hairpin RNA (shRNA), also called expressed RNAi activators, is a plasmid-coded RNA that needs to be transcribed in the nucleus to down-regulate the expression of a desired gene. It can be transcribed through either RNA polymerase II or III. The first transcript generates a hairpin like stem-loop structure and is then processed in the nucleus by a complex containing the RNase II enzyme Drosha. The individual pre-shRNAs generated are finally transported to the cytoplasm by exportin 5. Once in the cytoplasm, the complex Dicer processes the loop of the hairpin to form a double-stranded siRNA[38]. Figure IV features a scheme with the mechanism of action of shRNA. Since shRNA is constantly synthesized in the target cells, more durable gene silencing is achieved in comparison to other forms of RNAi[39]. shRNAs represent an important tool in the assessment of gene function in mammals and are largely used as a research tool. Although

36 Laffont B, Rayner KJ. MicroRNAs in the Pathobiology and Therapy of Atherosclerosis. Can. J. Cardiol. 2017; 33:313-324. https://doi.org/10.1016/j.cjca.2017.01.001.

37 Kristen AV, Ajroud-Driss S, Conceição I, Gorevic P, Kyriakides T, Obici L. Patisiran, an RNAi therapeutic for the treatment of hereditary transthyretin-mediated amyloidosis. Neurodegener. Dis. Manag. 2019; 9:5-23. https://doi.org/10.2217/nmt-2018-0033.

38 Moore CB, Guthrie EH, Huang MT, Taxman DJ. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. Methods Mol. Biol. 2010; 629:141-58. https://doi.org/10.1007/978-1-60761-657-3_10.

39 Torrecilla J, del Pozo-Rodríguez A, Solinís MÁ, Apaolaza PS, Berzal-Herranz B, Romero-López C et al. Silencing of hepatitis C virus replication by a non-viral vector based on solid lipid nanoparticles containing a shRNA targeted to the internal ribosome entry site (IRES). Colloids Surf. B. Biointerfaces. 2016; 146:808-17. https://doi.org/10.1016/j.colsurfb.2016.07.026.

shRNA has been assayed to develop new therapies for retinal diseases[40,41,42], or cancer[43], no therapeutic product based on shRNA has been approved.

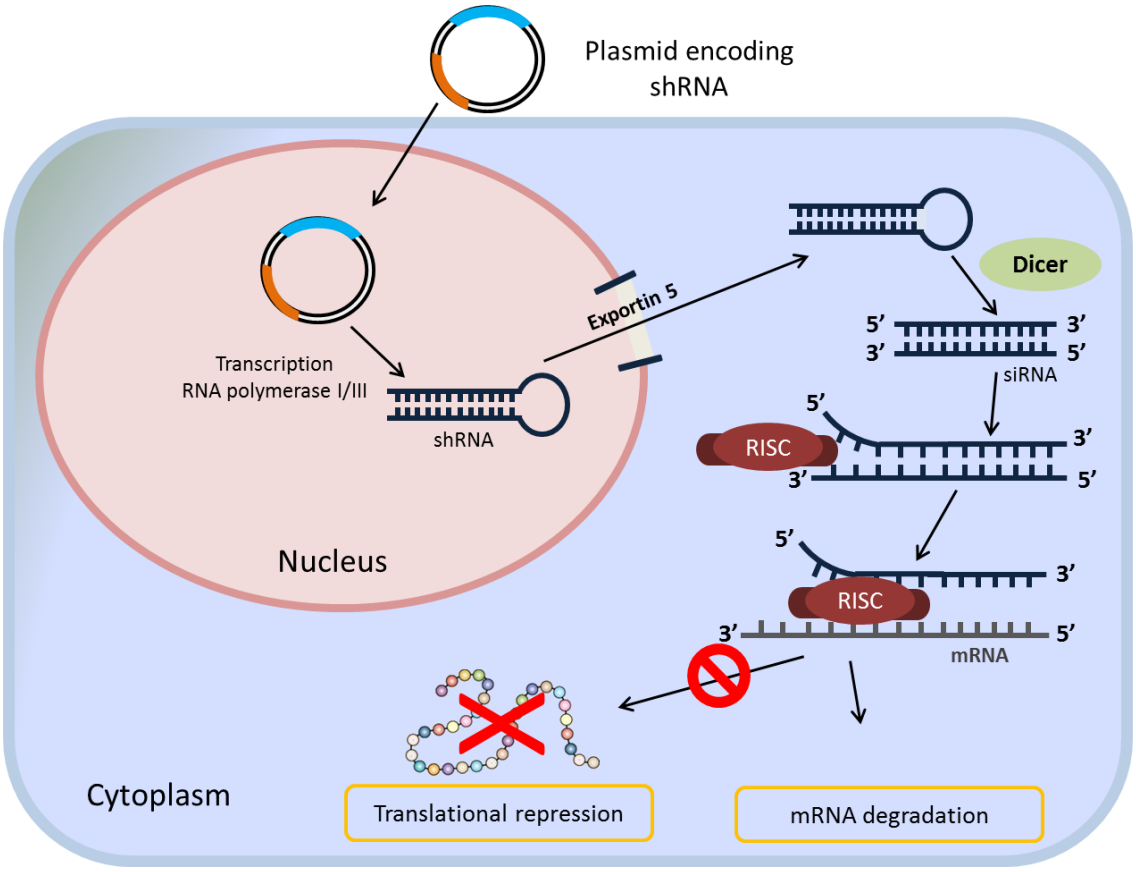

Figure IV. Mechanism of action of shRNA. shRNA: short-hairpin RNA.

1.1.3. Gene editing

Gene-editing technology has recently emerged as a new treatment modality for a variety of diseases, including hereditary, infectious, and neoplastic diseases. This technology is based on programmable nucleases, which consist of a nuclease that can be reprogrammed to cleave a

40 Hardcastle AJ, Sieving PA, Sahel JA, Jacobson SG, Cideciyan AV, Flannery JG et al. Translational Retinal Research and Therapies. Transl. Vis. Sci. Technol. 2018; 7:8. https://doi.org/10.1167/tvst.7.5.8.

41 Rozenblum GT, Lopez VG, Vitullo AD, Radrizzani M. Aptamers: current challenges and future prospects. Expert Opin. Drug Discov. 2016; 11:127-35. https://doi.org/10.1517/17460441.2016.1126244.

42 Scarborough RJ, Gatignol A. RNA Interference Therapies for an HIV-1 Functional Cure. Viruses. 2017; 10. pii: E8. https://doi.org/10.3390/v10010008.

43 Mues M, Karra L, Romero-Moya D, Wandler A, Hangauer MJ, Ksionda O et al. High-complexity shRNA libraries and PI3 kinase Inhibition in cancer: high-fidelity synthetic lethality predictions. Cell Rep. 2019; 27:631-47.e5. https://doi.org/10.1016/j.celrep.2019.03.045.

precise target sequence[44,45]. Consequently, they induce a double strand break (DSB) at a specific and desired location. Once the target gene is cleaved by the programmed nuclease, the reparation of the DSB can be done by two different mechanisms: non-homologous end joining (NHEJ), and homology-dependent repair (HDR)[46]. In the NHEJ, the target region is eliminated by joining the DSB, and it can be used to silence or correct a pathogenic gene. On the contrary, with the HDR modality, a homologous sequence can be introduced into the DSB, enabling donor DNA to be inserted to either correct an existing gene or add a new one. Figure V shows the different modalities of DSB reparation.

There are four types of gene editing nucleases: meganucleases, zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)/associated nuclease Cas9 (Cas9) (CRISPR/Cas9)[47]. Meganucleases, also called homing endonucleases, stimulate the cellular recombination and repair of DNA to fix the break by simply copying the gene encoding them (the homing endonuclease gene) and flanking DNA into the broken chromosome[48]. ZFN contains zinc finger proteins, the most common class of DNA binding proteins across all of biology, and *Fok*I nuclease as the DNA-binding and cleavage domains. ZFNs have now been widely used for genome editing in many species and cell types for basic science, biotechnology, and medical applications, such as targeted disruption of the CCR5 gene for HIV-1 therapy[49]. One important disadvantage of ZFN is that it is an expensive and time-consuming technology. TALEN uses the same *Fok*I-derived nuclease domain than ZFN, but differs in that they employ distinctive DNA-binding arrays: TALE effector repeat arrays[50]. TALEN technology for the recognition of a wider range or target gene sequences requires complicated engineering that is a matter of concern. The CRISPR/Cas9

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44 Moore CBT, Christie KA, Marshall J, Nesbit MA. Personalised genome editing - The future for corneal dystrophies. Prog. Retin. Eye Res. 2018; 65:147-65. https://doi.org/10.1016/j.preteyeres.2018.01.004.

45 Blighe K, DeDionisio L, Christie KA, Chawes B, Shareef S, Kakouli-Duarte T et al. Gene editing in the context of an increasingly complex genome. BMC Genomics. 2018; 19:595. https://doi.org/10.1186/s12864-018-4963-8.

46 Gersbach CA, Gaj T, Barbas CF 3rd. Synthetic zinc finger proteins: the advent of targeted gene regulation and genome modification technologies. Acc Chem Res. 2014; 47:2309-18. https://doi.org/10.1021/ar500039w.

47 Moore CB, Guthrie EH, Huang MT, Taxman DJ. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. Methods Mol. Biol. 2010; 629:141-58. https://doi.org/10.1007/978-1-60761-657-3_10.

48 Belfort M, Bonocora RP. Homing endonucleases: from genetic anomalies to programmable genomic clippers. Methods Mol. Biol. 2014; 1123:1-26. http://dx.doi.org/10.1007/978-1-62703-968-0_1.

49 Gersbach CA, Gaj T, Barbas CF 3rd. Synthetic zinc finger proteins: the advent of targeted gene regulation and genome modification technologies. Acc Chem Res. 2014; 47:2309-18. https://doi.org/10.1021/ar500039w.

50 Kim Y, Kweon J, Kim A, Chon JK, Yoo JY, Kim HJ et al. A library of TAL effector nucleases spanning the human genome. Nat. Biotechnol. 2013; 31:251-8. https://doi.org/10.1038/nbt.2517.

system is based on a prokaryotic antiviral mechanism in which the bacteria insert a partial gene sequence from an infection source, such as bacteriophage, into their own genomes to defend against repeat infection[51]. This system includes a RNA guide to bind to a complementary sequence in a target gene, which is recognized and cut by the Cas9[52].

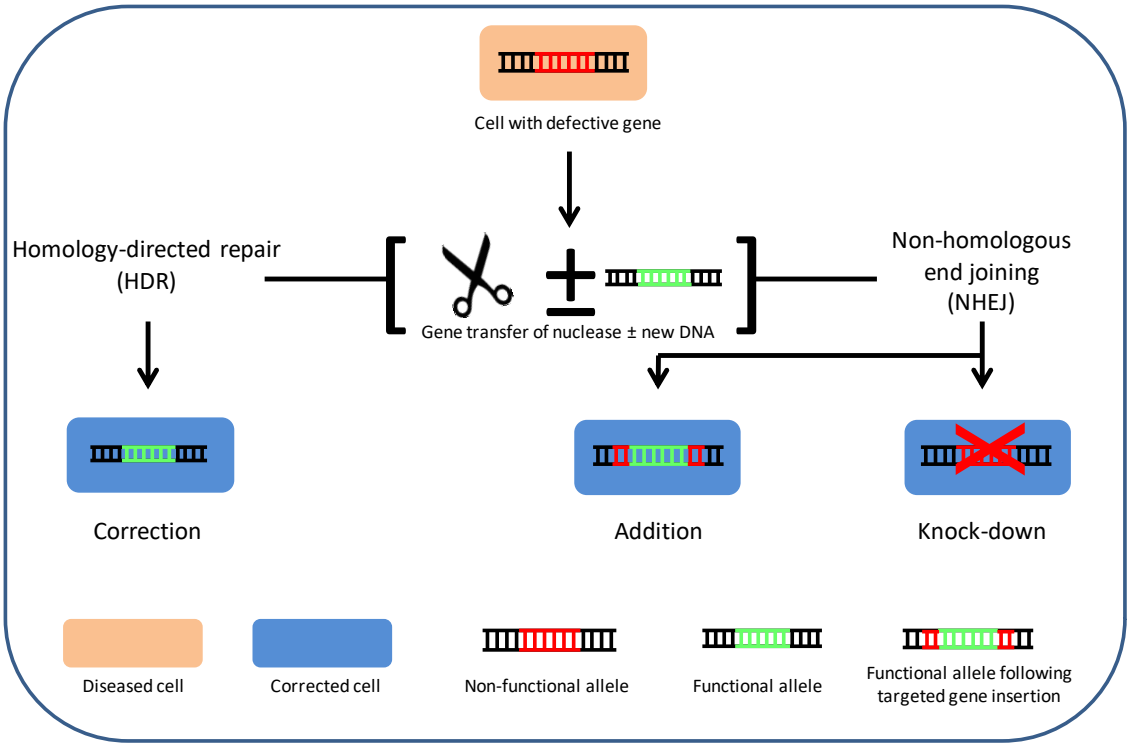

Figure V. Mechanisms of gene repair with nucleases.

Contrary to gene augmentation and suppression, therapeutics based on gene editing can lead to a permanent effect at the genome, and therefore, this recent technology represents a key development of gene therapy[53].

In the last years, there has been a tremendous progress in gene editing development, mainly with CRISPR/Cas9, thanks to the simplicity of the manufacturing procedures in comparison to the earlier tools, meganucleases, ZFN and TALEN. However, it is important to remark that in comparison to standard gene transfer approaches, genome editing, particularly that based on

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51 Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, et al. An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol. 2015; 13:722-36. https://doi.org/10.1038/nrmicro3569.

52 Shim G, Kim D, Park GT, Jin H, Suh SK, Oh YK. Therapeutic gene editing: delivery and regulatory perspectives. Acta Pharmacol. Sin. 2017; 38:738-53. https://doi.org/10.1038/aps.2017.2.

53 Torrecilla J, del Pozo-Rodríguez A, Solinís MÁ, Apaolaza PS, Berzal-Herranz B, Romero-López C et al. Silencing of hepatitis C virus replication by a non-viral vector based on solid lipid nanoparticles containing a shRNA targeted to the internal ribosome entry site (IRES). Colloids Surf. B. Biointerfaces. 2016; 146:808-17. https://doi.org/10.1016/j.colsurfb.2016.07.026.

CRISPR/Cas9, is in its translational and clinical infancy. In spite of that, several clinical trials with gene editing technologies have been completed or are undergoing[54]. At present, there is just one clinical trial for gene editing in ocular diseases (Clinicaltrials.gov Identifier: NCT03872479). A homozygous or compound heterozygous mutation on the CEP290 gene is responsible of retinal degeneration in Leber Congenital Amaurosis 10. AGN-151587 (EDIT-101), which is a gene editing product aimed to eliminate the mutation on CEP290 gene administered via subretinal injection, is now in phase 2 of a study with adult and pediatric patients who suffer this disease. This study is the first one employing CRISPR-based medicine *in vivo* in human. Also engineering ZFN have been assayed in clinical trials to disrupt CCR5 (C-C motif chemokine receptor type 5) expressed in human T cells and hematopoietic stem cells to provide them resistance to HIV infection[55]. Other clinical trials with ZFN have been approved for delivering the factor IX gene for hemophilia, the α-iduronidase gene for mucopolysaccharidosis I and the iuronidate-2-sulfatase gene for mucopolysaccharidosis II. The first-in-human use of TALEN gene-edited T cells in two infants with refractory relapsed B cell acute lymphoblastic leukemia led to a successful induction of molecular remission ahead of allogeneic stem cell transplantation[56].

The rapid technological advances in genome editing have allowed manipulating germ cells, gametes, zygotes, or embryos. In a recent study[57], by using CRISPR/Cas9, an induced DSB at the mutant paternal allele were predominantly repaired using homologous-wild type maternal gene instead of a synthetic DNA template. The authors were able to avoid mosaicism in cleaving embryos and achieve a high yield of homozygous embryos carrying the wild type *MYBPC*3 gene (whose mutation causes hypertrophic cardiomyopathy, without evidence of off-target mutation). In spite of the potential use for the correction of heritable mutations in human embryos, much remains to be considered before clinical applications, including the reproducibility of the technique with other heterozygous mutations.

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54 Dunbar CE, High KA, Joung JK, Kohn DB, Ozawa K, Sadelain M. Gene therapy comes of age. Science. 2018; 359:6372. https://doi.org/10.1126/science.aan4672.

55 Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G et al. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N. Engl. J. Med. 2014; 370:901-10. https://doi.org/10.1056/NEJMoa1300662.

56 Qasim W, Zhan H, Samarasinghe S, Adams S, Amrolia P, Stafford S et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Sci. Transl. Med. 2017; 9. pii: eaaj2013. https://doi.org/10.1126/scitranslmed.aaj2013.

57 Ma H, Marti-Gutierrez N, Park SW, Wu J, Lee Y, Suzuki K et al. Correction of a pathogenic gene mutation in human embryos. Nature. 2017; 548:413-9. https://doi.org/10.1038/nature23305.

1.2. Delivery systems for gene therapy

One of the main challenges of gene therapy is the development of safe and effective administration systems that are able to overcome the main limitations of nucleic acids when they are administered in the body[58]. Therefore, a fundamental aspect for the success of gene therapy is the availability of delivery systems capable of protecting the genetic material from degradation, facilitating its internalization in target cells and releasing them inside. The ideal delivery system depends on the target cells, the kind of nucleic acid to be delivered and the duration of expression[59]. The delivery systems are classified into two large groups: viral and non-viral vectors.

Viral vectors are prepared from genetically modified viruses so that they are not able to replicate in the target cells, but they express the therapeutic gene they transport. Viral vectors allow high transfection efficiencies; however, they present important safety limitations due to the oncogenic and immunogenic potential (due to viral proteins). Another problem associated to viral vectors is the inability to transport large nucleic acids.

Non-viral vectors are safer, simpler, cheaper and more reproducible systems. In addition, they do not present limitations regarding the size of the genetic material they can incorporate. Nevertheless, a disadvantage of non-viral systems is that their transfection efficiency is lower compared to viral vectors, although in recent years new non-viral systems have been developed with materials that exhibit higher transfection efficiencies. In fact, the number of clinical trials with products based on non-viral vectors (Figure VI) has increased in the last decade and those based on lipid nanocarriers (lipofection) are used in 3.9% of all trials[60].

58 Rodríguez-Gascón A, del Pozo-Rodríguez A, Solinís MÁ. Non-Viral Delivery Systems in Gene Therapy. In: Gene Therapy - Tools and Potential Application. Martín F, (Ed.), IntechOpen, London, United Kingdom. 2013; 3-33. https://doi.org/10.5772/50194.

59 Ramamoorth M, Narvekar A. Non-Viral Vectors in Gene Therapy- An Overview. J. Clin. Diagn. Res. 2015; 9:GE01–6. https://doi.org/10.7860/JCDR/2015/10443.5394.

60 Gene Therapy Clinical Trials Worldwide. Provided by the Journal of Gene Medicine. Jon Wiley and Sons Ltd, 2019. http://www.abedia.com/wiley/index.html. Accessed 30 Mar 2020.

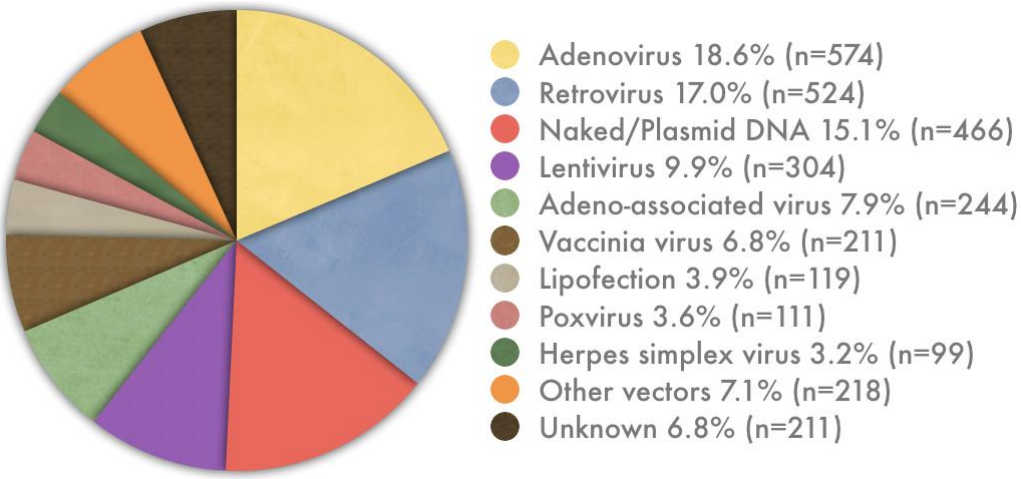

Figure VI. Vectors used in gene therapy clinical trials. Data consulted in Gene Therapy Clinical Trials Worldwide 2019.

1.2.1. Viral vectors

Viruses used as delivery systems of genetic material include adenoviruses, adeno-associated viruses (AAV), retroviruses and lentiviruses among the most evaluated in clinical trials. Other viruses, such as those derived from herpes virus or poxvirus have also been studied as possible viral vectors.

The selection of the most suitable viral system in each case depends on different factors: the organ or the target cell, the ability to integrate the genetic material carried by the vector in the genome of the host cell, the duration of expression gene over time (short term or long term response) or the size of therapeutic nucleic acid. Table I shows the main characteristics of the most frequently studied viral vectors, to be taken into account for their application in gene therapy.

Table I. Features of the most studied viral vectors in gene therapy.

	Retroviruses	**Lentiviruses**	**Adenoviruses**	**AAV**
Viral genome	RNA	RNA	DNA	DNA
Target cells	Dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells	Dividing and non-dividing cells
Integration in the host genome	Yes	Yes	No	Yes
Response	Long-term	Long-term	Short-term	Long-term
Size of the genetic material to be carried	8 kb	8 kb	7.5 - 30 kb	4.5 kb

Abbreviations. AAV: adeno-associated viruses.

1.2.1.1. Retroviral vectors

Retroviruses are RNA viruses that contain 2 strands of RNA enveloped by an icosahedral capsid of peptide nature (Figure VII). The capsid is surrounded by a phospholipid envelope, in which different types of glycoproteins act as ligands for specific receptors of cell surfaces, and therefore determine the tropism of the virus.

The genome of retroviruses (Figure VII) contains 3 types of genes: *gag* genes that encode capsid proteins, *pol* genes that encode the enzymes necessary for the replicative cycle of the virus (protease, integrase, reverse transcriptase) and *env* genes that encode the envelope glycoproteins. This genome also contains a packaging signal, ψ, thanks to which the RNA molecules bind to the capsid proteins and are effectively packaged, and the long terminal repeats (LTR) at each end of the viral genome. The left LTR contains a region for the start of transcription (U3 promoter), and a first binding site (pbs) for the start of reverse transcription. The right LTR contains a poly-purine sequence (ppt) for replication of the second strand. For application in gene therapy, the retroviral vectors are generated by the substitution of the gag, pol and env sequences of the viral genome by the therapeutic gene. The therapeutic sequence in this case can not be greater than 7-8 kb.

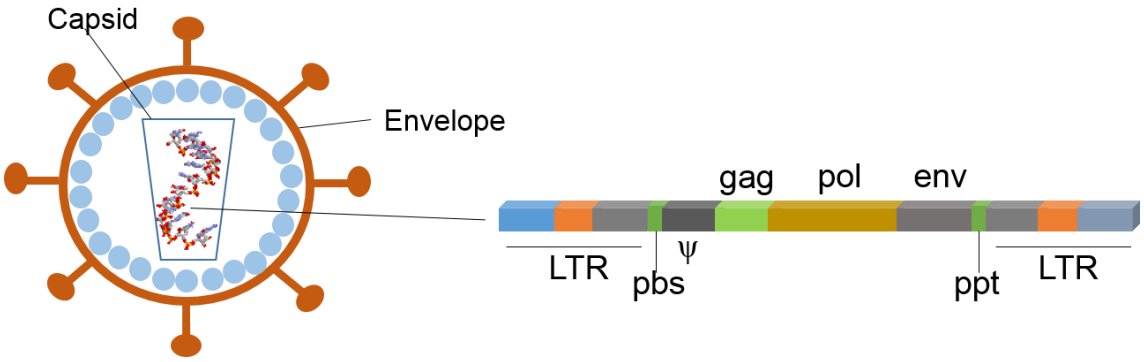

Figure VII. General structure of retroviruses and retroviral genome.

The replicative cycle of a retrovirus begins with entry into the cell, which is mediated by receptors[61]. Once inside the cell, the viral reverse transcriptase enzyme produces a DNA molecule from the viral RNA. Subsequently, the DNA is integrated into the genome of the host cell, and the transcription yields different RNAs, which are exported to the cellular cytoplasm, where they are translated into structural proteins of the virus, and directs synthesis of new virion

61 Baum C, Schambach A, Bohne J, Galla M. Retrovirus vectors: toward the plentivirus? Mol. Ther. 2006; 13:1050-63. https://doi.org/10.1016/j.ymthe.2006.03.007.

nucleocapsids. The nucleocapsids leave the cell, and keep enveloped by a plasma membrane-derived outer coat.

It is important to point out that the DNA obtained by reverse transcription from the RNA is not able to cross the nuclear membrane of the cell to be treated. Therefore, retroviral vectors only transfect efficiently dividing cells, because the DNA takes advantage of the disruption of the nuclear envelope during the mitosis, to reach the interior of the nucleus[62].

On the other hand, the integrase enzyme allows the integration of the genetic material in the genome of the host cell. Thanks to this, it is possible to obtain a long-lasting expression of the therapeutic sequence; however, the insertion into the genome of the target cell is also one of the major problems of viral gene therapy, since if it takes place in an unwanted region of the genome of the transfected cells, there is a risk of mutagenesis and oncogenesis. In fact, in clinical trials with these vectors several patients developed leukemia and dysplasia of the bone marrow due to insertional mutagenesis [63,64]. These adverse effects were partially reduced by the design of so-called self-inactivating vectors in which the genome sequences of the virus identified as responsible for mutagenesis, 3'LTR, are deleted[65]. Another limitation of retroviral vectors is that they are recognized and inactivated by the complement system, which means that they are mainly used in *ex vivo* gene therapy.

Despite the mentioned limitations, and due to its high transfection efficiency, since 1989, the year in which the first clinical trial with gene therapy was launched, 524 clinical trials with retroviral vectors have been started (17% of the total of clinical trials with gene therapy)[66].

One of the most commonly used retroviruses in gene therapy is the murine leukemia virus (MLV), which has shown efficacy in different types of immunodeficiency. In fact, recently the

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62 Matuskova M, Durinikova E. Retroviral Vectors in Gene Therapy. In: Advances in Molecular Retrovirology. Saxena SK (Ed.), IntechOpen, London, United Kingdom. 2016; 143-166. https://doi.org/10.5772/61844.

63 Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J. Clin. Invest. 2008; 118:3132–42. https://doi.org/10.1172/JCI35700.

64 Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, Kempski H et al. Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J. Clin. Invest. 2008; 118: 3143–50. https://doi.org/10.1172/JCI35798.

65 Yu SF, von Rüden T, Kantoff PW, Garber C, Seiberg M, Rüther , et al. Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 1986; 83:3194-8. https://doi.org/10.1073/pnas.83.10.3194.

66 Gene Therapy Clinical Trials Worldwide. Provided by the Journal of Gene Medicine. Jon Wiley and Sons Ltd, 2019. http://www.abedia.com/wiley/index.html. Accessed 30 Mar 2020.

EMA has approved a drug based on *ex vivo* gene therapy with this retroviral vector for the treatment of patients with severe combined immunodeficiency due to adenosine deaminase (ADA-SCID) deficiency that cannot be treated with bone marrow transplant (Strimvelis; GSK GlaxoSmithKline Pharmaceuticals)[67].

1.2.1.2. Lentiviral vectors

Lentiviruses also belongs to the Retroviridae family. However, they have certain differential genes with respect to the rest of retroviruses that facilitate the entry of genetic material into the cell nucleus, so lentiviral vectors can also transfect non-dividing cells. One of the most known and studied viruses of this subfamily is the HIV.

The general structure of lentiviruses is the same as that described for retroviruses: two RNA strands included in a protein capsid, surrounded by a phospholipid envelope that includes different types of glycoproteins.

The genome of the lentiviruses (Figure VIII) shares with the retroviruses the *gag*, *pol* and *env* genes, as well as the LTR, pbs and ppt sequences. However, it presents other specific genes: *tat* genes (transcription regulators), *rev* genes (regulators of the expression of viral proteins), *vif* genes (necessary for the infection of different cell types), *vpr* genes (participate in the entrance to the nucleus), *vpu* genes (involved in the release of viral particles from infected cells) and *nef* genes (increase the infectivity of the virus).

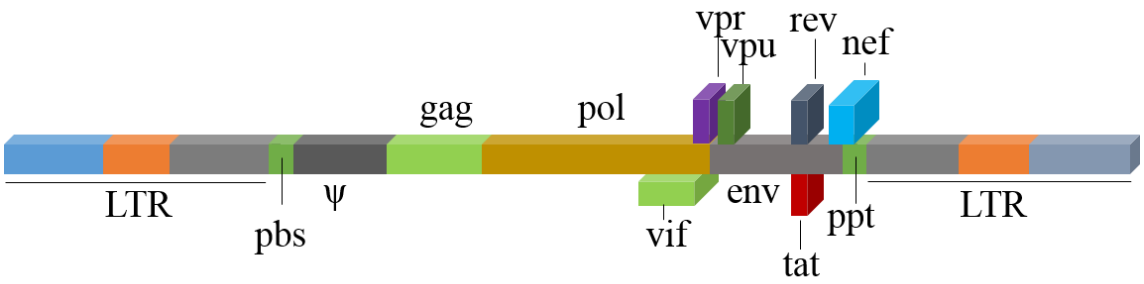

Figure VIII. General structure of lentiviral genome.

The cycle of life is similar to that described for retroviral vectors, with the difference that lentiviruses present genes encoding nuclear localization signals that favour the entry of DNA (synthesized by the reverse transcription process) into the nucleus.

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67 Stirnadel-Farrant H, Kudari M, Garman N, Imrie J, Chopra B, Giannelli S et al. Gene therapy in rare diseases: the benefits and challenges of developing a patient-centric registry for Strimvelis in ADA-SCID. Orphanet J. Rare Dis. 2018; 13:49. https://doi.org/10.1186/s13023-018-0791-9.

In order to use lentiviruses as gene delivery systems, the *tat* gene is removed, and the *gag* and *pol* genes are encoded on a different plasmid from that of the *rev* or *env* genes. The final vector results from three separate plasmids containing the necessary viral sequences for packaging[68]. In addition, it has been reported the generation of self-inactivating (SIN) lentiviral vectors by the introduction of deletions into the 3'LTR of the viral genome[69], as previously mentioned in the case of retroviral vectors.

Due to the tropism of lentiviruses and their ability to transfect cells that are not in division, the main application of lentiviral vectors is directed to introduce genetic material *in vivo* in cells of the central nervous system[70] (for example, for the treatment of Parkinson's disease[71]), or in cells of the retina[72] (suitable for the treatment of retinitis pigmentosa). Furthermore, lentiviral vectors efficiently transfect *ex vivo* cells of the hematopoietic system, which are difficult to transfect with other vectors. In fact, *ex vivo* gene therapy with lentiviral vectors to genetically modify CD34+ cells has been evaluated in more than 100 clinical trials in recent years for the treatment of monogenic diseases (i.e. β-thalassemia[73], X-linked adrenoleukodystrophy[74],

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68 Milone MC, O'Doherty U. Clinical use of lentiviral vectors. Leukemia. 2018; 32:1529-41. https://doi.org/10.1038/s41375-018-0106-0.

69 Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D et al. A third-generation lentivirus vector with a conditional packaging system. J. Virol. 1998; 72:8463–71.

70 Hutson TH, Foster E, Moon LD, Yáñez-Muñoz RJ. Lentiviral vector-mediated RNA silencing in the central nervous system. Hum. Gene Ther. Methods. 2014; 25:14-32. https://doi.org/10.1089/hgtb.2013.016.

71 Palfi S, Gurruchaga JM, Lepetit H, Howard K, Ralph GS, Mason S et al. Long-Term Follow-Up of a Phase I/II Study of ProSavin, a Lentiviral Vector Gene Therapy for Parkinson's Disease. Hum. Gene Ther. Clin. Dev. 2018; 29:148-55. https*://doi.org/10.1089/humc.2018.081.*

72 Matet A, Kostic C, Bemelmans AP, Moulin A, Rosolen SG, Martin S et al. Evaluation of tolerance to lentiviral LV-RPE65 gene therapy vector after subretinal delivery in non-human primates. Transl. Res. 2017; 188:40-57. https://doi.org/10.1016/j.trsl.2017.06.012.

73 Cavazzana-Calvo M, Payen E, Negre O, Wang G, Hehir K, Fusil F et al. Transfusion independence and HMGA2 activation aftergene therapy of human [bgr]-thalassaemia. Nature. 2010;467:318–22. https://doi.org/doi: 10.1038/nature09328.

74 Cartier N, Hacein-Bey-Abina S, Bartholomae CC, Veres G, Schmidt M, Kutschera I et al. Hematopoietic stem cell genetherapy with a lentiviral vector in X-linked adrenoleukodystro-phy. Science. 2009; 326:818–23. https://doi.org/10.1126/science.1171242.

metachromatic-leukodystrophy[75] or Wiskott-Aldrich Syndrome[76]), of different types of cancer[77], and of infectious diseases such as HIV infection[78].

1.2.1.3. Adenoviral vectors

Adenoviruses encompass more than 50 different virus serotypes, and are responsible for 5-10% of respiratory infections in children and adults. In gene therapy, serotypes 2 and 5 are the most used. Adenoviruses are non-enveloped viruses that consist of a double strand of DNA within an icosahedral capsid (Figure IX).

The genome of an adenovirus (Figure IX) has a size of approximately 35 kb. At the ends are the inverted terminal repeats (ITR), and close to the left ITR the packing signal, ψ, is arranged. The numerous genes it contains differ in the early (E) and late (L) regions. The latter, responsible for the coding of structural proteins, are transcribed after replication of the viral genome, and the E regions are transcribed before synthesizing the viral DNA, since they give rise to regulatory proteins. These proteins alter the expression of host cell proteins that are necessary for DNA synthesis, intervene in viral replication, and also prevent the death of infected cells by blocking the apoptosis or avoiding recognition by the immune system.

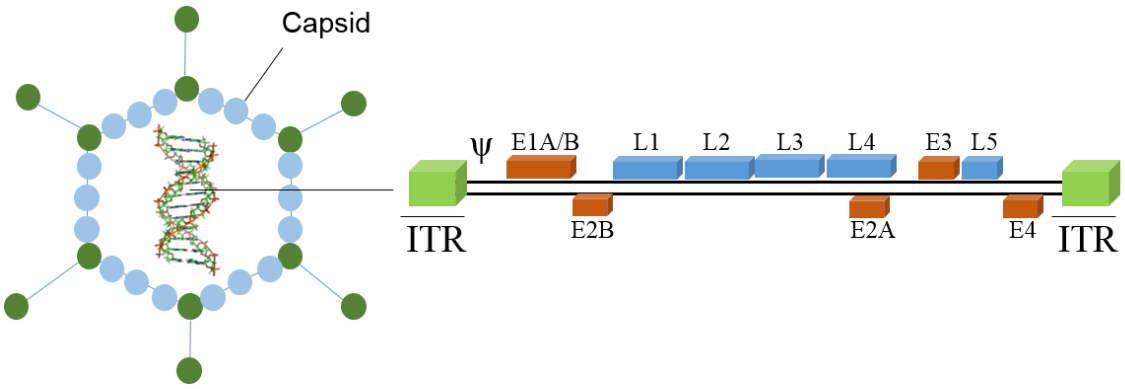

Figure IX. General structure of adenoviruses and adenoviral genome.

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75 Sessa M, Lorioli L, Fumagalli F, Acquati S, Redaelli D, BaldoliC et al. Lentiviral haemopoietic stem-cell gene therapy in early-onset metachromatic leukodystrophy: an ad-hoc analysis of anon-randomised, open-label, phase 1/2 trial. Lancet. 2016; 388:476–87. https://doi.org/10.1016/S0140-6736 (16)30374-9.

76 Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Bar-icordi C et al. Lentiviral hematopoietic stem cell gene therapy inpatients with Wiskott-Aldrich syndrome. Science. 2013; 341:1233151 https://doi.org/10.1126/science.1233151.

77 Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci. Transl. Med. 2011; 3:95ra73. https://doi.org/10.1126/scitranslmed.3002842.

78 Symonds G, Bartlett JS, Kiem HP, Tsie M, Breton L. Cell-Delivered Entry Inhibitors for HIV-1: CCR5 Downregulation and Blocking Virus/Membrane Fusion in Defending the Host Cell Population. AIDS Patient Care STDS. 2016; 30:545-50. https://doi.org/10.1089/apc.2016.0245.

Adenovirus are endocytosized into the cell after binding to the CAR receptor (Coxackie Adenovirus Receptor). The nucleocapsids are released into the cytoplasm, and with the help of cellular microtubules, they reach the nuclear membrane, and the genetic material is introduced to the nucleus of the cell through the nuclear pores. Replication then begins, and once all the components of the virus have been synthesized, they are assembled and released from the cell by cell lysis induced by the virus itself. During this process the adenoviral genome does not integrate into the genome of the host cell. This is one of the advantages of adenoviruses which makes them safer compared to other viral systems, although this also means that the viral life cycle is not adapted for long-term transgene expression.

To be used as vectors for gene therapy, E regions are deleted from the genome, and various levels of attenuation can be achieved by removal of different numbers of genes: only one E1B gene (first generation vectors), the majority of early genes (second generation vectors), and even full deletion of all genetic information of an adenovirus (so-called gutless vectors)[79]. The large size of the genome and the possibility to delete a major part of it provide high coding capacity for these vectors: 1-2 kb can be inserted in early generation vectors, and up to 30 kb in gutless vectors. However, they need another helper virus that replicates normally and expresses all the proteins needed to assemble the adenoviral vector[80]. Despite its advantages, the total elimination of impurities from helper viruses is complicated and limits its clinical application. In fact, at high doses adenoviruses are toxic[81] and one of their main limitations is that they are very immunogenic[82], which decreases their effectiveness.

In spite of their limitations, the advantages of adenoviral vectors have meant that they have been evaluated in more than 500 clinical trials[83], most of them aimed at the treatment of cancer.

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79 Giacca M. Gene Therapy. Springer-Verlag Mailand. 2010. https://doi.org/10.1007/978-88-470-1643-9.

80 Brunetti-Pierri N. Helper-dependent adenoviral vectors for liver-directed gene therapy. Hum. Mol. Genet. 2011; 20:7–13. https://doi.org/10.1093/hmg/ddr143.

81 Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP et al. Fatal systemic inflammatory response syndrome in an ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol. Genet. Metab. 2003; 80:148-58. https://doi.org/10.1016/j.ymgme.2003.08.016.

82 Wold WS, Toth K. Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. Curr. Gene Ther. 2013; 13:421-33. https://doi.org/10.2174/15665232136661311250950 46.

83 Lee CS, Bishop ES, Zhang R, Yu X, Farina EM, Yan S et al. Adenovirus-Mediated Gene Delivery: Potential Applications for Gene and Cell-Based Therapies in the New Era of Personalized Medicine. Genes Dis. 2017; 4:43-63. https://doi.org/10.1016/j.gendis.2017.04.001.

1.2.1.4. Adeno-associated viral (AAV) vectors

Adeno-associated viruses (AAV) are small non-enveloped viruses with single-stranded DNA (Figure X). The genome has a size of about 4.7 kb and is composed of three regions called rep, cap and aap, flanked by the corresponding ITR. The rep region encodes non-structural proteins involved in viral replication, packaging and integration into the host genome, genes in the cap region encode the structural proteins of the capsid and *aap* gene encodes the assembly-activating protein[84]. AAV vectors for clinical application are generated by replacing the *rep*, *cap* and *aap* genes with the gene of interest.

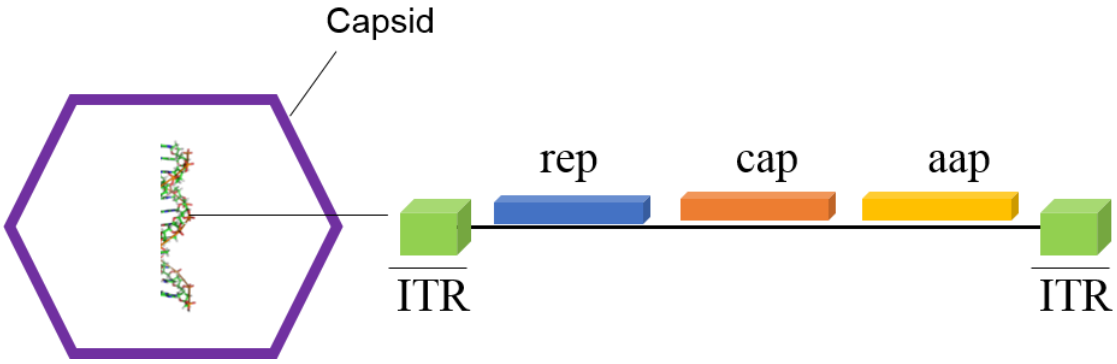

Figure X. General structure of AAV and AAV genome. AAV: adeno-associated viruses.

The entry of AAV into the cell takes place by endocytosis after binding to the receptor-mediated cellular surface. Once the nucleocapsid escapes from the endosome and is transported to the nucleus, the viral genome is able to cross the nuclear membrane. In the nucleus a second strand of DNA, necessary for the replication of the virus, will be synthesized. In the presence of a helper virus (co-infection by an adenovirus or herpes virus) the double-helix DNA generated can be integrated into the genome of the host cell, and replication of the virus will take place. In the absence of a helper virus, the AAV genome usually remains latent in the form of an episome. Replication of the viral genome and subsequent packaging result in the generation of viral particles that will escape from the host cell by lysis thereof[85].

These vectors are quite safe, can transfect cells with or without capacity of division and provide long-term gene expression, up to 6 years. Another advantage of AAV is the possibility of selecting

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84 Naumer M, Sonntag F, Schmidt K, Nieto K, Panke C, Davey NE et al. Properties of the adeno-associated virus assembly-activating protein. J. Virol. 2012; 86:13038–48. https://doi.org/10.1128/JVI.01675-12.

85 Hastie E, Samulski RJ. Adeno-associated virus at 50: a golden anniversary of discovery, research, and gene therapy success--a personal perspective. Hum. Gene Ther. 2015; 26:257-65. https://doi.org/10.1089/hum.2015.025.

the most suitable serotype depending on the target tissue[86]. Table II shows the most suitable AAV serotypes for different tissues.

Table II. AAV serotypes suitable for specific target tissues.

Serotype	Target tissue
AAV1	Nervous system, skeletal muscle
AAV2	Nervous system, kidney, photoreceptors, retinal pigment epithelium
AAV4	Nervous system, retinal pigment epithelium
AAV5	Nervous system, lungs, photoreceptors, retinal pigment epithelium
AAV6	Skeletal muscle, lungs
AAV7	Skeletal muscle
AAV8	Nervous system, photoreceptors, retinal pigment epithelial, liver, skeletal muscle, heart, pancreas
AAV9	Nervous system, heart, liver, skeletal muscle, lungs

Abbreviations. AAV: adeno-associated viruses.

The main disadvantage of AAV is the limited size of the genetic material they can transport, which must not exceed 4.5 kb. However, viral vectors based on AAV have been evaluated in more than 180 clinical trials, most of them aimed at the treatment of monogenic diseases. In fact, a medicine product called Luxturna and based on AAV2 has reached the market. Luxturna (voretigene neparvovec) is a gene transfer vector that employs an AAV2 as a delivery vehicle for the human retinal pigment epithelium 65kDa protein (hRPE65) cDNA to the retina[87]. This medicine is indicated for the treatment of adult and paediatric patients with vision loss due to inherited retinal dystrophy caused by confirmed biallelic RPE65 mutations and who have sufficient viable retinal cells.

1.2.1.5. Manufacturing of viral vectors

Viral vector production for clinical application requires viral propagation in suitable animal cell lines, viral recovery, concentration purification and formulation[88]. To meet commercial and regulatory requirements, each process must be scalable and reproducible and must yield high virus titers. For large-scale manufacturing, suspension-adapted cell lines cultured in bioreactors

86 Naso MF, Tomkowicz B, Perry WL 3rd, Strohl WR. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. BioDrugs. 2017; 31:317-34. https://doi.org/10.1007/s40259-017-0234-5.

87 EMA (European Medicine Agency). Luxturna: Summary of product characteristics. 2018b. https://www.ema.europa.eu/en/documents/product-information/luxturna-epar-product-information_en.pdf. Accessed 30 Mar 2020.

88 Walsh G. Nucleic acid and cell-based therapeutics. In: Pharmaceutical biotechnology: concepts and applications. Walsh G (Ed.), Chichester: John Wiley & Sons, Ltd., West Sussex, UK. 2007; 419-64.

are more appropriate than adherent-cells systems, conventionally used at laboratory-scale[89]. Depending on the viral vector and the cell clone used, the most suitable bioreactor must be chosen[90].

The production of viral vectors may be carried out by transiently transfecting the producing cells with vector and helper or packaging plasmids, or by generating stable producer cell lines. In the first case, culture cells are co-transfected with multiple plasmids, one containing the expression cassette for the transgene and other plasmids encoding regulatory and structural viral proteins. The main limitations of transient transfected cells are the amount of transfected cells achieved and the variability in transfection[91]. In addition, transient transfection results in contaminations of the final product due to excess plasmids[92] and residual transfection reagent. In the second case, cells are genetically modified, so that they contain inserted in the cellular genome the genes that encode the structural proteins necessary for the formation of viral particles containing the transgene of interest[93]. Processes using stable producer cell lines are easier to scale-up and result in less contaminated vectors, although some drawbacks have to be also considered: the gene products necessary to produce vectors are toxic to cells, each vector-produced cell line requires specific certifications as master cell bank and, upon cell expansion high titer vectors are not always ensured[94].

One of the limitations of viral vectors manufacturing is to purify a sufficient amount of viral particles even to start a clinical trial. The downstream processing or purification of viral vectors aims to eliminate contaminants either process or product-related. Process-related impurities derive from starting materials (residual DNA and residual host cell protein from each cell bank) or raw materials (culture reagents, purification reagents and equipment materials, helper viruses and helper virus nucleic acid used in production), whereas product-related impurities

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89 Brindley DA, Fuerstenau-Sharp M, Smith JA, Bure K, Pettitt D, Mitrophanous K et al. Emerging Platform Bioprocesses for Viral Vectors and Gene Therapies. BioProcess International. 2016; 8-14.

90 Grein TA, Weidner T, Czermak P. Concepts for the Production of Viruses and Viral Vectors in Cell Cultures. In: New Insights into Cell Culture Technology. Gowder SJT (Ed.), IntechOpen, London, United Kingdom. 2017; 173-192. https://doi.org/10.5772/66903.

91 Van der Loo JC, Wright JF. Progress and challenges in viral vector manufacturing. Hum. Mol. Genet. 2016; 25:R42-52. https://doi.org/10.1093/hmg/ddv451.

92 Sanber KS, Knight SB, Stephen SL, Bailey R, Escors D, Minshull J et al. Construction of stable packaging cell lines for clinical lentiviral vector production. Sci. Rep. 2015; 5:9021. https://doi.org/10.1038/srep09021.

93 Rodrigues GA, Shalaev E, Karami TK, Cunningham J, Slater NKH, Rivers HM. Pharmaceutical Development of AAV-Based Gene Therapy Products for the Eye. Pharm. Res. 2018; 36:29. https://doi.org/10.1007/s11095-018-2554-7.

94 Van der Loo JC, Wright JF. Progress and challenges in viral vector manufacturing. Hum. Mol. Genet. 2016; 25:R42-52. https://doi.org/10.1093/hmg/ddv451.

include vectors with deleted, rearranged, hybrid or mutated sequences. The ultimate goal of the downstream processing is to obtain a product with high purity, potency, and quality that can meet the guidelines of the FDA[95] and EMA[96] regulatory agencies. With this aim, different concentration methods have been developed: centrifugation, tangential flow filtration, ultrafiltration, polyethylene glycol precipitation, two-phase extraction, membrane filtration, liquid chromatography or adsorption chromatography[97].

1.2.1.6. Quality control of viral vectors

Another aspect to take into account are the quality controls that these vectors must overcome to ensure that each batch manufactured meets the specifications of purity, power, safety and identity, and there is consistency and comparability between batches. This is a challenge given the high complexity of the viral systems due to the large number of protein subunits that make up the viral capsid, and the composition of the lipid membrane present in enveloped viruses. In addition, it is necessary to develop specific analytical methods for each type of virus, and even for each serotype. These methods can be divided into those that are similar to others already validated for recombinant proteins and vaccines, and those that are specific to each vector. The former include, for example, the analysis of impurities related to the production process, such as packaging cell proteins. Specific assays of viral vectors include the analysis of the activity of the resulting product of the therapeutic genetic material, as a measure of potency. It is also necessary to determine the impurities due to the presence of residual genetic material encapsulated in the vector. In any case, it must be considered that many of these methods developed to analyse the specific quality controls of viral vectors are not yet validated according to the standards established to license and market these products.

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95 FDA U.S. Food and Drug Administration Center for Biologics Evaluation and Research. Chemistry, manufacturing, and control (CMC) information for human gene therapy investigational new drug applications (INDs): draft guidance for industry. 2020. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/chemistry-manufacturing-and-control-cmc-information-human-gene-therapy-investigational-new-drug. Accessed 30 Mar 2020.

96 EMA (European Medicine Agency). Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal product. 2018a. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-quality-non-clinical-clinical-aspects-gene-therapy-medicinal-products_en.pdf. Accessed 30 Mar 2020.

97 Nestola P, Peixoto C, Silva RR, Alves PM, Mota JP, Carrondo MJ. Improved virus purification processes for vaccines and gene therapy. Biotechnol. Bioeng. 2015; 112:843-57. https://doi.org/10.1002/bit.25545.

1.2.2. Non-viral vectors

Non-viral systems can be defined as those physical or chemical methods that help in the process of transfer of exogenous genetic material to the cell, facilitating the entry and intracellular bioavailability thereof. Non-viral systems try to imitate the capacities of viruses as gene transfer vehicles, providing greater security from the point of view of biological risk and pathogenicity. However, reproducing with a non-viral system what a virus performs naturally is a great challenge that has led to the development of different strategies. The selection and design of the most appropriate non-viral system are conditioned by its efficacy and safety, by the tissue or target cell and by the type of therapeutic genetic material[98].

1.2.2.1. Physical methods

Physical methods are based on the application of physical forces to temporarily alter the permeability of the cell membrane, allowing the genetic material to cross the cytoplasmic membrane and reach the interior of the cell. It is important that there is a balance between the efficiency of cellular internalization and the damage exerted on the cell. These methods have been frequently evaluated as systems of administration of naked genetic material, without the need to formulate it or include it in a viral or non-viral vector, which gives them great simplicity. Nevertheless, physical methods are mainly evaluated and used in preclinical studies. Table III summarizes the main characteristics of physical delivery methods used in gene therapy[99].

98 Ramamoorth M, Narvekar A. Non-Viral Vectors in Gene Therapy- An Overview. J. Clin. Diagn. Res. 2015; 9:GE01–6. https://doi.org/10.7860/JCDR/2015/10443.5394.

99 Herrero MJ, Sendra L, Miguel A, Aliño SF. Physical Methods of Gene Delivery. In: Safety and Efficacy of Gene-Based Therapeutics for Inherited Disorders. Brunetti-Pierri N (Ed.), Springer, Cham. 2017; 113-135. https://doi.org/10.1007/978-3-319-53457-2.

Table III. Types of physical delivery systems in gene therapy and main features.

Method	Advantages	Limitations
Needle injection Direct needle injection on a specific tissue	Simple Safe	Low efficiency Local inflammation
Hydrofection or hydrodynamic injection Intravascular injection of high volumes of a solution containing the nucleic acids	High efficacy in liver	Hemodynamic changes
Microinjection Direct injection into host cell by microneedles	High efficiency	Cell by cell administration Time consuming Need of specialist
Biolistic injection or gene gun Administration of metal microparticles at high velocity	Simple and fast Reproducibility	Low efficiency Low tissue penetration Cell damage High cost
Electroporation Application of electric pulses that open pores on cell membrane	Non-invasive Simple High efficiency Low cost Widely employed	Risk of tissue damage Surgery necessary to target internal organs
Sonoporation Application of ultrasounds (combined with microbubbles or nanocarriers) to permeabilize temporally cell membrane	Non-invasive Safe Targeting to specific tissues	Low reproducibility Tissue damage
Magnetofection Application of external magnetic fields combined with magnetic particles	Non-invasive Effective in primary cells (difficult to transfect)	Effective only on surface areas Mainly applied *in vitro*
Optofection Application of laser pulses combined with nucleic acid complexes or nanoparticles	Nucleic acids release from endosomes	Tissue damage Inflammation Restricted to single cells or small areas

1.2.2.2. Chemical carriers

Chemical vectors are based on the use of different types of compounds capable of encapsulating or binding, electrostatically or covalently, the genetic material.

In order to develop a suitable non-viral delivery system the selected vector must have the capacity to enter the cell and to overcome different barriers maintaining the stability of the nucleic acid throughout the entire transfection process. Once within the cell, it must guarantee the proper intracellular distribution of genetic material. Therefore, the main steps that these delivery systems have to overcome to reach the cytoplasm (in the case of RNAs) or the nucleus (in the case of DNAs) are the following: interaction with cell membrane, entry into the cytoplasm, intracellular distribution and entry into the nucleus (Figure XI). Up to date, different strategies for overcoming these limitations have been proposed, and the evolution of non-viral vector transfection has been significantly improved in recent years.

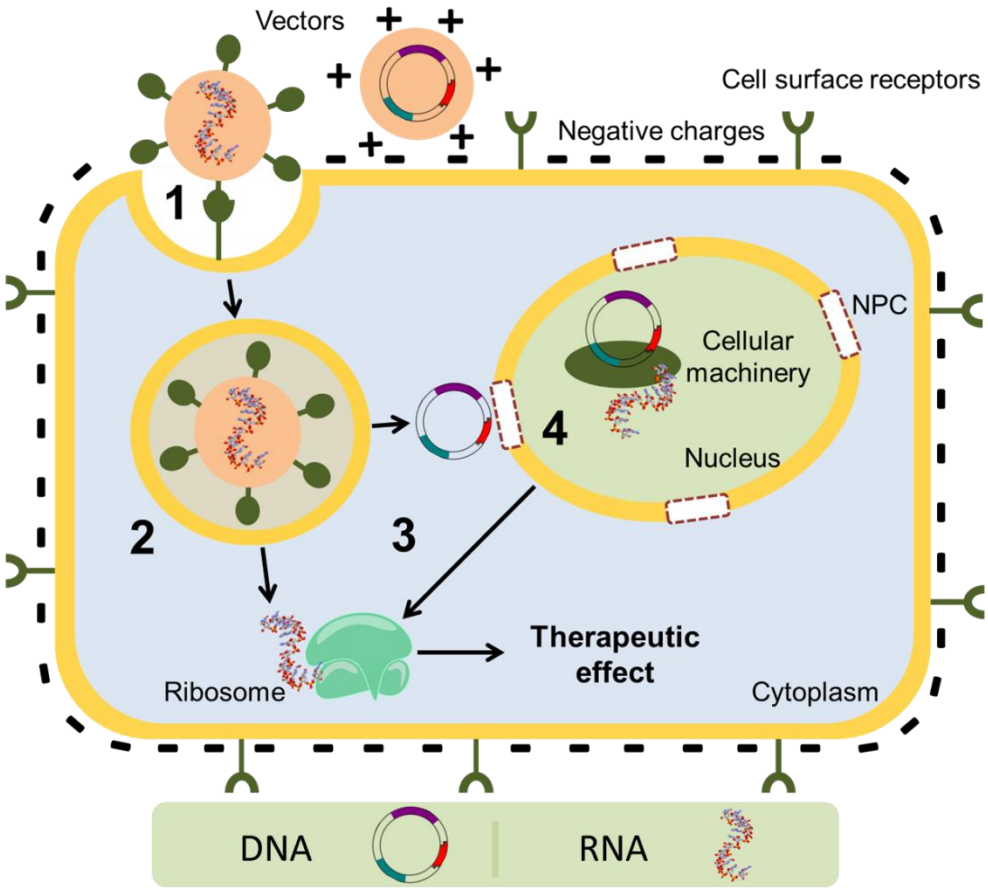

Figure XI. Main stages during transfection process: (1) interaction with cell membrane, (2) entry into the cytoplasm, (3) intracellular distribution and (4) entry into the nucleus. NPC = Nuclear pore complex.

The first step in the genetic transfer process is the interaction between vectors and cell membranes. Cationic vectors interact electrostatically with the negative charge of the cell membrane surface, and the internalization process is started. Additionally, in order to enhance the interaction with specific cells, different ligands can be added to the vectors to improve binding to surface receptors[100,101].

Once the vector has bound to the cell surface it must penetrate into the cytoplasm. The internalization or entry into the cell can take place through two mechanisms: fusion with cell membrane or endocytosis. These two entry routes are not exclusive, and depending on the type of cell and vector, one or the other may predominate. However, in most cases vectors penetrate

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100 Apaolaza PS, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez JM, Friedrich U, Torrecilla J et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. Biomaterials. 2016; 90:40-9. https://doi.org/10.1016/j.biomaterials.2016.03.004.

101 Gan L, Wang J, Zhao Y, Chen D, Zhu C, Liu J et al. Hyaluronan-modified core-shell liponanoparticles targeting CD44-positive retinal pigment epithelium cells via intravitreal injection». Biomaterials. 2013; 34:5978-87. https://doi.org/10.1016/j.biomaterials.2013.04.035.

into the cells mainly through endocytic pathways. Endocytosis begins with the formation of a vesicle from the invagination of the plasma membrane, called endosome. These endosomes fuse with lysosomes by creating endolysosomes, and their hydrolytic enzymes can degrade genetic material. Therefore, to achieve efficient gene transfer, it is necessary the release of nucleic acids to the cytoplasm before their lysosomal degradation occurs. In fact, the endosomal escape represents an important barrier to achieve efficient transfection in the case of non-viral gene therapy[102].

In the case of DNA, once it is cytoplasm, it must be able to enter to the nucleus. However, the nuclear membrane is a selective barrier for macromolecules, such as DNA. The transport trough this membrane is a highly regulated process, facilitated by a series of water channels of about 10 nm, called nuclear pore complexes (NPCs). The genetic material transported by non-viral vectors penetrates the nucleus through two main routes: NPCs or during cellular mitosis, when the nuclear membrane is temporally disrupted. The passage through the NPCs is carried out by means of an energy-dependent process that generally involves the recognition of specific nuclear localization signals (NLS)[103]. The NLS consist of one or more short sequences of amino acids with positive charges containing arginines and lysines[104]. The formulation of DNA with compounds containing NLS is a strategy commonly used in non-viral gene therapy.

Chemical delivery systems or non-viral vectors are broadly categorized into inorganic, polymeric, lipidic or peptidic particles. In many cases, the combination of some of different kinds of chemical compounds is used in order to improve their profile of efficiency, cellular specificity and safety, giving rise to hybrid systems[105].

1.2.2.2.1. Inorganic particles

Inorganic particles are nanostructured systems with different sizes, shapes and porosity, designed to protect the genetic material from degradation and to escape from the reticuloendothelial system after its systemic administration. They can be composed of different

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102 del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. Applications of lipid nanoparticles in gene therapy. Eur. J. Pharm. Biopharm. 2016; 109:184-93. https://doi.org/10.1016/j.ejpb.2016.10.016.

103 Kim YH, Han ME, Oh SO. The molecular mechanism for nuclear transport and its application. Anat Cell Biol. 2017; 50:77-85. https://doi.org/10.5115/acb.2017.50.2.77.

104 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: the importance of the entry pathway. Eur. J. Pharm. Biopharm. 2011; 79:495-502. https://doi.org/10.1016/j.ejpb.2011.06.005.

105 Ramamoorth M, Narvekar A. Non-Viral Vectors in Gene Therapy- An Overview. J. Clin. Diagn. Res. 2015; 9:GE01–6. https://doi.org/10.7860/JCDR/2015/10443.5394.

elements, being the most used in gene therapy calcium phosphate[106], silica[107], gold[108], or magnetic compounds such as iron oxide[109].

These inorganic particles are of interest since they are easy to produce and ligands can be added to their surface that facilitate the union to the genetic material through electrostatic interactions. Cationic components are usually incorporated to the surface of the particle. An example of this type of system consists of combining iron oxide particles with polyethyleneimine (PEI), which favours the condensation of nucleic acids, with polyethylene glycol (PEG), which favours the colloidal stability of the particles, and with cell penetration peptides, which favour cellular internalization[110]. In the case of gold particles, nucleic acids are previously thiolated to covalently bound to the delivery system[111].

Other types of inorganic materials used to develop inorganic particles that are showing encouraging results *in vitro* and *in vivo* in animal models are graphene[112] or fullerene[113]. However, it is still necessary to study in greater depth the long-term safety and the influence of functionalization, size and shape in transfection efficiency to facilitate the clinical application of these newer compounds.

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106 Mostaghaci B, Loretz B, Lehr CM. Calcium Phosphate System for Gene Delivery: Historical Background and Emerging Opportunities. Curr. Pharm. Des. 2016; 22:1529-33. https://doi.org/10.2174/1381612822666151210123859.

107 Kesse S, Boakye-Yiadom KO, Ochete BO, Opoku-Damoah Y, Akhtar F, Filli MS et al. Mesoporous Silica Nanomaterials: Versatile Nanocarriers for Cancer Theranostics and Drug and Gene Delivery. Pharmaceutics. 2019; 11: pii: E77. https://doi.org/10.3390/pharmaceutics11020077.

108 Bishop CJ, Tzeng SY, Green JJ. Degradable polymer-coated gold nanoparticles for co-delivery of DNA and siRNA. Acta Biomater. 2015; 11:393-403. https://doi.org/ 10.1016/j.actbio.2014.09.020.

109 Eslaminejad T, Nematollahi-Mahani SN, Ansari M. Glioblastoma Targeted Gene Therapy Based on pEGFP/p53-Loaded Superparamagnetic Iron Oxide Nanoparticles. Curr. Gene Ther. 2017; 17:59-69. https://doi.org/10.2174/1566523217666170605115829.

110 Stephen ZR, Dayringer CJ, Lim JJ, Revia RA, Halbert MV, Jeon M et al. Approach to Rapid Synthesis and Functionalization of Iron Oxide Nanoparticles for High Gene Transfection. ACS Appl. Mater. Interfaces. 2016; 8:6320-8. https://doi.org/10.1021/acsami.5b10883.

111 Hu Y, Wen C, Song L, Zhao N, Xu FJ. Multifunctional hetero-nanostructures of hydroxyl-rich polycation wrapped cellulose-gold hybrids for combined cancer therapy. J. Control. Release. 2017; 255:154-63. https://doi.org/10.1016/j.jconrel.2017.04.001.

112 Vincent M, de Lázaro I, Kostarelos K. Graphene materials as 2D non-viral gene transfer vector platforms. Gene Ther. 2017; 24:123-32. https://doi.org/ 10.1038/gt.2016.79.

113 Clancy KFA, Hardy JG. Gene Delivery with Organic Electronic Biomaterials. Curr. Pharm. Des. 2017; 23:3614-25. https://doi.org/10.2174/1381612823666170710124137.

1.2.2.2.2. Polymeric particles

The main component of these vectors is a cationic polymer that binds and condenses the genetic material, giving rise to the so-called polyplexes[114]. Cationic polymers bind by electrostatic interactions the negatively charged genetic material, so that the nucleic acid is adsorbed to the surface of the nanoparticulate system or is encapsulated in its interior. In addition, these systems allow the incorporation of different ligands that improve the transfection efficiency in the target tissue. In general, polymeric vectors are more stable than lipid vectors, and even in some cases, the progressive degradation of the polymer allows controlling the rate of release of the genetic material once it is inside the cell.

The polymers used in the preparation of non-viral vectors can be subdivided into synthetic and natural polymers (also called biopolymers).

The synthetic polymers most used in gene therapy are PEI, the dendrimers[115], the polyesters (i.e., poly(lactic-co-glycolic) or PLGA)[116], or polymethacrylates[117]. Among them, PEI has been evaluated in various clinical trials for the treatment by local gene therapy of different types of cancer[118], but the high toxicity of this polymer has limited its application.

In the group of the biopolymers applied in gene therapy are polysaccharides, such as chitosan, cyclodextrins, alginate, pullulan or dextran. Some of these polysaccharides are used by

114 Sung YK, Kim SW. Recent advances in the development of gene delivery systems. Biomater. Res. 2019; 23:8. https://doi.org/10.1186/s40824-019-0156-z.

115 Palmerston Mendes L, Pan J, Torchilin VP. Dendrimers as Nanocarriers for Nucleic Acid and Drug Delivery in Cancer Therapy. Molecules. 2017; 22: pii: E1401. https://doi.org/10.3390/molecules22091401.

116 Ramezani M, Ebrahimian M, Hashemi M. Current Strategies in the Modification of PLGA-based Gene Delivery System. Curr. Med. Chem. 2017; 24:728-39. https://doi.org/10.2174/0929867324666161205130416.

117 Xie Y, Yu F, Tang W, Alade B, Peng Z-H, Wang Y et al. Chloroquine-containing DMAEMA copolymers as efficient anti-miRNA delivery vectors with improved endosomal escape and anti-migratory activity in cancer cells. Macromol. Biosci. 2018; 18:1. https://doi.org/10.1002/mabi.201700194.

118 Chen J, Guo Z, Tian H, Chen X. Production and clinical development of nanoparticles for gene delivery. Mol. Ther Methods Clin Dev. 2016; 3:16023. https://doi.org/10.1038/mtm.2016.23.

themselves as delivery systems, but most of them are generally used in combination with other non-viral vectors to improve their efficacy, safety or biodistribution[119,120,121].

1.2.2.2.3. Lipidic particles

Lipid-based systems are the most studied non-viral vectors at the clinical level. Up to 119 clinical trials have been documented, most of them in phases I and II, in which lipid vectors have been used as delivery systems for the genetic material. In most cases, vectors have been designed for the treatment of different types of cancer, but also for the treatment of cardiovascular diseases, hepatitis C virus infection or monogenic diseases such as cystic fibrosis[122]. Recently, a lipid-based siRNA delivery system called Patisiran (Alnylam® Pharmaceuticals) has reached the market, as treatment of hereditary transthyretin-induced amyloidosis[123].

The main components of the lipid-based vectors are cationic lipids, formed by hydrophobic alkyl chains, linked through an intermediate binding structure to a polar group. The most used cationic lipids are: 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA), 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP), 1,2-dimyristyloxypropyl-3-dimethyldhydroxyethylammonium bromide (DMRIE) or 3ß-[N- (N', N'-dimethylaminoethane)-carbamoyl]-cholesterol (DC-cholesterol), although derivatives of these lipids are also being studied in order to improve their efficacy and safety properties[124]. Thanks to their cationic nature, these lipids are able to condense and protect the genetic material, as well as to bind to the negative charges of the cell membranes. The main limitations of non-viral vectors based on cationic lipids are the low efficacy *in vivo* due to the fact that they are not stable and that they

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119 Apaolaza PS, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez JM, Friedrich U, Torrecilla J et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. Biomaterials. 2016; 90:40-9. https://doi.org/10.1016/j.biomaterials.2016.03.004.

120 Kim YH, Han ME, Oh SO. The molecular mechanism for nuclear transport and its application. Anat Cell Biol. 2017; 50:77-85. https://doi.org/10.5115/acb.2017.50.2.77.

121 Tabasum S, Noreen A, Maqsood MF, Umar H, Akram N, Nazli Z, et al. A review on versatile applications of blends and composites of pullulan with natural and synthetic polymers. Int. J. Biol. Macromol. 2018; 120(Pt A):603-32. https://doi.org/10.1016/j.ijbiomac.2018.07.154.

122 Gene Therapy Clinical Trials Worldwide. Provided by the Journal of Gene Medicine. Jon Wiley and Sons Ltd, 2019. http://www.abedia.com/wiley/index.html. Accessed 30 Mar 2020.

123 Kristen AV, Ajroud-Driss S, Conceição I, Gorevic P, Kyriakides T, Obici L. Patisiran, an RNAi therapeutic for the treatment of hereditary transthyretin-mediated amyloidosis. Neurodegener. Dis. Manag. 2019; 9:5-23. https://doi.org/10.2217/nmt-2018-0033.

124 Kulkarni JA, Cullis PR, van der Meel R. Lipid Nanoparticles Enabling Gene Therapies: From Concepts to Clinical Utility. Nucleic Acid Ther. 2018; 28:146-57. https://doi.org/10.1089/nat.2018.0721.

undergo rapid clearance, as well as the possibility of generating inflammatory or anti-inflammatory responses.

Cationic lipids can be used by themselves to form complexes, known as lipoplexes, by mixing them directly with the negatively charged genetic material, but they are normally used to prepare colloidal systems that are then bound to the genetic material to obtain the lipoplexes. The preparation of these colloidal systems can involve other lipid components, which may improve the transfection efficiency of cationic lipids, such as the phospholipid 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), which has fusogenic function and facilitates endosomal escape, or PEG, which forms a steric coating that makes vectors more stable *in vivo*. The colloidal lipid systems used in gene therapy are liposomes, nanoemulsions and solid lipid nanoparticles (SLNs)[125].

Nanoemulsions consist of a dispersion of an oil phase stabilized in an aqueous phase by means of a third component that acts as a surfactant, so that droplets of about 200 nm are formed. From the technological point of view, nanoemulsions are simple to manufacture, and they are very stable during storage. In spite of this, the application of cationic nanoemulsions in gene therapy is still quite reduced[126].

SLNs are spherical particles in the range of nanometres, formed by a core composed of a solid lipid at room temperature surrounded by a layer of surfactants. In the case of SLNs designed to be applied in gene therapy, cationic lipids exert part of the surfactant effect and, in turn, confer positive charge to the surface of the particles. SLNs have shown efficacy as systems for administering different types of genetic material at preclinical level *in vitro* and *in vivo*, after their systemic or local administration, showing promising results especially in ocular

125 Del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. Applications of lipid nanoparticles in gene therapy. Eur. J. Pharm. Biopharm. 2016; 109:184-93. https://doi.org/10.1016/j.ejpb.2016.10.016.

126 Teixeira HF, Bruxel F, Fraga M, Schuh RS, Zorzi GK, Matte U et al. Cationic nanoemulsions as nucleic acids delivery systems. Int. J. Pharm. 2017; 534:356-67. https://doi.org/10.1016/j.ijpharm.2017.10.030.

pathologies[127,128,129,130], as well as in infectious diseases[131], lysosomal storage disorders[132] and various types of cancer[133].

Liposomes are spherical vesicles composed of one or more lipid bilayers surrounding an aqueous core, which show a size ranging from 20 nm to a few microns. Cationic liposomes are effective transfection systems in very varied types of cells *in vitro*, and also *in vivo* after their local or systemic administration. In fact, in most clinical trials using lipid vectors, these are cationic liposomes.

1.2.2.2.4. Peptidic particles

Some peptides are capable of condensing nucleic acids by themselves resulting in the formation of nanoparticulate systems. These include cationic peptides composed of short sequences of positively charged amino acids such as histidine, arginine or lysine; in fact, poly-L-lysine is one of the peptide vectors with the highest transfection efficiency[134]. Proteins of natural origin, such as collagen or albumin[135], are also used as peptide vectors. In addition, it is very common to use

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127 Apaolaza PS, del Pozo-Rodríguez A, Torrecilla J, Rodríguez-Gascón A, Rodríguez JM, Friedrich U et al. Solid lipid nanoparticle-based vectors intended for the treatment of X-linked juvenile retinoschisis by gene therapy: In vivo approaches in Rs1h-deficient mouse model. J. Control. Release. 2015; 217:273-83. https://doi.org/10.1016/j.jconrel.2015.09.033.

128 Torrecilla J, del Pozo-Rodríguez A, Vicente-Pascual M, Solinís MÁ, Rodríguez-Gascón A. Targeting corneal inflammation by gene therapy: Emerging strategies for keratitis. Exp. Eye Res. 2018; 176:130-40. https://doi.org/10.1016/j.exer.2018.07.006.

129 Torrecilla J, Gómez-Aguado I, Vicente-Pascual M, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. MMP-9 Downregulation with Lipid Nanoparticles for Inhibiting Corneal Neovascularization by Gene Silencing. Nanomaterials (Basel). 2019; 9: pii: E631. https://doi.org/10.3390/nano9040631.

130 Vicente-Pascual M, Albano A, Solinís MÁ, Serpe L, Rodríguez-Gascón A, Foglietta F et al. Gene delivery in the cornea: in vitro & ex vivo evaluation of solid lipid nanoparticle-based vectors. Nanomedicine (Lond). 2018; 13:1847-54. https://doi.org/10.2217/nnm-2018-0112.

131 Torrecilla J, del Pozo-Rodríguez A, Solinís MÁ, Apaolaza PS, Berzal-Herranz B, Romero-López C et al. Silencing of hepatitis C virus replication by a non-viral vector based on solid lipid nanoparticles containing a shRNA targeted to the internal ribosome entry site (IRES). Colloids Surf. B. Biointerfaces. 2016; 146:808-17. https://doi.org/10.1016/j.colsurfb.2016.07.026.

132 Ruiz de Garibay AP, Solinís MÁ, del Pozo-Rodríguez A, Apaolaza PS, Shen JS, Rodríguez-Gascón A. Solid Lipid Nanoparticles as Non-Viral Vectors for Gene Transfection in a Cell Model of Fabry Disease. J. Biomed. Nanotechnol. 2015; 11:500-11. https://doi.org/10.1166/jbn.2015.1968.

133 Del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. Applications of lipid nanoparticles in gene therapy. Eur. J. Pharm. Biopharm. 2016; 109:184-93. https://doi.org/10.1016/j.ejpb.2016.10.016.

134 Mandal H, Katiyar SS, Swami R, Kushwah V, Katare PB, Kumar Meka A et al. ε-Poly-l-Lysine/plasmid DNA nanoplexes for efficient gene delivery in vivo. Int. J. Pharm. 2018; 542:142-52. https://doi.org/10.1016/j.ijpharm.2018.03.021.

135 Look J, Wilhelm N, von Briesen H, Noske N, Günther C, Langer K et al. Ligand-Modified Human Serum Albumin Nanoparticles for Enhanced Gene Delivery. Mol. Pharm. 2015; 12:3202-13. https://doi.org/10.1021/acs.molpharmaceut.5b00153.

peptides as ligands to functionalize some of the previously described polyplexes and lipoplexes. In this sense, peptides may be use to target non-viral vectors to a specific tissue (i.e. transferrin to target tumor tissue or hepatocytes[136], or RGD (arginine-glycine-aspartic acid) sequences to target specific tissues[137]). Another application of peptide as ligands is to improve their effectiveness by helping the genetic material to overcome some of the barriers of the transfection process: cell penetrating peptides[138] to improve cell entry, fusogenic peptides[139] to increase endosomal escape or NLS[140] to entry into the nucleus.

1.2.2.2.5. Manufacturing and quality control of non-viral vectors

The production of nanoparticles for clinical gene therapy shows important hurdles, which are still hampering the translation from laboratory to patients. Non-viral vectors are complex formulations that must be customized depending on the nucleic acid to be delivered, the variety of target diseases and the administration route[141]. Due to their complexity, nanoparticulate systems show unique Chemistry, Manufacturing and Controls (CMC) challenges[142]. In fact, suitable methods for large-scale production of simple nanosystems, such as liposomes, have been developed[143]. However, when formulation becomes more complex, for example with the addition of surface modification or ligands, the number of steps in the production process as well as the cost of the final product increase, and quality control is also more difficult[144].

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136 Tros de Ilarduya C, Düzgüneş N. Delivery of therapeutic nucleic acids via transferrin and transferrin receptors: lipoplexes and other carriers. Expert Opin. Drug Deliv. 2013; 10:1583-91. https://doi.org/10.1517/17425247.2013.837447.

137 Mohammed-Saeid W, Chitanda J, Al-Dulaymi M, Verrall R, Badea I. Design and Evaluation of RGD-Modified Gemini Surfactant-Based Lipoplexes for Targeted Gene Therapy in Melanoma Model. Pharm. Res. 2017; 34:1886-96. https://doi.org/10.1007/s11095-017-2197-0.

138 Layek B, Lipp L, Singh J. Cell Penetrating Peptide Conjugated Chitosan for Enhanced Delivery of Nucleic Acid. Int. J. Mol. Sci. 2015; 16:28912-30. https://doi.org/10.3390/ijms161226142.

139 Alipour M, Hosseinkhani S, Sheikhnejad R, Cheraghi R. Nano-biomimetic carriers are implicated in mechanistic evaluation of intracellular gene delivery. Sci. Rep. 2017; 7:41507. https://doi.org/10.1038/srep41507.

140 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: the importance of the entry pathway. Eur. J. Pharm. Biopharm. 2011; 79:495-502. https://doi.org/10.1016/j.ejpb.2011.06.005.

141 Chen J, Guo Z, Tian H, Chen X. Production and clinical development of nanoparticles for gene delivery. Mol. Ther Methods Clin Dev. 2016; 3:16023. https://doi.org/10.1038/mtm.2016.23.

142 Havel H, Finch G, Strode P, Wolfgang M, Zale S, Bobe I et al. Nanomedicines: From Bench to Bedside and Beyond. AAPS J. 2016; 18:1373-8. https://doi.org/10.1208/s12248-016-9961-7.

143 Kraft JC, Freeling JP, Wang Z, Ho RJ. Emerging research and clinical development trends of liposome and lipid nanoparticle drug delivery systems. J. Pharm. Sci. 2014;103:29-52. https://doi.org/10.1002/jps.23773.

144 Tinkle S, McNeil SE, Mühlebach S, Bawa R, Borchard G, Barenholz YC et al. Nanomedicines: addressing the scientific and regulatory gap. Ann. N. Y. Acad. Sci. 2014;1313:35-56. https://doi.org/10.1111/nyas.12403.

Regarding quality attributes, parameters that must be especially considered because of their impact on biological yield are size, shape, surface charge, presence of ligands to provide effective targeting, surface modification with PEG, impurities associated to starting materials and the production process, and stability during manufacturing, long-term storage and upon administration[145]. Batch to batch variability of non-viral vectors can potentially led to changes in all these parameters. Therefore, small changes in manufacturing process variables (such as temperature, pH, time, agitation speed, quality of starting materials, etc) can significantly affect the quality, efficacy and safety of the final vector[146]. It is important to stablish procedures to assess nanotherapeutics not only at final steps but also at intermediate ones. Moreover, the application of concepts of quality by design (QbD) based on quality guidelines introduced by the International Conference on Harmonisation[147] has been proposed to address questions related to manufacturing processes and CMC complexities[148,149]. The aim of QbD fundamentally aims at building quality and safety from the first design steps of the product[150]. This methodology intends for establishing a multi-dimensional design that defines process input requirements and operational ranges necessary to ensure that the product meets critical quality attributes. Designers of new nanotherapeutics will gain an understanding of these concepts and the role their preliminary data plays in preparing and positioning a potential nanoparticulate system for a gene therapy product development.

1.3. Applications of gene therapy

Despite gene therapy entered clinical trials in the early 1990s, the first nucleic acid based product registered in the European Union was Glybera in 2012, for the lipoprotein lipase deficiency. Currently, only nine GTMPs have received approval worldwide; nevertheless, since 1989 almost 2,700 gene therapy-based clinical trials have been completed, are ongoing or have

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145 Tyner KM, Zou P, Yang X, Zhang H, Cruz CN, Lee SL. Product quality for nanomaterials: current U.S. experience and perspective. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 2015;7:640-54. https://doi.org/10.1002/wnan.1338.

146 Tyagi P, Santos JL. Macromolecule nanotherapeutics: approaches and challenges. Drug Discov. Today. 2018;23:1053-61. https://doi.org/10.1016/j.drudis.2018.01.017.

147 ICH (International Conference on Harmonisation). Quality guidelines. https://www.ich.org/page/quality-guidelines. Accessed 30 Mar 2020.

148 Havel H, Finch G, Strode P, Wolfgang M, Zale S, Bobe I et al. Nanomedicines: From Bench to Bedside and Beyond. AAPS J. 2016;18:1373-8. https://doi.org/10.1208/s12248-016-9961-7.

149 Tyagi P, Santos JL. Macromolecule nanotherapeutics: approaches and challenges. Drug Discov. Today. 2018;23:1053-61. https://doi.org/10.1016/j.drudis.2018.01.017.

150 Bastogne T. Quality-by-design of nanopharmaceuticals - a state of the art. Nanomedicine. 2017;13:2151-7. https://doi.org/10.1016/j.nano.2017.05.014.

been approved for a broad range of applications, as can be seen in Figure XII[151]. Therefore, it is expected that nucleic acid based products have a substantial impact on the biopharmaceutical market in a near future.

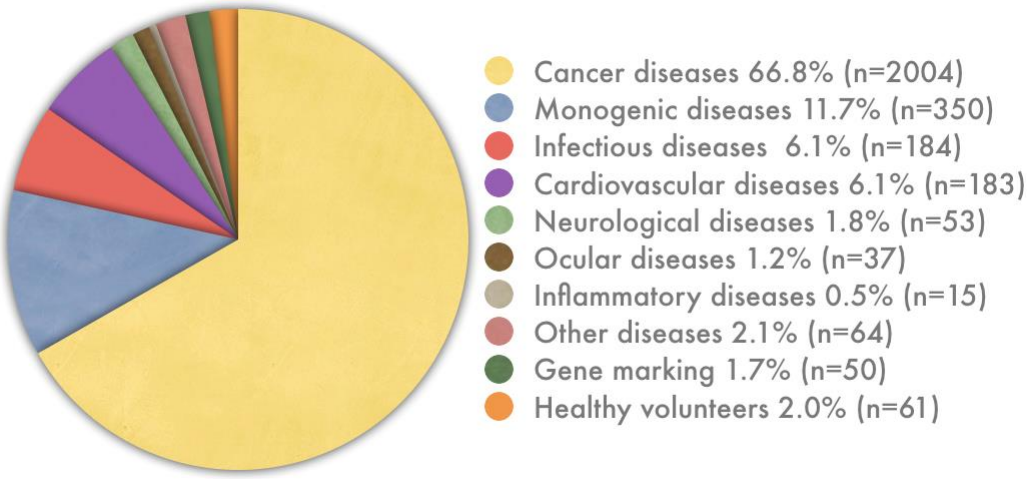

Figure XII. Indications addressed by Gene Therapy Clinical trials (Gene Therapy Clinical Trials Worldwide 2019).

The first major clinical advance in the state of the field of gene therapy was in 1990, when the Adenosine deaminase (ADA) gene was administered to a 4-year old girl to treat the severe combined immunodeficiency (SCID) she suffered[152]. This clinical trial fostered the launching of additional studies, one of them in 2000 for patients with the X-linked form of SCID[153], which supposed an important landmark for gene therapy. On the one hand, it provided for the first time a demonstration of therapeutic effect of gene transfer for the treatment of a genetic disease. On the other hand, two of the patients developed T cell leukemia as a result of insertional oncogenesis related to the retroviral vector used[154], which dampened the perspectives on gene therapy. Nonetheless, other potential hazards derived from the use of viral

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151 Gene Therapy Clinical Trials Worldwide. Provided by the Journal of Gene Medicine. Jon Wiley and Sons Ltd, 2019. http://www.abedia.com/wiley/index.html. Accessed 30 Mar 2020.

152 Anderson WF, Blaese RM, Culver K. The ADA human gene therapy clinical protocol: Points to consider response with clinical protocol. Hum. Gene Ther. 1990; 1:331-62. https://doi.org/10.1089/hum.1990.1.3-331.

153 Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science. 2000; 288:669–72. https://doi.org/10.1126/science.288.5466.669.

154 Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E et al. Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J. Clin. Invest. 2008; 118:3132–42. https://doi.org/10.1172/JCI35700.

vectors to administer the genetic material were already known. In 1999, a systemic inflammatory response to the dosage of adenoviral vector administered into bloodstream for the treatment of ornithine transcarbamylase (OTC) deficiency caused the death of Jesse Gelsinger, an 18-year-old male with partial OTC deficiency who participated in a pilot (safety) study of gene therapy[155].

Gene therapy has survived its previous failures and it has emerged thanks to the improvements of viral and non-viral vectors, the management of immune reactions and the use of new mechanisms of action.

The extensive research activity in cancer has not led to a significant number of gene therapy based approvals. Since 2003, when Gencidine®, indicated for the treatment of head and neck squamous cell carcinoma, received approval in China as the first GTMP marketed worldwide[156], only eight new products have been approved. However, GTMPs are becoming an emerging and expanding class of innovative medicinal products that can offer a more specific and causal/targeted treatment of many rare diseases, including rare cancers[157]. Gene therapy may be initially approved for patients who are lacking other therapeutic options, including conditions that in absence of treatment can cause disability or early death, and conditions that require intensive and onerous maintenance therapy in form of enzyme or protein replacement. For these patients, gene therapy could offer long-term stabilization or improvement of their health, with the ultimate objective of obtaining a cure[158]. Table IV shows nucleic acid–based products,

155 Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol. Genet. Metab. 2003; 80:148-58. https://doi.org/10.1016/j.ymgme.2003.08.016.

156 Pearson S, Jia H, Kandachi K. China approves first gene therapy. Nat. Biotechnol. 2004; 22:3-4. https://doi.org/10.1038/nbt0104-3.

157 Farkas AM, Mariz S, Stoyanova-Beninska V, Celis P, Vamvakas S, Larsson K et al. Advanced Therapy Medicinal Products for Rare Diseases: State of Play of Incentives Supporting Development in Europe. Front Med (Lausanne). 2017; 4:53. https://doi.org/10.3389/fmed.2017.00053.

158 Sinclair A, Islam S, Jones S. Gene therapy: an overview of approved and pipeline technologies. Ottawa: CADTH; 2018. (CADTH issues in emerging health technologies; issue 171).

including antisense oligonucleotides (ASOs) and gene-engineered cells, commercialized until present[159,160,161].

Apart from the five gene therapy products approved (Gencidine®, Oncorine®, Glybera®, Imlygic® and Luxturna®), products based on ASOs, small interfering RNAs (siRNA) or aptamers have been also authorized, which have yet to exert a profound influence on the biopharma product landscape.

Kymriah®, Yescarta®, Zalmoxis® and Strimvelis™ may be categorized as both cell and gene therapies. In all cases, genetic modification is undertaken *ex vivo* using a viral vector to achieve transduction, followed by infusion of the genetically modified cells into the patient. Kymriah® Yescarta® and Strimvelis™ use autologous cells, whereas Zalmoxis® uses allogeneic cells as a starting point. Strimvelis™ is a hematopoietic stem cell therapy and the other three are T-cell therapies, being Kymriah® and Yescarta® the first chimeric antigen receptor (CAR)-T cell-based products. All four products have orphan status or target niche conditions and either are under additional monitoring or require further post-authorization safety studies.

Out of the total of GTMPs approvals, Vitravene® and Glybera® were withdrawn from market in 2002 and 2017, respectively. Moreover, the authorization for use in the EU of Kynamro® and Exondis 51® were refused in 2012 and 2018, respectively.

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159 Walsh G. Biopharmaceutical benchmarks 2018. Nat. Biotechnol. 2018; 36:1136-45. https://doi.org/10.1038/nbt.4305.

160 EMA (European Medicine Agency). https://www.ema.europa.eu/en/medicines/human. 2020. Accessed 30 Mar 2020.

161 FDA U.S. Food and Drug Administration. Approved Cellular and Gene Therapy Products. https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products. 2019. Accessed 30 Mar 2020.

Table IV. Nucleic acid–based products approved.

Product	Year/Agency (First approval)	Company	Indication / Administration route	Strategy / Vector
Vitravene® (Fomivirsen)	1998/FDA	Isis Pharmaceuticals	CMV retinitis in AIDS patients/ Intravitreal injection	*In vivo*-ASO
Gencidine®	2003/SDFA	SiBiono GeneTech	Head and neck carcinoma/Intratumoral	*In vivo*-Gene augmentation/AD5
Macugen® (Pegaptanib)	2004/FDA	Eyetech	Wet form of AMD / Intravitrea linjection	*In vivo*-Aptamer
Oncorine®	2005/SDFA	Sunway Biotech	Nasopharyngeal cancer/Local	*In vivo*-Oncolitic virus/AD5
Glybera® (Alipogene tiparvovec)	2012/EMA	UniQure	Lipoprotein lipase deficiency/Intramuscular	*In vivo*-Gene augmentation/AAV1
Kynamro® (Mipomersen)	2013/FDA	Kastle Therapeutics	Familial hypercholesterolemia/ Subcutaneous Injection	*In vivo*-ASO
Imlygic® (Talimogene laherparepvec)	2015/FDA	BioVex	Melanoma/Local	*In vivo*-Oncolitic virus/HSV-1
Spinraza® (Nusinersen)	2016/FDA	Biogen	Spinal muscular atrophy/Intrathecal	*In vivo*-ASO
Exondys 51® (Eteplirsen)	2016/FDA	Sarepta Therapeutics	Duchenne muscular dystrophy/ Intravenous infusion	*In vivo*-ASO
Zalmoxis® (Nalotimagene carmaleucel)	2016/EMA	MolMed	Haploidentical HSC Transplant/ Intravenous infusion	*Ex vivo*-Allogeneic T cells genetically modified
StrimvelisTM	2016/EMA	MolMed	ADA-SCID/Intravenous infusion	*Ex vivo*-Autologous CD34+ genetically modified
Luxturna® (Voretigene neparvovec)	2017/FDA	Spark Therapeutics	Retinal dystrophy (RPE65)/Intraocular	*In vivo*-Gene augmentation/AAV2
Kymriah® (Tisagenlecleucel)	2017/FDA	Novartis	ALL and DLBCL/Intravenous infusion	*Ex vivo*-Autologous CAR T cell
Yescarta® (Axicabtagene ciloleucel)	2018/FDA	Kite Pharma	DLBCL and PMBCL/Intravenous infusion	*Ex vivo*-Autologous CAR T cell
Tegsedi® (Inotersen)	2018/EMA	Akcea Therapeutics	hATTR / Subcutaneous injection	*In vivo*-ASO
Onpattro® (Patisiran)	2018/FDA	Alnylam Pharmaceuticals	hATTR / Intravenous infusion	*In vivo* siRNA/Lipid nanoparticles

Abbreviations. FDA: Food and Drug Administration. CMV: Cytomegalovirus. AIDS: Acquired immuno-deficiency syndrome. ASO: Antisense oligonucleotides. SDFA: China State Food & Drug Administration. AD5: Adenovirus serotype 5. AMD: Age-related macular degeneration. EMA: European Medicines Agency. AAV1: Adeno-Associated Virus serotype 1. HSV-1: Herpes Simplex Virus type 1. HSCT: Haematopoietic stem cell. ADA-SCID: Severe Combined Immunodeficiency due to Adenosine Deaminase deficiency. AAV2: Adeno-Associated Virus serotype 2. ALL: B-cell acute lymphoblastic leukaemia. DLBCL: Diffuse large B-cell lymphoma. PMBCL: Primary mediastinal large B-cell lymphoma. CAR: Chimeric antigen receptor. hATTR: Hereditary transthyretin amyloidosis. siRNA: Small interfering ribonucleic acid.

A major challenge that faces most of the GTMPs is high development and production cost, which has led to pricing and refund issues, being the implementation of innovative reimbursement models a critical aspect at present. A representative example is Glybera®, an adeno-associated virus serotype 1-based gene therapy for intramuscular administration in adult patients with familiar lipoprotein lipase deficiency, a rare autosomal recessive disorder. Glybera® was commercialized in 2012 with a price of $1m per treatment and it was pulled from market in 2017 despite being therapeutically successful[162].

As challenging as the generally high price is GTMPs developmental timeline, that typically span two or even three decades from concept introduction to commercialization[162]. For instance, Kymriah®, the first CAR-T cell therapy, was approved by the FDA in 2017, after almost 30 years since the concept of redirecting T cells' potential to kill cancerous cells was introduced[163]. In the same way, in the case of Strimvelis™ it took nearly 15 years since the onset of the preclinical *in vivo* study[164] before its developer Fondazione Telethon (Italy) received orphan designation status from the European Commission in 2005. In this sense, there is already a similar precedent with the monoclonal antibodies, the most relevant biopharmaceutical products currently; it took more than three decades to get the commercialization of these products to become the primary drivers of the pharmaceutical market.

Besides the economic factors, other aspects have contributed to the low level of commercialization of GTMPs, such as the complexity of the technologies, difficulties in manufacturing processes and regulatory barriers[162]. GTMPs face significant additional regulatory challenges when pursuing market approval due to the risks and concerns gene therapies, which must be accounted during the regulatory process[165]. Moreover, the mismatch between the capacity of manufacturing vectors and the requirement of these emerging therapies is an important hurdle that is slowing down gene therapy progress[166].

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162 Yu TTL, Gupta P, Ronfard V, Vertès AA, Bayon Y. Recent Progress in European Advanced Therapy Medicinal Products and Beyond. Front. Bioeng. Biotechnol. 2018; 6: article 130. https://doi.org/10.3389/fbioe.2018.00130.

163 Eshhar Z. Tumor-specific T-bodies: towards clinical application. Cancer Immunol. Immunother. 1997; 45:131–6. https://doi.org/10.1007/s002620050415.

164 Ferrari G, Rossini S, Giavazzi R, Maggioni D, Nobili N, Soldati M et al. An in vivo model of somatic cell gene therapy for human severe combined immunodeficiency. Science. 1991; 251:1363–6. https://doi.org/10.1126/science.1848369.

165 Halioua-Haubold CL, Peyer JG, Smith JA, Arshad Z, Scholz M, Brindley DA et al. Regulatory considerations for gene therapy products in the US, EU, and Japan. Yale J. Biol. Med. 2017; 90:683-93.

166 Kaemmerer WF. How will the field of gene therapy survive its success? Bioeng. Transl. Med. 2018; 3:166–77. https://doi.org/10.1002/btm2.10090.

Nevertheless, considering not only the intensive investigation performed but also the recent advances in the gene editing field and T-cell based therapies, GTMPs will undoubtedly have a substantial contribution on the biopharmaceutical market over the years to come. Furthermore, with several product candidates now undergoing regulatory review, it appears likely that clinicians will have increasing opportunities to generate their own assessments of gene therapy as a treatment modality[167].

1.3.1. Gene therapy medicinal products for cancer

Cancer diseases that have been targeted by gene therapy are primarily those that do not respond well to conventional treatment such as metastatic melanoma or glioblastoma. As mentioned above, the first gene therapy marketed was Gencidine®, approved in 2003 for the treatment of head and neck squamous cell carcinoma by the China State Food & Drug Administration (SDFA), although it is not available in the United States of America or Europe. Gencidine is a type 5 recombinant adenovirus, which has the E1 region replaced by a Rous sarcoma virus promoter linked with the human wildtype p53 gene and a poly (A) tail[168]. The tumor suppressor p53 and its target genes are essential regulators of cell cycle control and induction of apoptosis. The p53 signaling cascade modulates cell cycle and DNA repair to maintain the genetic integrity of cells. If irreparable DNA damages occur, p53 activates cellular apoptotic pathways to eliminate genetically damaged cells[169]. Gencidine®, administered by intratumoural injection, induces the expression of the tumor suppressor protein p53 causing growth arrest and apoptosis in tumor cells. However, the antitumor effects depend on the expression level of transduced p53 and on the integrity in p53-mediated cascades in the target tumors[170].

Another approach to address the treatment of cancer is the use of oncolytic viruses (OV) that selectively replicate in tumor cells without harming normal cells. Recombinant virus technology has allowed the development of conditionally replicating viruses, being Oncorine® (H101) the first OV marketed, which received approval in China in 2005 for treatment of nasopharyngeal

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167 Anguela XM, High KA. Entering the Modern Era of Gene Therapy. Annu. Rev. Med. 2019; 70:273-88. https://doi.org/10.1146/annurev-med-012017-043332.

168 Ma G, Shimada H, Hiroshima K, Tada Y, Suzuki N, Tagawa M. Gene medicine for cancer treatment: Commercially available medicine and accumulated clinical data in China. Drug Des. Devel. Ther. 2009; 2:115-22. https://doi.org/10.2147/dddt.s3535.

169 Pflaum J, Schlosser S, Müller M. p53 family and cellular stress responses in cancer. Front. Oncol. 2014; 4:285. https://doi.org/10.3389/fonc.2014.00285.

170 Cheng P-H, Wechman SL, McMasters KM, Zhou HS. Oncolytic Replication of E1b-Deleted Adenoviruses. Viruses 2015; 7:5767–79; https://doi.org/10.3390/v7112905.

cancer after intratumoral administration[171]. Oncorine® is a type 5 adenovirus with E1B-55KD and partial E3 deletion that cannot replicate in normal cells where p53 is active; therefore, it can selectively infect and kill tumor cells via the targeting of pro-apoptosis. The first OV to gain approval by the FDA and EMA as an anticancer therapy was Talimogene laherparepvec (Imlygic®), approved in 2015 for melanoma treatment. It is a modified type 1 Herpes simplex virus (HSV-1) engineered to express human granulocyte-macrophage colony-stimulating factor (GM-CSF). The insertion of GM-CSF in place of both loci of the ICP34.5 gene, as well as by the deletion of the ICP47 gene increased the selective replication within tumor cells, enhancing the tumor-specific immune response[172,173]. The treatment is administered as a series of subcutaneous or intranodal injections over at least six months, and it has an estimated average cost of US$65,000[174].

Targeting a sufficient number of cells, even when the vector could be injected into the tumors directly and repeatedly, represented a serious obstacle to achieving full efficacy. Furthermore, considering that metastasis is the source of mortality for most cancers, systemic gene therapy is of considerable interest, and nowadays it is available with the *ex vivo* infusion of genetically modified hematopoietic T cells. In this sense, genetically modified immune T cells represent a new class of therapeutics that has shown encouraging success for the treatment of some types of cancer. However, specialized manufacturing facilities and personal trained to conduct customized procedures for such therapies are vital to ensure accessibility and quality of care[174].

Zalmoxis® (Nalotimagene carmaleucel) is an *ex vivo* GTMP approved by EMA in 2016 as adjunctive treatment in haploidentical haematopoietic stem cell transplantation (Haplo-HSCT) of adult patients with high-risk haematological malignancies. Haplo-HSCT can be associated with prolonged immunodeficiency post-transplantation, and Zalmoxis® aids immune reconstitution and reduce the risk of graft versus host disease[175]. This GTMP is based on allogenic somatic T-

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171 Castellanos MR, Pan Q. Novel p53 therapies for head and neck cancer. World J. Otorhinolaryngol. Head Neck Surg. 2016; 2:68–75. https://doi.org/10.1016/j.wjorl.2016.05.005.

172 Rehman H, Silk AW, Kane MP, Kaufman HL. Into the clinic: Talimogene laherparepvec (T-VEC), a first-in-class intratumoral oncolytic viral therapy. J. Immunother. Cancer. 2016; 4:53. https://doi.org/10.1186/s40425-016-0158-5.

173 Eissa IR, Bustos-Villalobos I, Ichinose T, Matsumura S, Naoe Y, Miyajima N et al. The Current Status and Future Prospects of Oncolytic Viruses in Clinical Trials against Melanoma, Glioma, Pancreatic, and Breast Cancers. Cancers (Basel). 2018; 10: pii: E356. https://doi.org/10.3390/cancers10100356.

174 Sinclair A, Islam S, Jones S. Gene therapy: an overview of approved and pipeline technologies. Ottawa: CADTH; 2018. (CADTH issues in emerging health technologies; issue 171).

175 EMA (European Medicine Agency). Zalmoxis: Summary of product characteristics. 2016. https://www.ema.europa.eu/en/documents/product-information/zalmoxis-epar-product-information_en.pdf. Accessed 16 Apr 2019.

cells genetically modified with a retroviral vector to express the Herpes Simplex Thymidine Kinase (HSV-TK) suicide gene and a truncated form of the human Low Affinity Nerve Growth Factor Receptor (ΔLNGFR) genes (for identification of transduced cells). The expression of the HSV-TK gene allows the selective killing of T cells that have this suicide gene, upon administration of ganciclovir or valganciclovir, preventing further development if patient develops graft-versus-host disease[176]

Eventually, the most recent therapies approved by FDA and EMA against cancer are Kymriah® (Tisagenlecleucel) and Yescarta® (Axicabtagene ciloleucel), the first chimeric antigen receptor (CAR)-T cell-based products, being both CD19-directed genetically modified autologous CAR-T cell immunotherapies. CARs consist of an antigen-binding domain, either from an immunoglobin molecule or from a T cell receptor, fused to an intracellular signaling domain, from receptors such as CD28, OX40, and CD137, that mediates activation and costimulation to enhance T cell function and persistence[177]. CARs recognize antigens independently of the major histocompatibility complex (MHC), which endows the CAR-T cell with a fundamental antitumor advantage, because a major mechanism of immunoevasion by cancer is loss of MHC-associated antigen presentation by tumor cells. Another advantage is that CARs target non-protein surface molecules, like carbohydrates and glycolipids. One limitation of current CAR-T cell strategies is that they require extracellular surface targets on the tumor cells[178].

CD19 is at present the most common CAR target; CD19 displays frequent and high-level expression in B cell malignancies, it is required for normal B cell development in humans, and it is not expressed outside of the B cell lineage, which make CD19 a nearly ideal target. For CAR-T cell therapy process, T cells are isolated from blood of the patient, activated, and then genetically engineered to express the CAR construct. T cells are modified by using a lentiviral or a retroviral vector for Kymriah® and Yescarta®, respectively. After *ex vivo* expansion of the CAR T cells, they are formulated into the final product for direct infusion[179]. However, CAR-T cell

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176 Mohty M, Labopin M, Velardi A, van Lint MT, Bunjes D, Bruno B et al. Allogeneic Genetically Modified T Cells (HSV-TK) As Adjunctive Treatment in Haploidentical Hematopoietic Stem-Cell Transplantation (haplo-HSCT) of Adult Patients with High-Risk Hematological Malignancies: A Pair-Matched Analysis from the Acute Leukemia Working Party of EBMT. Blood. 2016; 128:672. https://doi.org/10.1182/blood.V128.22.672.672.

177 Dunbar CE, High KA, Joung JK, Kohn DB, Ozawa K, Sadelain M. Gene therapy comes of age. Science. 2018; 359:6372. https://doi.org/10.1126/science.aan4672.

178 June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. Science. 2018; 359:1361–5. https://doi.org/10.1126/science.aar6711.

179 Hartmann J, Schüßler-Lenz M, Bondanza A, Buchholz CJ. Clinical development of CAR T cells—challenges and opportunities in translating innovative treatment concepts. EMBO Mol Med. 2017; 9:1183-97. https://doi.org/10.15252/emmm.201607485.

administration has been associated with serious systemic toxicities that often require intensive care and in some instances have caused patient deaths. To date, the most prevalent adverse effects following infusion of CAR-T cells result from on-target T cell activation, including cytokine release syndrome, macrophage activation syndrome, and tumor lysis syndrome[180].

Kymriah® and Yescarta® have a list price of US$475,000 and of US$373,000, respectively[181]. One of the indications of Kymriah® is the treatment of paediatric and young adult patients up to 25 years of age with B-cell acute lymphoblastic leukaemia (ALL) that is refractory, in relapse post-transplant or in second or later relapse. The other indication is the treatment of adult patients with relapsed or refractory diffuse large B-cell lymphoma (DLBCL), after two or more lines of systemic therapy. Yescarta® is indicated for the treatment of adult patients with relapsed or refractory DLBCL and primary mediastinal large B-cell lymphoma (PMBCL), after two or more lines of systemic therapy[182].

1.3.2. Gene therapy medicinal products for other applications

The resurgence of gene therapy in recent years for the treatment of genetic diseases is largely due to successes in trials that utilized both *ex vivo* strategies (for X-linked SCID and adrenoleukodystrophy) and *in vivo* approaches (Leber congenital amaurosis type 2 and hemophilia B)[183]. Gene therapies to treat rare disorders caused by single-gene mutations have made the most progress towards market availability. It has to be considered that in many of these diseases there are few treatment options apart from supportive and symptomatic care. Development of gene therapies has also been influenced by ease of administration in target tissues i.e. diseases of the eye and hematopoietic system.

Among the organs targeted by gene therapy, the eye has been at the forefront of translational gene therapy largely due to appropriate disease targets and its suitable anatomic features[184]. In fact, formiversen (Vitravene®) indicated for the treatment of cytomegalovirus retinitis (CMV) in

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180 Anguela XM, High KA. Entering the Modern Era of Gene Therapy. Annu. Rev. Med. 2019; 70:273-88. https://doi.org/10.1146/annurev-med-012017-043332.

181 Sinclair A, Islam S, Jones S. Gene therapy: an overview of approved and pipeline technologies. Ottawa: CADTH; 2018. (CADTH issues in emerging health technologies; issue 171).

182 EMA (European Medicine Agency). Yescarta: Summary of product characteristics. 2018d. https://www.ema.europa.eu/en/documents/product-information/zalmoxis-epar-product-information_en.pdf. Accessed 06 Apr 2020.

183 Anguela XM, High KA. Entering the Modern Era of Gene Therapy. Annu. Rev. Med. 2019; 70:273-88. https://doi.org/10.1146/annurev-med-012017-043332.

184 Solinís MÁ, del Pozo-Rodríguez A, Apaolaza PS, Rodríguez-Gascón A. Treatment of ocular disorders by gene therapy. Eur. J. Pharm. Biopharm. 2015; 95(Pt B):331-42. doi: https://doi.org/10.1016/j.ejpb.2014.12.022.

patients with AIDS was the first therapeutic ASO approved by FDA in 1998. It was administered intraocularly and its target was the mRNA that encoded the CMV immediate-early (IE)-2 protein, which is required for viral replication. EMA also approved this product in 1999, however, Novartis stopped marketing the drug in 2002 in Europe and in 2006 in the United States[185].

Likewise, pegaptanib (Macugen®), indicated for the treatment of age-related macular degeneration, was the first therapeutic aptamer approved by FDA in 2004. Macugen® is an RNA aptamer for intravitreal administration that consists of 28 nucleotides that binds to 165 isoform of VEGF (Vascular Endothelial Growth Factor). Its anti-angiogenic effect not only stops the excessive growth of blood vessels, but also prevents the formation of defective blood vessels[186].

Moreover, FDA and EMA approved Luxturna® in 2017 and 2018, respectively, for the treatment of adult and pediatric patients with vision loss due to inherited retinal dystrophy caused by confirmed biallelic *RPE65* mutations and who have sufficient viable retinal cells. Biallelic mutations in the *RPE65* gene, which encodes the all-trans retinyl ester isomerase, in this gene, can be described as Leber congenital amaurosis type 2, retinitis pigmentosa type 20, early-onset retinal dystrophy, and other clinical labels for severe rod-mediated inherited retinal dystrophies, which all eventually progress to complete blindness[187]. Luxturna® was designated an orphan medicine by EMA for two forms of the disease, retinitis pigmentosa in 2015 and Leber's congenital amaurosis in 2012. Voretigene neparvovec (Luxturna®) consists of a recombinant adeno-associated virus serotype 2 vector carrying a functional *RPE65* gene. This gene augmentation therapy is given by bilateral subretinal injection, and has a list price of US$425,000 (per eye treatment).

Ex vivo gene therapy approaches have mainly targeted hematopoietic system, being Strimvelis™ the first *ex vivo* GTMP approved for the treatment of an inherited disorder, ADA-SCID. This recessive immune disorder is caused by mutations in the *ADA* gene and characterized by the absence of cellular and humoral immune function and a fatal outcome very early in life. Strimvelis™, with orphan designation and approved in 2016 by EMA under conditional monitoring, saw the first clinical application on a single patient in March 2017. This

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185 Stein CA, Castanotto D. FDA-Approved Oligonucleotide Therapies in 2017. Mol. Ther. 2017; 25:1069-75. https://doi.org/10.1016/j.ymthe.2017.03.023.

186 Parashar A. Aptamers in Therapeutics. J. Clin. Diagn. Res. 2016; 10:BE01-6. https://doi.org/10.7860/JCDR/2016/18712.7922.

187 Russell S, Bennett J, Wellman JA, Chung DC, Yu ZF, Tillman A et al. Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. Lancet. 2017; 390: 849–60. https://doi.org/10.1016/S0140-6736 (17)31868-8.

hematopoietic stem cell therapy is indicated for the treatment of patients with ADA-SCID, for whom no suitable human leukocyte antigen (HLA)-matched related stem cell donor is available[188]. Patients receive CD34+ cells transduced with retroviral vector that encodes for the human ADA cDNA sequence. The genetically modified autologous CD34+ cells act by repopulating the hematopoietic system with cells that express active levels of the ADA enzyme. Strimvelis™, with a list price of €594,000, should be administered by intravenous infusion in a specialized transplant center. These *ex vivo* therapies require complex procedures and trained personnel to harvest, transduce, and reinfuse the hematopoietic target cells, and in the case of Strimvelis™, is currently available only at a single center in Milan[189].

Apart from gene therapy medicines, different nucleic acid–based products have also been approved for the treatment of diverse genetic diseases, including spinal muscular atrophy (SMA), Duchenne muscular dystrophy (DMD), familial hypercholesterolemia and hereditary transthyretin amyloidosis (hATTR).

SMA is an autosomal recessive neurodegenerative rare disease that, in most cases, involves homozygous deletion of the survival of motor neuron 1 (*SMN1*) gene on chromosome 5q. As consequence, patients suffer the deficiency of the survival of motor neuron (SMN) protein which plays a critical role in motor neuron development. *SMN1* is one of two nearly identical genes that encode SMN, the other is the survival of motor neuron 2 (*SMN2*). Infants with a more severe form of the disease (type 1 SMA) die before 2 years of age; later onset of the disease in infants is referred to as SMA2. Nusinersen (Spinraza®) is an ASO approved in 2016 by FDA and in 2017 by EMA (orphan medicine in 2012) for treating patients with SMA. Biogen has priced Spinraza at $750,000 for the first year's treatment ($125,000 per injection) and $350,000 per year subsequently[190].

SMN protein is mainly produced from *SMN1*, whereas *SMN2* produces a small amount of full-length SMN protein. Typically, a higher number of copies of *SMN2*, is associated with a less severe phenotype of the pathology. However, since the amount of protein formed is low, even multiple copies of *SMN2* do not fully stop the disease. The intron 7 in *SMN2* contains an intronic splicing silencer (termed ISS-N1) with binding sites for negative splicing factors (NSFs). Binding

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188 Sinclair A, Islam S, Jones S. Gene therapy: an overview of approved and pipeline technologies. Ottawa: CADTH; 2018. (CADTH issues in emerging health technologies; issue 171).

189 Anguela XM, High KA. Entering the Modern Era of Gene Therapy. Annu. Rev. Med. 2019; 70:273-88. https://doi.org/10.1146/annurev-med-012017-043332.

190 Stein CA, Castanotto D. FDA-Approved Oligonucleotide Therapies in 2017. Mol. Ther. 2017; 25:1069-75. https://doi.org/10.1016/j.ymthe.2017.03.023.

of these NSFs to intron 7 pre-mRNA precludes the recognition of exon 7 during the splicing process. The ASO nusinersen blocks the ISS-N1 site preventing the binding of the NSFs. As a result, Spinraza® administered via intrathecal injections, modulates the splicing of the *SMN2* mRNA transcript to include exon 7, thereby increasing the production of full-length SMN functional protein[191].

DMD is a rare X-linked disease characterized by loss-of-function mutations in the *DMD* gene coding for dystrophin, which disrupt the reading frame of the dystrophin mRNA and cause the introduction of premature stop codons, leading to mRNA degradation and the loss of protein synthesis in striated muscle. It is a fatal disorder characterized by progressive muscle weakening and wasting, with boys losing ambulation by 12 years of age or earlier; death often occurs within the 20s, usually due to respiratory or cardiac complications[192,193]. Eteplirsen (Exondys 51®) is a 30-nucleotide phosphorodiamidate morpholino oligomer and was approved as an ASO drug in 2016 by FDA, although authorization for use in the EU was refused by EMA in 2018. Eteplirsen promotes dystrophin production by restoring the translational reading frame of DMD through specific skipping of exon 51 in defective gene variants. The therapeutic strategy of antisense-mediated "exon skipping" is developed to force exon exclusion from mature mRNA of DMD with the purpose of restoring reading frame. Eteplirsen is suitable for 14% of DMD patients with *DMD* mutations, it is administered by intravenous injection and it has a price of $300,000/patient/year[194]

Familial hypercholesterolemia is an autosomal dominant genetic condition resulting from mutations of the low-density lipoprotein-cholesterol (LDL-C) receptor, apolipoprotein B (ApoB) or pro-protein convertase subtilisin/kexin 9 (PCSK9)[195]. Mipomersen (Kynamro®) is an orphan medicine approved in 2013 by FDA but with refused authorization for use in the EU by EMA in 2012. Mipomersen is a single-stranded synthetic DNA ASO targeting ApoB-100, resulting in suppression of the hepatic production of the ApoB, total cholesterol, LDL-C, and non-high-

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191 Talbot K, Tizzano EF. The clinical landscape for SMA in a new therapeutic era. Gene Ther. 2017; 24:529-33. https://doi.org/10.1038/gt.2017.52.

192 Lim KRQ, Maruyama R, Yokota T. Eteplirsen in the treatment of Duchenne muscular dystrophy. Drug Des. Devel. Ther. 2017; 11:533–45. https://doi.org/10.2147/DDDT.S97635.

193 Stein CA, Castanotto D. FDA-Approved Oligonucleotide Therapies in 2017. Mol. Ther. 2017; 25:1069-75. https://doi.org/10.1016/j.ymthe.2017.03.023.

194 Chen Ch, Yang Z, Tang X. Chemical modifications of nucleic acid drugs and their delivery systems for gene-based therapy. Med Res Rev. 2018; 38:829–69. https://doi.org/10.1002/med.21479.

195 Wong E, Goldberg T. Mipomersen (Kynamro) A Novel Antisense Oligonucleotide Inhibitor for the Management of Homozygous Familial Hypercholesterolemia. P T. 2014; 39:119–22.

density lipoprotein (HDL) cholesterol lipoproteins in rare genetic disorder patients with homozygous familial hypercholesterolaemia. Kynamro® is available as a solution for injection under the skin. Due to the serious risk of liver toxicity, mipomersen is labelled a black box warning hepatoxicity by FDA[194].

hATTR is a rare, autosomal dominantly inherited, progressively debilitating and life-threatening disease. Misfolded transthyretin (TTR) proteins accumulate as amyloid deposits at multiple sites culminating in intractable peripheral sensorimotor neuropathy and, in many cases, autonomic neuropathy and/or cardiomyopathy. Recently, two different nucleic acid–based products have been approved for the treatment of hATTR, by using two different strategies[196]. Inotersen (Tegsedi®) is an ASO designed to suppress the expression of both wild type and mutant forms of TTR. It has recently gained marketing authorization approval by EMA in 2018 (orphan designation in 2014) under additional monitoring for the treatment of stage 1 or stage 2 polyneuropathy in adult patients with hATTR, and regulatory approval from the FDA for the treatment of the polyneuropathy of hATTR. It is available as a solution for injection under the skin in pre-filled syringes and the recommended dose is one injection once a week[197]. Onpattro® (Patisiran) is a siRNA designed to target TTR to reduce the levels of both wild type and mutant TTR. Patisiran is formulated in lipid nanoparticles that direct the siRNA to the liver, the primary site of TTR production. Patisiran, with the same indications that Inotersen, is available as a solution for infusion and received regulatory approval in 2018 from the FDA and EMA (orphan designation in 2011)[198].

1.4. Challenges of gene therapy

As it has been commented along this introduction, gene therapy has still many challenges to overcome: the science is complex, treatment is technically difficult, and the regulatory approval process is necessarily different to that for conventional therapies. Actually, it has been considered as the most complex “drugs” ever developed[199]. Efficacy and safety, and

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196 Kristen AV, Ajroud-Driss S, Conceição I, Gorevic P, Kyriakides T, Obici L. Patisiran, an RNAi therapeutic for the treatment of hereditary transthyretin-mediated amyloidosis. Neurodegener. Dis. Manag. 2019; 9:5-23. https://doi.org/10.2217/nmt-2018-0033.

197 EMA (European Medicine Agency). Onpattro: Summary of product characteristics. 2018c. https://www.ema.europa.eu/en/documents/product-information/onpattro-epar-product-information_en.pdf. Accessed 06 Apr 2020.

198 Kristen AV, Ajroud-Driss S, Conceição I, Gorevic P, Kyriakides T, Obici L. Patisiran, an RNAi therapeutic for the treatment of hereditary transthyretin-mediated amyloidosis. Neurodegener. Dis. Manag. 2019; 9:5-23. https://doi.org/10.2217/nmt-2018-0033.

199 Dunbar CE, High KA, Joung JK, Kohn DB, Ozawa K, Sadelain M. Gene therapy comes of age. Science. 2018; 359:6372. https://doi.org/10.1126/science.aan4672.

manufacturing issues are important challenges that must be faced. There are also difficult questions about cost, accessibility, and social justice that will need answers once the methods are shown to be effective and safe.

1.4.1. Efficacy issues

The low efficacy, which may lead to treatment failure, is one of the most important challenges of gene therapy[200]. The development of new delivery systems with higher transduction rates and higher affinity to a specific cell or tissue is necessary to increase the number of products that reach clinical evaluation. Another reason that may explain the low efficacy of a gene therapy product is the presence of endogenous natural antibodies against viral vectors or the transgene product. This is of particular importance because it prevents transduction, and limits the gene therapy product administration more than once.

1.4.2. Safety issues

Potential immunogenicity and oncogenicity are the main challenges concerning safety. When a gene vector integrates into the host genome, there is a risk of unwanted tumor formation. This occurs as a consequence of activation/upregulation of oncogenes or inactivation or downregulation of tumor/suppressing genes[201]. To overcome the potential oncogenicity of viral vectors, it is crucial to design new vectors that prevent the activation of oncogenic genes at the integrations sites. The use of non-integrating vectors or highly targeted genomic integration at the desired chromosomal loci is also helpful.

Immunogenicity reactions may be due to both the viral vector and the transgene product due to the unpredictability of innate and antigen-dependent immune responses in humans[201]. One additional problem is that these responses are difficult to detect in animal models. Some alternatives to prevent immunogenicity includes the administration of immunosuppressive agents prior or after the administration of the gene therapy product, the modification of the capsid proteins of the vector, or the elimination of viral genes.

1.4.3. Drug development and manufacture issues

Because of its unique set of characteristics, the non-clinical development package of a GTMP is rather more complex than conventional medicinal products. One important difference with

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200 Carvalho M, Martins AP, Sepodes B. Hurdles in gene therapy regulatory approval: a retrospective analysis of European Marketing Authorization Applications. Drug Discov. Today. 2019; 24:823-8. https://doi.org/10.1016/j.drudis.2018.12.007.

201 Carvalho M, Martins AP, Sepodes B. Hurdles in gene therapy regulatory approval: a retrospective analysis of European Marketing Authorization Applications. Drug Discov. Today. 2019; 24:823-8. https://doi.org/10.1016/j.drudis.2018.12.007.

respect to a conventional drug product is that the approval of a gene therapy product must be based not only on data of the therapeutic transgene, but also of data on the vector/delivery system.

Manufacturing of gene therapy products is an additional complexity factor. In the near future, there is a challenge for the development manufacturing capacities, which must be sufficient to meet the coming demand, specifically the AAV production. In this sense, several companies of viral vector production are amplifying their manufacturing facilities to face the future increase demand for viral production[202].

1.4.4. Ethical issues

Currently, at least in the Western countries, clinical use of gene therapy is limited to somatic cells for the treatment of a specific disease. As it has been explained above, germline gene therapy lead to hereditary modifications that pass on to subsequent generations, and therefore, it is the object of a heated discussion[203]. The recent announcement by a Chinese research of the birth of twins whose genomes were edited by CRISPR/Cas9 during *in vitro* fertilization has engendered broad condemnation for the premature clinical deployment of a still experimental biomedical area[204]. The organizing committee of "The Second International Summit on Human Genome Editing" held in Hong Kong in November 2018 under the auspices of the U.S. National Academies of Science and Medicine, the U.K. Royal Society, and the Hong Kong Academy of Sciences, reiterated that "the scientific understanding and technical requirements for clinical practice remain too uncertain and the risks too great to permit clinical trials of germline editing at this time"[203].

The potential use of gene therapy for purposes other than diseases treatment is another important topic to be address. For instance, the application to eugenetics, that is, the attempt to change or improve complex human traits related to a broader number of genes; for example, personality, intelligence, or character[205].

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202 Kaemmerer WF. How will the field of gene therapy survive its success? Bioeng. Transl. Med. 2018; 3:166–77. https://doi.org/10.1002/btm2.10090.

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205 Carvalho M, Martins AP, Sepodes B. Hurdles in gene therapy regulatory approval: a retrospective analysis of European Marketing Authorization Applications. Drug Discov. Today. 2019; 24:823-8. https://doi.org/10.1016/j.drudis.2018.12.007.

1.4.5. Affordability

Gene therapy-based medicines have a high cost of development, production, product storage, and transportation, which lead to very high prices. For instance, the price of Glybera was around 1,000,000 €, Strimvelis, 600,000 €, and Yescarta and Kymriah 300,000 and 400,000 €, respectively. GTMP are expensive to develop and to manufacture, and sometimes, they are one time treatments; these reasons, among others, justify the high cost[206]. Affordability of novel innovative and high budget impact therapies has become an important topic in Europe and so far, each country has come up with individual approaches to improve affordability[207].

1.4.6. Intellectual property complexity

The complexity of the intellectual property "territories" that can surround a given gene therapy development also justifies the slow down progress of gene therapy[208]. In fact, a new gene therapy product under development may be conditioned by patents involving not only the therapeutic transgene itself, but also its mechanism of action, the non-viral or viral vector used as the delivery system, and the method for delivery of the nucleic acid therapy to the patient (e.g., the delivery devices, surgical techniques, and treatment protocols to be used).

Despite the numerous challenges, in the last years, important unified efforts by research and clinical scientists in academic, translational and industry settings have resulted in tangible outcomes, with several marketing authorizations and approved commercial products. Initiatives for willingness to participate in clinical trials, and equitative access of patient population to somatic gene editing as a treatment option in clinical care are necessary to increase the opportunities to successful of gene therapy.

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206 Daley GQ, Lovell-Badge R, Steffann J. After the Storm - A Responsible Path for Genome Editing. N. Engl. J. Med. 2019; 380:897-9. https://doi.org/10.1056/NEJMp1900504.

207 Flume M, Bardou M, Capri S, Sola-Morales O, Cunningham D, Levin LA et al. Approaches to manage 'affordability' of high budget impact medicines in key EU countries. J Mark Access Health Policy. 2018; 6:1478539. https://doi.org/10.1080/20016689.2018.1478539.

208 Kaemmerer WF. How will the field of gene therapy survive its success? Bioeng. Transl. Med. 2018; 3:166–77. https://doi.org/10.1002/btm2.10090.

2. Corneal inflammation

The eye is an extremely specialized organ, with individual structures that work together to capture and process visual information. It is broadly divided into two compartments, the anterior and the posterior segment[209]. The cornea, a transparent window at the anterior segment of the eye, performs a significant function in eyesight by refracting the light to focus a visual image. Three layers and two membranes compose the cornea: an external stratified epithelium followed by a transparent Bowman's membrane, a middle stromal layer and, finally, Descemet's basement membrane of the inner corneal endothelium (Figure XIII). The tear film covers the convex anterior surface of the cornea since concave posterior surface is circular and is in direct contact with the aqueous humour that fills the anterior chamber of the eye. The cornea, together with the sclera, forms the entire outer coat of the eye.

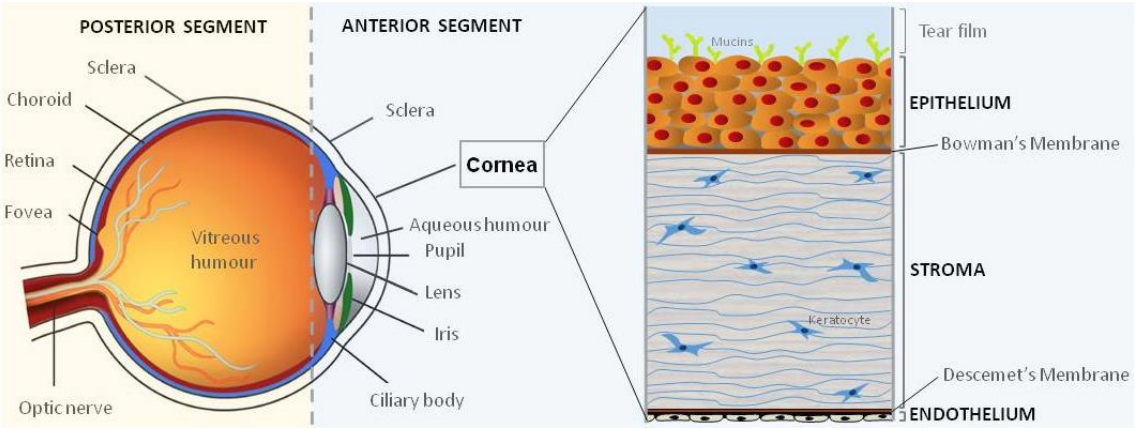

Figure XIII. Schematic representation of the human eyeball and corneal layer structure.

A wide range of factors may be responsible of corneal inflammation or keratitis, including infections, dry eye, disorders of the eyelids, physical and chemical injury, and a large variety of underlying diseases. According to the location, time, severity and relationship to inflammation of other parts of the eye, keratitis can be classified as included in Table V.

209 Dias MF, Joo K, Kemp JA, Fialho SL, da Silva Cunha A, Woo SJ, Kwon YJ. Molecular genetics and emerging therapies for retinitis pigmentosa: Basic research and clinical perspectives. Prog. Retin. Eye Res. 2017; 1–25. https://doi.org/10.1016/j.preteyeres.2017.10.004.

Table V. Types of keratitis depending on its location, time, severity and relationship to inflammation of other parts of the eye.

Location			Inflammation of other parts	
Corneal epithelium		Corneal stroma	Conjunctiva	Uveal tract
Superficial Keratitis		Stromal keratitis or interstitial keratitis	Keratoconjunctivitis	Keratouveitis
Filamentary	Punctate / Ulcerative			
Severity			**Evolution**	
Mild	Moderate	Severe	Acute	Chronic

Some cases of keratitis result from unknown factors, but major risk factors to develop it include any break or disruption of the surface layer of the cornea. The risk of developing keratitis is also increased by the use of contact lenses, or by disturbances of immune function through diseases, such as acquired immune deficiency syndrome (AIDS), or the use of medications, for instance, corticosteroids or chemotherapy[210,211]. Infections are the main cause of developing corneal inflammation, and among them, those caused by the HSV-1 are the most frequent, with an incidence of about 1.5 million, including 40,000 new cases of related blindness each year[212]. In developing countries, it is the leading cause of infectious blindness[213].

Keratitis, regardless of its origin, presents common symptoms and signs, including eye pain, blurred vision, photophobia, tearing, and eye redness. Furthermore, chronic inflammation of the cornea induces visual disturbance, and often results in tissue destruction that leads to corneal ulceration, scarring and, even, perforation, causing visual impairment and blindness[214].

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210 Utz VM, Kaufman AR. Allergic eye disease. Pediatr. Clin. North Am. 2014; 61:607–620. https://doi.org/10.1016/j.pcl.2014.03.009.

211 Boersma PM, Haarsma LD, Schotanus MP, Ubels JL. TNF-R1 and FADD mediate UVB-Induced activation of K+channels in corneal epithelial cells. Exp. Eye Res. 2017; 154:1–9. https://doi.org/10.1016/j.exer.2016.11.003.

212 Asim V, Farooq AV, Shukla D. Herpes simplex epithelial and stromalk: an epidemiologic update. Surv. Ophthalmol. 2012; 57:448–462. doi:10.1016/j.survophthal.2012.01.005.

213 Kwon MS, Carnt NA, Truong NR, Pattamatta U, White AJ, Samarawickrama C, Cunningham AL. Dendritic cells in the cornea during Herpes simplex viral infection and inflammation. Surv. Ophthalmol. 2018. https://doi.org/10.1016/j.survophthal.2017.11.001.

214 Lee SH, Kim KW, Joo K, Kim JC. Angiogenin ameliorates corneal opacity and neovascularization via regulating immune response in corneal fibroblasts. BMC Ophthalmol. 2016; 16:1–13. https://doi.org/10.1186/s12886-016-0235-z.

2.1. Inflammation process

After an inflammatory stimulus, a complex and dynamic wound healing process may start, in which, while the cause persists, a lot of different molecules including extracellular matrix (ECM) proteins, cytokines and growth factors, among others, are involved. All of them modulate subsequent events in response to the inflammatory stimulus. In this process, different phases occur, which can temporally overlap. The first phase (inflammatory phase) is characterized by the recruitment of neutrophils and macrophages to the injured site in response to chemokines. In the following phase (proliferative phase), there is deposition of ECM, which involves proliferation and migration of the cells within the scaffold matrix, and includes formation of new blood vessels, fibroblast proliferation, and production of ECM. While normal corneal keratocytes are quiescent and contribute to the maintenance of corneal transparency, disruption of the integrity of the cornea induces differentiation of quiescent cells into fibroblasts and/or myofibroblasts[215]. In the final phase (tissue remodeling or maturation phase), proteolitic enzymes degrade the matrix stroma, and vascular regression and granulation tissue remodeling occurs in addition to new formation of ECM components[216,217].

An important group of enzymes involved in all stages of the inflammatory process, from the beginning of the epithelial defect to its resolution and repair, are matrix metalloproteinases (MMPs)[218,219,220,221,222]. These enzymes participate in remodeling the ECM, in the epithelial

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215 Kim JW, Lim CW, Kim B. Effects of nicotine on corneal wound healing following acute alkali burn. PLoS One. 2017; 12, e0179982. https://doi.org/10.5061/dryad.mk4r5.

216 Pakyari M, Farrokhi A, Maharlooei MK, Ghahary A. Critical role of transforming growth factor beta in different phases of woundh. Adv. Wound Care. 2013; 2:215–224. https://doi.org/10.1089/wound.2012.0406.

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218 Coleman CM, Hannush S, Covello SP, Smith FJ, Uitto J, McLean WH. A novel mutation in the helix termination motif of keratin K12 in a US family with Meesmann corneal dystrophy. Am. J. Ophthalmol. 1999; 128:687–691. https://doi.org/10.1016/S0002-9394(99)00317-7.

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220 Afonso AA, Sobrin L, Monroy DC, Selzer M, Lokeshwar B, Pflugfelder SC. Tear fluid gelatinase B activity correlates with IL-1alpha concentration and fluorescein clearance in ocular rosacea. Invest. Ophthalmol. Vis. Sci. 1999; 40:2506–2512.

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barrier function[223] and in the migration of corneal epithelial cells to the underlying stroma[224,225,226,227,228,229]. Immune response cells secrete cytokines and regulatory proteins with proinflammatory or anti-inflammatory functions, which modulate the process and maintain the physiological condition of cells[230,231]. Almost twenty five cytokines that keep the normal physiological condition of the ocular surface have been detected in tears of healthy subjects (Higuchi et al. 2011[231]). Categories of cytokines include interferon-gamma (IFN-γ), transforming growth factor beta (TGF-β), interleukins (IL), and chemokines (small cytokines) involved in cellular and humoral immune responses[232]. Proinflammatory cytokines such as IL13, IL-6, IL-1b and tumour necrosis factor (TNF)-alpha are directly involved in the process, by increasing various

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224 Goktas S, Erdogan E, Sakarya R, Sakarya Y, Yilmaz M, Ozcimen M et al. Inhibition of corneal neovascularization by topical and subconjunctival tigecycline. J. Ophthalmol. 2014; ID 452685. https://doi.org/10.1155/2014/452685.

225 Gordon GM, Ledee DR, Feuer WJ, Fini ME. Cytokines and signaling pathways regulating matrix metalloproteinase-9 (MMP-9) expression in corneal epithelial cells. J. Cell. Physiol. 2009; 221, 402–411. https://doi.org/10.1002/jcp.21869.

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228 Sivak JM, Fini ME. MMPs in the eye: Emerging roles for matrix metalloproteinases in ocular physiology. Prog. Retin. Eye Res. 2002; 21, 1–14. https://doi.org/10.1016/S1350-9462(01)00015-5.

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231 Higuchi A, Kawakita T, Tsubota K. IL-6 induction in desiccated corneal epithelium in vitro and in vivo. Mol. Vis. 2011; 17, 2400–2406.

232 Zhang JM, An J. Cytokines, inflammation and pain. Int. Anesth. Clin. 2007; 45, 27–37. https://doi.org/10.1097/AIA.0b013e318034194e.

angiogenic factors, such as VEGF and MMP-9 in corneal cells[233,234,235,236,237,238]. In the group of anti-inflammatory cytokines, IL-10 is a potent immunomodulatory cytokine that interacts with antigen presenting cells inhibiting the production of monokines such as IL-1, IL-6, IL-8 and TNF-alpha[239,240,241,242,243].

2.2. Inflammation and corneal neovascularization

Angiogenesis is an essential process for tissue reproduction and development, and it is also necessary for wound healing. However, the establishment and maintenance of an avascular stroma by angiogenic privilege (by the production of higher level of angiostatic factors to offset of angiogenic factors), is an important aspect for corneal development and physiology. When an imbalance between angiogenic and antiangiogenic factors occurs, the normally avascular cornea

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235 Hayashi K, Hooper LC, Detrick B, Hooks JJ. HSV immune complex (HSV-IgG: IC) and HSV-DNA elicit the production of angiogenic factor VEGF and MMP-9. Arch. Virol. 2009; 154, 219–226. https://doi.org/10.1007/s00705-008-0303-7.

236 Zahir-Jouzdani F, Atyabi F, Mojtabavi N. Interleukin-6 participation in pathology of ocular diseases. Pathophysiology. 2017; 24, 123–131. https://doi.org/10.1016/j.pathophys.2017.05.005.

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241 de Waal Malefyt R, Haanen J, Spits H, Roncarolo M, te Velde A, Figdor C et al. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J. Exp. Med. 1991b; 174, 915–924. https://doi.org/10.1084/jem.174.4.915.

242 Ralph P, Nakoin I, Sampson-Johannes A, Fong S, Lowe D, Min H, Lin L. IL-10, T lymphocyte inhibitor of human blood cell production of IL-1 and tumor necrosis factor. J. Immunol. 1992; 148, 808–814.

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may become vascularized during inflammation[244]. Table VI summarizes the main angiogenic and antiangiogenic factors involved in corneal neovascularization (CNV).

The main angiogenic factors involved in corneal neovascularization are:

- Vascular endothelial growth factor (VEGF): it specifically interacts with vascular endothelial cells, promotes endothelial cell proliferation, increases the permeability of blood vessels, and promotes new blood vessel formation[245,246,247].
- Cell surface membrane-bound VEGF receptor 1 (VEGFR1, also known as sflt-1): it is a transmembrane receptor that binds VEGF with high affinity, initiating intracellular signaling[248].
- Cell surface membrane-bound VEGF receptor 2 (sVEGFR2): it is a broad and non-redundant physiological regulator of lymphatic vessels[249].
- Fibroblast growth factor (FGF): it stimulates VEGF expression in endothelial cells and stromal cells[250,251,252,253,254].

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244 Azar DT. Corneal angiogenic privilege: angiogenic and antiangiogenic factors in corneal avascularity, vasculogenesis, and wound healing (an American Ophthalmological Society thesis). Trans. Am. Ophthalmol. Soc. 2006; 104, 264–302.

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- Angiogenin: a component of tears involved in the innate immune system related with inflammatory disease [255,256].
- Prostaglandins: critical and early response mediators that initiate or amplify inflammation. In particular, prostaglandin E_2 has traditionally been identified as a prevalent inflammatory mediator in many tissues and inflammatory diseases [257,258].
- Platelet-derived growth factor (PDGF): it induces growth of corneal epithelial cells, stromal fibroblasts, and endothelial cells, by surface receptor specific binding[259].
- Matrix metalloproteinases (MMPs): expression of MMPs in corneal tissue results in excessive degradation of the extracellular matrix facilitating the migration of endothelial cells and the formation of CNV[260].
- Hypoxia-inducible factor (HIF): one of essential mediators of the cellular oxygen-signaling pathway, enhanced also the expression of the VEGF[261].
- Tumor necrosis factor (TNF)-α: a cytokine that induces inflammatory responses and binds to TNFR1 and TNFR2 receptors. TNF-α plays a critical role in corneal inflammation by

255 Lee SH, Kim KW, Joo K, Kim JC. Angiogenin ameliorates corneal opacity and neovascularization via regulating immune response in corneal fibroblasts. BMC Ophthalmol. 2016; 16, 1–13. https://doi.org/10.1186/s12886-016-0235-z.

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261 Rankin E, Giaccia A. The role of hypoxia-inducible factors in tumorigenesis. Cell Death Differ. 2008; 15, 678–685. https://doi.org/10.110910.1038/cdd.2008.21.

activation of nuclear factor-κB (NF-κB) signaling pathway that controls several genes involved in immune inflammatory responses[262,263,264,265].

On the contrary, the main antiangiogenic factors involved in CNV are:

- Pigment epithelium–derived factor (PEDF): it interacts with receptors on the cell surface and activates the necessary signal transduction events for neurotrophic activities to maintain avascularity of ocular tissues by pharmacologic inhibition against multiple inducers of angiogenesis, including VEGF and IL-8[266].
- Angiostatin: it binds to adenosine triphosphate (ATP) synthase and downregulates vascular endothelial cell proliferation and migration, affecting angiogenesis as well as developmental neovascularization[267,268,269].
- Endostatin: it associates with tropomyosins, integrins, VEGF receptor, MMPs, and glypicans in the antimigratory and proliferative effects on vascular endothelial cells[266] and inhibits both FGF-induced and VEGF-induced CNV[270,271,272].

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267 Gabison E, Chang JH, Hernández-Quintela E, Javier J, Lu PC, Ye H et al. Anti-angiogenic role of angiostatin during corneal wound healing. Exp. Eye Res. 2004; 78, 579–589. https://doi.org/10.1016/j.exer.2003.09.005.

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- Restin: it is the 22 kDa carboxyl terminal of collagen XV that possesses antiangiogenic properties by inhibiting the migration of endothelial cells[266,273].
- Arrestin: the type IV collagen derivative negatively regulates angiogenesis maintaining corneal angiogenic privilege[274,275,276].
- Vasohibin-1: negative feedback mediator of angiogenesis whose expression increases during neovascularization[277].
- Peroxisome proliferator-activated receptor gamma (PPARγ): suppressor of inflammation-mediated neovascularization by negatively regulating proinflammatory responses from macrophages[278,279].
- Decorin: leucine-rich proteoglycan expressed in the cornea that plays a major role in angiogenesis regulation by suppressing endothelial cell migration and tube formation[280].
- Brain-specific angiogenesis inhibitor1: transmembrane protein with antiproliferative function by blocking αvβ5 integrin in vascular endothelial cells[281].

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275 Mundel T, Kalluri R. Type IV collagen-derived angiogenesis inhibitors. Microvasc. Res. 2007; 74, 85–89. https://doi.org/10.1016/j.mvr.2007.05.005.

276 Wang Y, Yin H, Chen P, Xie L, Wang Y. Inhibitory effect of canstatin in alkali burn-induced corneal neovascularization. Ophthalmic. Res. 2011; 46, 66–72. https://doi.org/10.1159/000322804.

277 Sato Y. The vasohibin family: a novel family for angiogenesis regulation. J. Biochem. 2013; 153, 5-11. https://doi.org/10.1093/jb/mvs128.

278 Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature. 1998; 391(6662):82-6. https://doi.org/10.1038/34184.

279 Saika S, Yamanaka O, Okada Y, Miyamoto T, Kitano A, Flanders KC et al. Effect of overexpression of PPARgamma on the healing process of corneal alkali burn in mice. AJP Cell Physiol. 2007; 293, C75–C86. https://doi.org/10.1152/ajpcell.00332.2006.

280 Mohan RR, Tovey JCK, Sharma A, Schultz GS, Cowden JW, Tandon A. Targeted decorin gene therapy delivered with adeno-associated virus effectively retards corneal neovascularization in vivo. PLoS One. 2011; 6. https://doi.org/10.1371/journal.pone.0026432.

281 Yoon KC, Ahn KY, Lee JH, Chun BJ, Park SW, Seo MS et al. Lipid-mediated delivery of brain-specific angiogenesis inhibitor 1 gene reduces corneal neovascularization in an in vivo rabbit model. Gene Ther. 2005; 12, 617–624. https://doi.org/10.1038/sj.gt.3302442.

- GA binding protein (GABP): a nuclear transcription factor that suppresses VEGF transcription[282].

A wide range of pathological insults to the cornea can disrupt this intricate equilibrium, and promote angiogenesis and CNV in any layer of the cornea[283], resulting in visual impairment[284,285]. The situations leading to CNV are mostly associated with acquired sources like hypoxia, infection, inflammation, trauma, and other causes that can induce loss of limbal barrier function[286,287].

282 Yoon KC, Bae JA, Park HJ, Im SK, Oh HJ, Lin XH et al. Subconjunctival gene delivery of the transcription factor GA-binding protein delays corneal neovascularization in a mouse model. Gene Ther. 2009; 16, 973–981. https://doi.org/10.1038/gt.2009.50.

283 Holland E, Brilakis G, Schwartz G. Herpes simplex keratitis. In: Cornea. Krachmer JH, Mannis MJ, Holland EJ (Eds.), Elsevier Mosby, Philadelphia, EEUU. 2005; II, Vol. 1; 1043–1074.

284 Hsu CC, Chang HM, Lin TC, Hung KH, Chien KH, Chen SY et al. Corneal neovascularization and contemporary antiangiogenic therapeutics. J. Chinese Med. Assoc. 2015; 78, 323–330. https://doi.org/10.1016/j.jcma.2014.10.002.

285 Menzel-Severing J. Emerging techniques to treat corneal neovascularisation. Eye. 2012; 26, 2–12. https://doi.org/10.1038/eye.2011.246.

286 Hsu CC, Chang HM, Lin TC, Hung KH, Chien KH, Chen SY et al. Corneal neovascularization and contemporary antiangiogenic therapeutics. J. Chinese Med. Assoc. 2015; 78, 323–330. https://doi.org/10.1016/j.jcma.2014.10.002.

287 Bakunowicz-ŁAzarczyk A, Urban B. Assessment of therapeutic options for reducing alkali burn-induced corneal neovascularization and inflammation. Adv. Med. Sci. 2016; 61, 101–112. https://doi.org/10.1016/j.advms.2015.10.003.

3. Gene therapy and corneal inflammation

As mentioned above, gene therapy consists in the use of nucleic acids to repair, replace, or regulate genes to prevent or treat a disease. It has emerged as a new approach for corneal diseases that leads to blindness, thanks to the advantages of the cornea in terms of accessibility, transparency, ease of vector administration and visual monitoring, and ability to perform frequent non-invasive corneal assessment[288,286]. In addition, the general circulation and the systemic immune system in the cornea are limited; it can tolerate foreign antigens without mounting a systemic immune response, a concept termed "immune privilege"[289,290,291]. From an experimental point of view, other important favouring circumstance is that the cornea can be maintained as an *ex vivo* organ cultured for several weeks, which allows to evaluate the efficacy and safety of gene therapy products, and to optimise gene transfer[292].

Several corneal diseases can potentially benefit from gene therapy. Some examples are corneal scarring, CNV, anterior and stromal dystrophies that are linked to genetic mutations, monogenic lysosomal storage disorders affecting the cornea such as mucopolysaccharidosis (MPS) type VII (Sly syndrome), corneal graft rejection and the maintenance of corneal endothelial cell density in eye-banked corneas[293].

Different strategies including gene supplementation and gene silencing through ASO or iRNA (mediated by miRNA, siRNA or shRNA) have been evaluated at preclinical level. Viral and non-viral vectors have been used to deliver nucleic acids to the cornea, and different routes of administration have been assayed, such as topical instillation, intrastromal, intracameral, and subconjuntival injection. The success of gene therapy to treat corneal diseases is conditioned by the ability of the vectors to target specific cell populations and to deliver adequate levels of the

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288 Mohan RR, Rodier JT, Sharma A. Corneal gene therapy: basic science and translational perspective. Ocul. Surf. 2013; 11, 150–164. https://doi.org/10.1016/j.jtos.2012.10.004.

289 Cursiefen C. Immune privilege and angiogenic privilege of the cornea. Chem. Immunol. Allergy. 2007; 92, 50–57. https://doi.org/https://doi.org/10.1159/000099253.

290 Taylor AW. Ocular immune privilege. Eye. 2009; 23, 1885–1889. https://doi.org/10.1038/eye.2008.382.

291 Solinís MÁ, Del Pozo-Rodríguez A, Apaolaza PS, Rodríguez-Gascón A. Treatment of ocular disorders by gene therapy. Eur. J. Pharm. Biopharm. 2015; 95, 331–342. https://doi.org/10.1016/j.ejpb.2014.12.022.

292 Ljubimov AV, Saghizadeh M. Progress in corneal wound healing. Prog. Retin. Eye Res. 2015; 49, 17-45. https://doi.org/10.1016/j.preteyeres.2015.07.002.

293 Rodríguez-Gascón A, del Pozo-Rodríguez A, Isla A, Solinís MÁ. Gene therapy in the cornea. In: eLS. John Wiley & Sons, Ltd: Chichester. 2016. https://doi.org/10.1002/9780470015902.a0024274.

therapeutic nucleic acid into corneal cells. Moreover, the identification of novel targets to interrupt or intercept pathological processes[294] is essential.

Gene therapy has a great potential to treat disorders affecting the cornea, although most studies are still at preclinical levels in animal models, and clinical trials in humans are scarce. Gene supplementation and different strategies of gene silencing are at advanced stages, whereas gene editing approaches are still incipient. Some limitations have to be addressed before a medicinal product based on gene therapy is approved to treat corneal keratitis, including the improvement of safety and efficacy of vectors, and the achievement of long-term therapeutic responses.

3.1. Delivery systems for gene therapy to the cornea

The ideal delivery system must be produced by an easy, simple and cheap method, and induce high levels of corneal transfection in the target cell without toxicity or immunogenicity. The vectors must be designed to safely and efficiently overcome several physical barriers that are obstacles to internalization, such as precorneal (tear turnover, nasolachrymal drainage) and corneal (tight junctions and hydrophobicity of epithelium) barriers, tissue-selective targeting, cell internalization, escape from endo-lysosomal vesicles, movement through the cytoplasm and, in the case of the DNA, entry into the nucleus[295].

3.1.1. Naked nucleic acids

Occasionally, naked or plasmid DNA have been used by topical administration to the surface of the eye, as well as by injection in the corneal stroma or in the subconjunctival space. This way to administer DNA has been applied in animal models of herpetic eye disease, and

294 Mohan RR, Rodier JT, Sharma A. Corneal gene therapy: basic science and translational perspective. Ocul. Surf. 2013; 11, 150–164. https://doi.org/10.1016/j.jtos.2012.10.004.

295 Rodríguez-Gascón A, del Pozo-Rodríguez A, Isla A, Solinís MÁ. Gene therapy in the cornea. In: eLS. John Wiley & Sons, Ltd: Chichester. 2016. https://doi.org/10.1002/9780470015902.a0024274.

CNV[296,297,298,299]. The efficacy of naked nucleic acids has been improved by using different physical methods (gene gun, electroporation or, iontophoresis, among others).

3.1.1.1. Gene gun delivery systems

Gene gun administration of DNA in healthy corneas of BALB/c, achieved transfection without tissue disruption, even when high pressures were applied[300]. However, in some cases, gene gun has shown to cause serious cell damage[301].

3.1.1.2. Electroporation

Ultrasound-enhanced drug delivery through the cornea has considerable therapeutic potential; however, the knowledge of how ultrasound enhances nucleic acid transport is poor, as well as the ability to predict the increased level of transport for given ultrasound parameters[302]. In cultured *ex vivo* human corneas, the electroporation of two plasmids containing the cytomegalovirus promoter and the reporter genes for Green Fluorescent Protein (GFP) or beta-galactosidase, induced the expression of both proteins. GFP was present for at least one month, and beta-galactosidase transfection was detected in up to 54% of endothelial cells[303].

3.1.1.3. Iontophoresis

Iontophoresis is a method to deliver compounds across the plasma membrane of cells by applying an electric field. Nucleic acids, which are highly negatively charged molecules, and show high charge to molecular weight ratios, are good candidates for iontophoresis. ASO, siRNA and

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296 Williams KA, Coster DJ. Gene therapy for diseases of the cornea - a review. Clin. Exp. Ophthalmol. 2010; 38, 93–103. https://doi.org/10.1111/j.1442-9071.2009.02179.

297 Daheshia M, Kuklin N, Kanangat S, Manickan E, Rouse B. Suppression of ongoing ocular inflammatory disease by topical administration of plasmid DNA encoding IL-10. J. Immunol. 1997; 159, 1945–1952.

298 Stechschulte SU, Joussen AM, Von Recum HA, Poulaki V, Moromizato Y, Yuan J et al. Rapid ocular angiogenic control via naked DNA delivery to cornea. Investig. Ophthalmol. Vis. Sci. 2001; 42, 1975–1979.

299 Yoon KC, Bae JA, Park HJ, Im SK, Oh HJ, Lin XH et al. Subconjunctival gene delivery of the transcription factor GA-binding protein delays corneal neovascularization in a mouse model. Gene Ther. 2009; 16, 973–981. https://doi.org/10.1038/gt.2009.50.

300 Bauer D, Wasmuth S, Lu M, Heiligenhaus A. Particle-mediated administration of plasmid DNA on corneas of BALB/c mice. Methods Mol. Biol. 2013; 940, 215–220. https://doi.org/10.1007/978-1-62703-110-3_18.

301 Klebe S, Stirling J, Williams K. Corneal endothelial cell nuclei are damaged after DNA transfer using a gene gun. Clin. Experiment. Ophthalmol. 2000; 28, 58–59. https://doi.org/10.1046/j.1442-9071.2000.00255.x.

302 Hariharan P, Nabili M, Guan A, Zderic V, Myers M. Model for porosity changes occurring during ultrasound-enhanced transcorneal drug delivery. Ultrasound Med. Biol. 2017; 43, 1223–1236. https://doi.org/10.1016/j.ultrasmedbio.2017.01.013.

303 He Z, Pipparelli A, Manissolle C, Acquart S, Garraud O, Gain P, Thuret G. Ex vivo gene electrotransfer to the endothelium of organ cultured human corneas. Ophthalmic. Res. 2010; 43, 43–55. https://doi.org/10.1159/000246577.

DNA up to 3 kb have been delivered to the cornea by iontophoresis in different experimental models[304,305,306].

3.1.2. Chemical carriers

Gene expression efficiency achieved by physical methods is still a limitation, and chemical non-viral vectors, generally based on nanoparticles, have gained more attention[307].

3.1.2.1. Magnetic nanoparticles

Among magnetic nanoparticles (MNPs), those composed by iron oxide (Fe_3O_4) are particularly interesting as gene delivery systems due to the magnetic features, very low toxicity, and excellent biocompatibility and biodegradability properties[308]. In a recent study, the application of oscillating magnetic fields, for 30 and 60 min, to MNPs in cultured mice corneas induced up to 23% of transfection efficiency[309].

3.1.2.2. Lipid nanoparticles

3.1.2.2.1. Liposomes

Liposomes have been applied to deliver nucleic acids to corneal cells; for instance, a plasmid containing the β-galactosidase gene formulated in liposomes was able to transfect the basal layer of the corneal epithelium[310].

3.1.2.2.2. SLNs

The generally recognized as safe status of formulation excipients, the scaling-up facilities and the possibility of sterilization, make SLNs suitable for industrial production. SLNs are effective to

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304 Berdugo M, Valamanesh F, Andrieu C, Klein C, Benezra D, Courtois Y, Behar-Cohen F. Delivery of antisense oligonucleotide to the cornea by iontophoresis. Antisense Nucleic Acid Drug Dev. 2003; 13, 107–114. https://doi.org/10.1089/108729003321629647.

305 Hao J, Li S, Kao W, Liu C. Gene delivery to cornea. Brain Res. Bull. 2010; 81, 256–261. https://doi.org/10.1016/j.brainresbull.2009.06.011.

306 Williams KA, Coster DJ. Gene therapy for diseases of the cornea - a review. Clin. Exp. Ophthalmol. 2010; 38, 93–103. https://doi.org/10.1111/j.1442-9071.2009.02179.

307 Solinís MÁ, Del Pozo-Rodríguez A, Apaolaza PS, Rodríguez-Gascón A. Treatment of ocular disorders by gene therapy. Eur. J. Pharm. Biopharm. 2015; 95, 331–342. https://doi.org/10.1016/j.ejpb.2014.12.022.

308 Shen L, Li B, Qiao Y. Fe3O4 nanoparticles in targeted drug/gene delivery systems. Materials (Basel). 2018; 11, 324. https://doi.org/10.3390/ma11020324.

309 Siene Ng W, Binley K, Song B, Morgan JE. Use of magnetic nanoparticles and oscillating magnetic field for non-viral gene transfer into mouse cornea. Lancet. 2015; 385, Suppl, S75. https://doi.org/10.1016/S0140-6736(15)60390-7.

310 Masuda L, Matsuo T, Yasuda T, Matsuo N. Gene transfer with liposomes to the intramuscular tissues by different routes of administration. Ophthalmol. Vis. Sci. 1996; 37, 1914–1920.

transfect *in vitro* and *in vivo* different eye cells[311,312,313], and they were able to transfect the cornea of rats after topical instillation[314]. Additionally, SLNs have been assayed as delivery systems of different drugs (corticosteroids and non-steroidal) to treat ocular inflammation[315], and, recently, SLNs have been tested in preclinical phases to potentially treat CNV by gene therapy[316].

3.1.2.3. Polymeric nanoparticles

Polymeric nanocarriers based on different materials (poly-(alkylcyanocrylate), chitosan, hyaluronic acid, poly-ε-caprolactone, poly-(lactic-co-glycolic acid), poly-(butylcyanocrylate)) have been also proposed as promising systems for administration of nucleic acids to treat corneal diseases. These materials have a great potential for gene delivery because they generally have good biocompatibility and biodegradability, related to their structural properties[317]. Moreover, nanoparticles made of cationic and bioadhesive materials exhibit greater retention time at the ocular surface after instillation[318]. In this sense, plasmid DNA-loaded hyaluronan/chitosan nanoparticles could be internalized by corneal epithelial and conjunctival cells, without causing any sign of toxicity[319].

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311 Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Rodríguez-Gascón A, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. Int. J. Pharm. 2014; 465, 413–426. https://doi.org/10.1016/j.ijpharm.2014.02.038.

312 Apaolaza PS, del Pozo-Rodríguez A, Torrecilla J, Rodríguez-Gascón A, Rodríguez JM, Friedrich U et al. Solid lipid nanoparticle-based vectors intended for the treatment of X-linked juvenile retinoschisis by gene therapy: In vivo approaches in Rs1h-deficient mouse model. J. Control. Release. 2015; 217, 273–283. https://doi.org/10.1016/j.jconrel.2015.09.033.

313 Apaolaza PS, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez JM, Friedrich U, Torrecilla J et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. Biomaterials. 2016; 90, 40–49. https://doi.org/10.1016/j.biomaterials.2016.03.004.

314 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Avilés-Triqueros M, Weber BHF, Fernández E, Rodríguez-Gascón A. Dextran and protamine-based solid lipid nanoparticles as potential vectors for the treatment of X-linked juvenile retinoschisis. Hum. Gene Ther. 2012; 23, 345–355. https://doi.org/10.1089/hum.2011.115.

315 Sánchez-López E, Espina M, Doktorovova S, Souto EB, García ML. Lipid nanoparticles (SLN, NLC): Overcoming the anatomical and physiological barriers of the eye – Part II - Ocular drug-loaded lipid nanoparticles. Eur. J. Pharm. Biopharm. 2017; 110, 58–69. https://doi.org/10.1016/j.ejpb.2016.10.013.

316 Torrecilla J, Gómez-Aguado I, Vicente-Pascual M, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. MMP-9 Downregulation with Lipid Nanoparticles for Inhibiting Corneal Neovascularization by Gene Silencing. Nanomaterials (Basel). 2019; 9: pii: E631. https://doi.org/10.3390/nano9040631.

317 Rodríguez-Gascón A, del Pozo-Rodríguez A, Isla A, Solinís MÁ. Gene therapy in the cornea. In: eLS. John Wiley & Sons, Ltd: Chichester. 2016. https://doi.org/10.1002/9780470015902.a0024274.

318 Reimondez-Troitiño S, Csaba N, Alonso MJ, de la Fuente M. Nanotherapies for the treatment of ocular diseases. Eur. J. Pharm. Biopharm. 2015; 95, 279-93. https://doi.org/10.1016/j.ejpb.2015.02.019.

319 de La Fuente M, Seijo B, Alonso MJ. Novel hyaluronic acid-chitosan nanoparticles for ocular gene therapy. Invest. Ophthalmol. Vis. Sci. 2008; 49, 2016–2024. https://doi.org/10.1167/iovs.07-1077.

3.1.3. Viral vectors

Several viral vectors have been applied to deliver genes to ocular tissues, such as retrovirus, adenovirus, AAV, and lentivirus[320]. The most appropriate viral vector must be selected taking into account the target cell and the duration of the response (long- or short-term response). Viral vectors have been used to efficiently transfect the corneal epithelium, the stroma, and the corneal endothelium[321,322,323]. However, the induction of immune response and inflammation, even considering the immune-privilege of the cornea, limits the application of viral-based vectors to inflammatory diseases.

3.2. Application of gene therapy to corneal inflammation

Current treatment options for corneal inflammation and related neovascularization are restricted by limited efficacy, adverse effects, and short duration of action[324,325]. In the late 1990s, for the first time successful transduction of corneal tissues was achieved and corneal gene therapy showed its potential in correcting acquired corneal inflammatory diseases[326,327]. Corneal gene delivery studies that used reporter genes, like GFP and β-galactosidase, laid the foundation for gene therapy studies of corneal diseases[328]. Later on, gene therapy has shown a great potential for the treatment of diseases affecting the ocular surface of the eye; in many

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320 Solinís MÁ, Del Pozo-Rodríguez A, Apaolaza PS, Rodríguez-Gascón A. Treatment of ocular disorders by gene therapy. Eur. J. Pharm. Biopharm. 2015; 95, 331–342. https://doi.org/10.1016/j.ejpb.2014.12.022.

321 Hippert C, Ibanes S, Serratrice N, Court F, Malecaze F, Kremer EJ, Kalatzis V. Corneal transduction by intra-stromal injection of AAV vectors in vivo in the mouse and ex vivo in human explants. PLoS One. 2012; 7. https://doi.org/10.1371/journal.pone.0035318.

322 Lu Y, Ai J, Gessler D, Su Q, Tran K, Zheng Q et al. Efficient transduction of corneal stroma by adeno-associated viral serotype vectors for implications in gene therapy of corneal diseases. Hum. Gene Ther. 2016; 27, 598–608. https://doi.org/10.1089/hum.2015.167.

323 Valtink M, Knels L, Stanke N, Engelmann K, Funk RHW, Lindemann D. Overexpression of human HMW FGF-2 but not LMW FGF-2 reduces the cytotoxic effect of lentiviral gene transfer in human corneal endothelial cells. Invest. Ophthalmol. Vis. Sci. 2012; 53, 3207–3214. https://doi.org/10.1167/iovs.12-9423.

324 Al-Debasi T, Al-Bekairy A, Al-Katheri A, Al Harbi S, Mansour M. Topical versus subconjunctival anti-vascular endothelial growt factor therapy (Bevacizumab, Ranibizumab and Aflibercept) for treatment of corneal neovascularization. Saudi J. Ophthalmol. 2017; 31, 99-105. https://doi: 10.1016/j.sjopt.2017.02.008.

325 Bertens CJF, Gijs M, van den Biggelaar FJHM, Nuijts RMMA. Topical drug delivery devices: A review. Exp. Eye Res. 2018; 168, 149–160. https://doi.org/10.1016/j.exer.2018.01.010.

326 Mashhour B, Couton D, Perricaudet M, Briand P. In vivo adenovirus-mediated gene transfer into ocular tissues. Gene Ther. 1994; 1, 122–126.

327 Budenz DL, Bennett J, Alonso L, Maguire A. In vivo gene transfer into murine corneal endothelial and trabecular meshwork cells. Invest. Ophthalmol. Vis. Sci. 1995; 36, 2211–2215.

328 Klausner EA, Peer D, Chapman RL, Multack RF, Andurkar SV. Corneal gene therapy. J. Control. Release. 2007; 124, 107–133. https://doi.org/10.1016/j.jconrel.2007.05.041.

occasions gene therapy has demonstrated to be better controlled and more efficient than protein based therapy[329].

Genome editing is being used as a tool to study the role of certain genes on eye formation, and to relate genes with corneal diseases[330,331,332]. Moreover, the unique qualities of the eye, previously mentioned, and the success of current retinal gene replacement studies, make gene editing in the eye a natural next step to target diseases for which gene replacement is not a viable option. The immuneprivileged status of the cornea is a very important advantage in the case of CRISPR/Cas9 system, since pre-existing humoral and cell-mediated adaptive immune responses to Cas9 in humans have been demonstrated[333].

Corneal dystrophies offer an ideal platform for personalized genome editing, since most of them are monogenic and highly penetrant with a known pattern of inheritance. Additionally, genome editing may also be applied to the treatment of other corneal diseases, such as HSV keratitis (HSK). Recent data suggest that HSV-1 may be an excellent target for treatment using a CRISPR/Cas9-based approach[334]. In a recent study, an effective abrogation of HSV-1 replication by targeting guide RNAs (gRNAs) to essential viral genes has been shown; additionally, the simultaneous targeting of HSV-1 with multiple gRNAs was able to completely abolish the production of infectious particles from human cells. The authors concluded that CRISPR/Cas9 is a potent prophylactic and therapeutic anti-viral strategy that may be used to impair viral replication and clear latent virus infections[335].

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329 Elbadawy HM, Gailledrat M, Desseaux C, Ponzin D, Ferrari S. Targeting herpetic keratitis by gene therapy. J. Ophthalmol. 2012, 594869 https://doi.org/10.1155/2012/594869.

330 Yasue A, Kono H, Habuta M, Bando T, Sato K, Inoue J et al. Relationship between somatic mosaicism of Pax6 mutation and variable developmental eye abnormalities-an analysis of CRISPR genome-edited mouse embryos. Sci. Rep. 2017; 7, 1–10. https://doi.org/10.1038/s41598-017-00088-w.

331 Hendee K, Sorokina E, Muheisen S, Reis L, Tyler R, Markovic V et al. PITX2 deficiency and associated human disease: insights from the zebrafish model. Hum. Mol. Genet. In press. 2018. https://doi.org/10.1093/hmg/ddy074.

332 Weh E, Takeuchi H, Muheisen S, Haltiwanger RS, Semina EV. Functional characterization of zebrafish orthologs of the human Beta 3-Glucosyltransferase B3GLCT gene mutated in Peters Plus Syndrome. PLoS One. 2017; 12, 1–19. https://doi.org/10.1371/journal.pone.0184903.

333 Charlesworth CT, Deshpande PS, Dever DP, Dejene B, Gomez-Ospina N, Mantri S et al. Identification of pre-existing adaptive immunity to Cas9 proteins in humans. 2018; bioRxiv 243345. https://doi.org/10.1101/243345.

334 Roehm PC, Shekarabi M, Wollebo HS, Bellizzi A, He L, Salkind J, Khalili K. Inhibition of HSV-1 replication by gene editing strategy. Sci. Rep. 2016; 6, 1–11. https://doi.org/10.1038/srep23146.

335 van Diemen FR, Kruse EM, Hooykaas MJG, Bruggeling CE, Schürch AC, van Ham PM et al. CRISPR/Cas9-mediated genome editing of herpesviruses limits productive and latent infections. PLoS Pathog. 2016; 12, 1–29. https://doi.org/10.1371/journal.ppat.1005701.

Most of the gene therapy working to develop a potential treatment for corneal inflammation are targeted to either inflammatory mediators or neovascularization, although other defects, such as fibrosis and opacity, may be also targeted by gene therapy[336,337]. Both gene supplementation with plasmid DNAs and gene silencing methods (ASO, miRNA, siRNA, or shRNA) have been evaluated.

3.2.1. Gene therapy targeted to corneal neovascularization

Inflammatory angiogenesis is the pathogenic mechanism of various sight-threating eye disease, among them CNV[338]. Although gene therapy for CNV seems to be effective, safety issues arising from the vectors and transgenic overexpression may slow down the clinical application.

The treatment of CNV by gene therapy can be carried out by either transgenic expression of an antiangiogenic factor or by inactivation of a proangiogenic factor via gene silencing. A recent review provides a comprehensive and extensive overview on therapeutic target genes and potential vectors to treat CNV[339]. Antiangiogenic factors (Table VI) studied as potential gene therapy-based strategies for CNV include: vasohibin-1, endostatin and angiostatin[340,341], peroxisome proliferator-activated receptor gamma (PPARγ)[342], decorin[343], brain-specific

336 Bargagna-Mohan P, Paranthan RR, Hamza A, Zhan CG, Lee DM, Kim KB et al. Corneal antifibrotic switch identified in genetic and pharmacological deficiency of vimentin. J. Biol. Chem. 2014; 287, 989-1006. https://doi.org/10.1074/jbc.M111.297150.

337 Das SK, Gupta I, Cho YK, Zhang X, Uehara H, Muddana SK et al. Vimentin knockdown decreases corneal opacity. Invest. Ophthalmol. Vis. Sci. 2014; 55, 4030-4040. https://doi.org/10.1167/iovs.13-13494.

338 Mirabelli P, Peebo BB, Xeroudaki M, Koulikovska M, Lagali N. Early effects of dexamethasone and anti-VEGF therapy in an inflammatory corneal neovascularization model. Exp. Eye Res. 2014; 125, 118–127. https://doi.org/10.1016/j.exer.2014.06.006.

339 Liu X, Wang S, Wang X, Liang J, Zhang Y. Recent drug therapies for corneal neovascularization. Chem. Biol. Drug Des. 2017; 90, 653–664. https://doi.org/10.1111/cbdd.13018.

340 Parker M, Bellec J, McFarland T, Scripps V, Appukuttan B, Hartzell M et al. Suppression of neovascularization of donor corneas by transduction with equine infectious anemia virus-based lentiviral vectors expressing endostatin and angiostatin. Hum. Gene Ther. 2014; 25, 408–418. https://doi.org/10.1089/hum.2013.079.

341 Murthy R, McFarland T, Yoken J, Chen S, Barone C, Burke D et al. Corneal transduction to inhibit angiogenesis and graft failure. Invest. Ophthalmol. Vis. Sci. 2003; 44, 1837–1842. https://doi.org/10.1167/iovs.02-0853.

342 Saika S, Yamanaka O, Okada Y, Miyamoto T, Kitano A, Flanders KC et al. Effect of overexpression of PPARgamma on the healing process of corneal alkali burn in mice. AJP Cell Physiol. 2007; 293, C75–C86. https://doi.org/10.1152/ajpcell.00332.2006.

343 Mohan RR, Tovey JCK, Sharma A, Schultz GS, Cowden JW, Tandon A. Targeted decorin gene therapy delivered with adeno-associated virus effectively retards corneal neovascularization in vivo. PLoS One. 2011; 6. https://doi.org/10.1371/journal.pone.0026432.

angiogenesis inhibitor 1[344], pigment epithelium-derived factor (PEDF)[345], and GA-binding protein (GABP)[346]. By topical administration or after subconjuctival injection, DNA expressing these factors packaged into either viral or non-viral vectors have shown to reduce neovascularization of the cornea in different animal models.

Aganirsen is an ASO that prevents insulin receptor substrate-1 expression; it has shown to inhibit CNV in a dose-finding phase II study. In a subsequent phase III clinical trial (I-CAN study), after topical administration (eye drops) to patients with keratitis, aganirsen inhibited CNV and demonstrated to be safe and well tolerated. Additionally, the need for transplantation was significantly reduced in patients with viral keratitis and central neovascularization[347].

Recent findings have highlighted the effectiveness of RNAi in therapeutically appropriate conditions for CNV[348]. VEGF[349,350,351], VEGFR1 *(VEGF receptor 1)*[352], HIF-1α (subunit of a heterodimeric transcription factor hypoxia-inducible factor 1)[353], cytochrome P450 enzyme

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344 Yoon KC, Ahn KY, Lee JH, Chun BJ, Park SW, Seo MS et al. Lipid-mediated delivery of brain-specific angiogenesis inhibitor 1 gene reduces corneal neovascularization in an in vivo rabbit model. Gene Ther. 2005; 12, 617–624. https://doi.org/10.1038/sj.gt.3302442.

345 Kuo CN, Yang LC, Yang CT, Lai CH, Chen MF, Chen CY et al. Inhibition of corneal neovascularization with plasmid pigment epithelium-derived factor (p-PEDF) delivered by synthetic amphiphile INTeraction-18 (SAINT-18) vector in an experimental model of rat corneal angiogenesis. Exp. Eye Res. 2009; 89, 678–685. https://doi.org/10.1016/j.exer.2009.06.021.

346 Yoon KC, Bae JA, Park HJ, Im SK, Oh HJ, Lin XH et al. Subconjunctival gene delivery of the transcription factor GA-binding protein delays corneal neovascularization in a mouse model. Gene Ther. 2009; 16, 973–981. https://doi.org/10.1038/gt.2009.50.

347 Cursiefen C, Viaud E, Bock F, Geudelin B, Ferry A, Kadlecová P et al. Aganirsen antisense oligonucleotide eye drops inhibit keratitis-induced corneal neovascularization and reduce need for transplantation: The I-CAN study. Ophthalmology. 2014; 121, 1683–1692. https://doi.org/10.1016/j.ophtha.2014.03.038.

348 Guzman-Aranguez A, Loma P, Pintor J. Small-interfering RNAs (siRNAs) as a promising tool for ocular therapy. Br. J. Pharmacol. 2013; 170, 730–747. https://doi.org/10.1111/bph.12330.

349 Singh N, Higgins E, Amin S, Jani P, Richter E, Patel A et al. Unique homologous siRNA blocks hypoxia-induced VEGF upregulation in human corneal cells and inhibits and regresses murine corneal neovascularization. Cornea. 2007; 26, 65–72. https://doi.org/10.1097/ICO.0b013e31802b4201.

350 Zuo L, Fan Y, Wang F, Gu Q, Xu X. A siRNA targeting vascular endothelial growth factor-A inhibiting experimental corneal neovascularization. Curr. Eye Res. 2010; 35, 375–384. https://doi.org/10.3109/02713681003597230.

351 Qazi Y, Stagg B, Singh N, Singh S, Zhang X, Luo L et al. Nanoparticle-mediated delivery of shRNA.VEGF-A plasmids regresses corneal neovascularization. Investig. Ophthalmol. Vis. Sci. 2012; 53, 2837–2844. https://doi.org/10.1167/iovs.11-9139.

352 Kim B, Tang Q, Biswas PS, Xu J, Schiffelers RM, Xie FY et al. Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. Am. J. Pathol. 2004; 165, 2177–2185. https://doi.org/10.1016/S0002-9440(10)63267-1.

353 Chen HCJ, Yeh LK, Tsai YJ, Lai CH, Chen CC, Lai JY et al. Expression of angiogenesis-related factors in human corneas after cultivated oral mucosal epithelial transplantation. Investig. Ophthalmol. Vis. Sci. 2012; 53, 5615–5623. https://doi.org/10.1167/iovs.11-9293.

CYP4B1[354], and cannabinoid CB1 receptor[355] are some of the target proteins whose expression has been downregulated by siRNA to treat CNV in experimental models.

Intrastromal delivery of a plasmid expressing shRNA against VEGF before murine corneal injury has shown to suppress 1 week after injury corneal VEGF by 55.7%, leukocyte infiltration by 69.5%, and neovascularization by 72.3%, and at the regression time point, treated corneas had 72.8% less neovascularization[356].

VEGFR1 (also called sflt-1) is essential for preserving the avascular ambit of the cornea. Normal human corneas strongly express sflt-1 in the corneal epithelium and weakly in the corneal stroma close to the limbus. Neovascularised human corneas have greatly reduced expression of sflt-1 and significantly less VEGF is bound by sflt-1[357]. The suppression of VEGFR1 by RNAi has been shown to abolish corneal avascularity in mice[358]. In a previous study[359], albumin nanoparticles containing cDNA encoding domains 2 and 3 of flt (the binding regions for VEGF) showed to be non-toxic to the cornea and induced the expression of intraceptors for extended periods that are effective in suppressing injury-induced CNV.

A VEGFR1-specific morpholino (synthetic molecule that is similar to DNA oligonucleotides that can bind mRNA to sterically block the molecular machinery of translation or alternative

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354 Seta F, Patil K, Bellner L, Mezentsev A, Kemp R, Dunn MW, Schwartzman ML. Inhibition of VEGF expression and corneal neovascularization by siRNA targeting cytochrome P450 4B1. Prostaglandins Other Lipid Mediat. 2007; 84, 116–127. https://doi.org/10.1016/j.prostaglandins.2007.05.001.

355 Pisanti S, Picardi P, Prota L, Proto MC, Laezza C, Mcguire PG et al. Genetic and pharmacologic inactivation of cannabinoid CB1 receptor inhibits angiogenesis. Blood. 2011; 117, 5541–5550. https://doi.org/10.1182/blood-2010-09-307355.

356 Singh N, Higgins E, Amin S, Jani P, Richter E, Patel A et al. Unique homologous siRNA blocks hypoxia-induced VEGF upregulation in human corneal cells and inhibits and regresses murine corneal neovascularization. Cornea. 2007; 26, 65–72. https://doi.org/10.1097/ICO.0b013e31802b4201.

357 Ambati BK, Patterson E, Jani P, Jenkins C, Higgins E, Singh N et al. Soluble vascular endothelial growth factor receptor-1 contributes to the corneal antiangiogenic barrier. Br. J. Ophthalmol. 2007; 91, 505-508. https://doi:10.1136/bjo.2006.107417.

358 Ambati BK, Nozaki M, Singh N, Takeda A, Jani PD, Suthar T et al. Corneal avascularity is due to soluble VEGF receptor-1. Nature. 2006; 443,993-997. https://doi.org/10.1038/nature05249.

359 Jani PD, Singh N, Jenkins C, Raghava S, Mo Y, Amin S et al. Nanoparticles sustain expression of Flt intraceptors in the cornea and inhibit injury-induced corneal angiogenesis. Invest. Ophthalmol. Vis. Sci. 2007; 48,2030-2036. https://doi.org/10.1167/iovs.06-0853.

splicing)[360] was able to decrease neovascularization in a murine corneal suture model[361], and also reduced angiogenesis and lymphangiogenesis, resulting in increased graft survival in a murine penetrating keratoplasty model[362].

It is known that several species of miRNAs are altered in neovascularization[363]. The miRNA-132 shows the largest change in the expression during angiogenesis[364], and it is upregulated in animals with CNV after infection by HSV[365]. miRNA-155 can also influence the expression of several immune events that contribute to tissue damage[366,367]. It has been suggested that manipulating miRNA-155 along with miRNA-132, may provide more therapeutic value than using each miRNA alone to achieve an effective resolution of ocular inflammatory reactions; however, further research is needed[368]. Another non-coding miRNA expressed in the cornea is miRNA206, which is upregulated after chemical injury; when this miRNA was silenced by an oligonucleotide, CNV was significantly reduced in a mice model of alkali burn[369]. miRNA-184, the most abundant in the corneal epithelium, negatively regulates proangiogenic factors, including PDGF, MMPs and VEGF. In a model of rat suture-induced neovascularization, in which a reduction of miRNA-184 expression was demonstrated, 7 days after topical administration of

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360 Summerton JE. Morpholino, siRNA, and S-DNA compared: impact of structure and mechanism of action on off-target effects and sequence specificity. Curr. Top. Med. Chem. 2007; 7,651-660. https://doi.org/10.2174/156802607780487740.

361 Cho YK, Uehara H, Young JR, Archer B, Zhang X, Ambati BK. Vascular endothelial growth factor receptor 1 morpholino decreases angiogenesis in a murine corneal suture model. Invest. Ophthalmol. Vis. Sci. 2012a; 53, 685-692. https://doi.org/10.1167/iovs.11-8391.

362 Cho YK, Zhang X, Uehara H, Young JR, Archer B, Ambati B. Vascular Endothelial Growth Factor Receptor 1 morpholino increases graft survival in a murine penetrating keratoplasty model. Invest. Ophthalmol. Vis. Sci. 2012b; 53, 8458-8471. https://doi.org/10.1167/iovs.12-10408.

363 Liu S, Romano V, Steger B, Kaye SB, Hamill KJ, Willoughby CE. Gene-based antiangiogenic applications for corneal neovascularization. Surv. Ophthalmol. 2018; 63, 193–213. https://doi.org/10.1016/j.survophthal.2017.10.006.

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365 Mulik S, Xu J, Reddy PBJ, Rajasagi NK, Gimenez F, Sharma S et al. Role of miR-132 in angiogenesis after ocular infection with herpes simplex virus. Am. J. Pathol. 2012; 181, 525–534. https://doi.org/10.1016/j.ajpath.2012.04.014.

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368 Bhela S, Mulik S, Gimenez F, Reddy PBJ, Richardson RL, Varanasi SK et al. Role of miR-155 in the pathogenesis of herpetic stromal keratitis. Am. J. Pathol. 2015; 185, 1073–1084. https://doi.org/10.1016/j.ajpath.2014.12.021.

369 Li X, Zhou H, Tang W, Guo Q, Zhang Y. Transient downregulation of microRNA-206 protects alkali burn injury in mouse cornea by regulating connexin 43. Int. J. Clin. Exp. Pathol. 2015; 8, 2719–2727.

this miRNA a decrease in neovascularization was observed. In a mice model of spontaneous corneal neovascular dystrophy, miRNA-204 was identified as a novel regulator of angiopoietin-1, a proangiogenic factor[370]. This miRNA was greatly downregulated during neovascularization, and it has been suggested as a potential tool to modulate CNV.

3.2.2. Gene therapy targeted to inflammatory mediators

A reasonable number of studies has been exploring the ability of gene therapy to deliver specific anti-inflammatory mediators to treat corneal diseases. Additionally, survival rates of corneal allografts can also be improved when this kind of molecules are overexpressed. Delivery of specific anti-inflammatory mediators by gene therapy has been mainly explored for herpetic keratitis infections. Ocular infection due to HSV-1 induces a chronic immunoinflammatory reaction in the cornea, responsible of the pathology of the blinding illness. In fact, around 10% of corneal transplantation operations is performed to replace HSV-1 damaged corneas[371]. Most of the therapeutic strategies based on gene therapy against HSK are directed to the inflammation process, although strategies directed to the viral genome itself are also under investigation[372].

The immunoregulatory cytokine IL-10 plays an important role in the pathogenesis of recurrent HSK[373]. Intracorneal injection of recombinant IL-10 after HSV infection prevented the development of necrotizing stromal keratitis and suppressed the severity of the corneal disease[374]. However, after topical instillation the bioavailability of this cytokine is very low. In addition, its very short half-life makes necessary repeated administrations to induce a therapeutic effect[375]. As an alternative, genetic supplementation allows the sustained production of IL-10 *de novo* in corneal cells. Different strategies to deliver the IL-10 gene have

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370 Kather JN, Friedrich J, Woik N, Sticht C, Gretz N, Hammes HP, Kroll J. Angiopoietin-1 is regulated by miR-204 and contributes to corneal neovascularization in KLEIP-deficient mice. Investig. Ophthalmol. Vis. Sci. 2014; 55, 4295–4303. https://doi.org/10.1167/iovs.13-13619.

371 Remeijer L, Osterhaus A, Verjans G. Human herpes simplex virus keratitis: the pathogenesis revisited. Ocul. Immunol. Inflamm. 2004; 12, 255–285. https://doi.org/10.1080/092739490500363.

372 Torrecilla J, Rodríguez-Gascón A, Solinís MÁ, del Pozo-Rodríguez A. Lipid nanoparticles as carriers for RNAi against viral infections: Current status and future perspectives. Biomed. Res. Int. 2014; 2014:161794. https://doi.org/10.1155/2014/161794.

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375 Li L, Elliott J, Mosmann T. IL-10 inhibits cytokine production, vascular leakage, and swelling during T helper 1 cell-induced delayed-type hypersensitivity. J. Immunol. 1994; 153, 3967–3978.

been assayed. For instance, electroporation was employed to produce a robust expression of IL-10 in murine corneas[376]. Gene therapy based on viral vectors has also been evaluated to transfer *in vitro* and *ex vivo* IL-10 to ovine and human corneas[377]; the efficiency obtained with lentiviruses was lower than with adenoviral vectors. In a recent study, the immunomodulatory and anti-inflammatory molecule human leukocyte anatigen-G (HLA-G) was engineered in an AAV vector and evaluated in rabbits. After a single intrastromal injection, the vector prevented CNV, inhibited trauma-induced T-lymphocyte infiltration (some of which were CD8+), and dramatically reduced myofibroblasts formation[378].

Non-viral vectors based on SLNs including a plasmid constructed with the IL-10 gene has also been evaluated *in vitro* and in rabbit explants, showing the capacity to transfect different layers of the cornea, depending on the composition of the vector[379].

Additionally to IL-10, other anti-inflammatory mediators such as IL-2, IL-4, IL-18, IL-21, IL-12p35, IL-12p40 IFN-β, IFN-α-1, IFN-γ, colony-stimulating factor (GM-CSF) and TNF-α, have been identified as possible targets for treatment of corneal inflammation by gene therapy[380]. For instance, siRNAs have been used to reduce the overexpression of TNF-α *in vitro* in cell cultures and *in vivo* in a mouse model for amelioration of chronic inflammation[381]. Although the objective of this study was directed to Behcet's disease, the siRNA could also be applied to corneal inflammation.

miRNAs have been minimally explored, especially in responses to infection. Nevertheless, the silencing of the miRNA-155 (up-regulated after ocular HSV-1 infection) in a HSV-1-infected mice

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376 Zhou R, Dean D. Gene transfer of interleukin 10 to the murine cornea using electroporation. Exp. Biol. Med. 2007; 232, 362–369.

377 Parker DG, Coster DJ, Brereton HM, Hart PH, Koldej R, Anson DS, Williams KA. Lentivirus-mediated gene transfer of interleukin 10 to the ovine and human cornea. Clin. Exp. Ophthalmol. 2010; 38, 405–413. https://doi.org/10.1111/j.1442-9071.2010.02261.

378 Hirsch ML, Conatser LM, Smith SM, Salmon JH, Wu J, Buglak NE et al. AAV vector-meditated expression of HLA-G reduces injury-induced corneal vascularization, immune cell infiltration, and fibrosis. Sci. Rep. 2017; 7, 1–11. https://doi.org/10.1038/s41598-017-18002-9.

379 Vicente-Pascual M, Albano A, Solinís MÁ, Serpe L, Rodríguez-Gascón A, Foglietta F et al. Gene delivery in the cornea: in vitro and ex vivo evaluation of solid lipid nanoparticle-based vectors. Nanomedicine (Lond). 2018; 13:1847-54. https://doi.org/10.2217/nnm-2018-0112.

380 Elbadawy HM, Gailledrat M, Desseaux C, Ponzin D, Ferrari S. Targeting herpetic keratitis by gene therapy. J. Ophthalmol. 2012, 594869 https://doi.org/10.1155/2012/594869.

381 Choi B, Hwang Y, Kwon HJ, Lee ES, Park KS, Bang D et al. Tumor necrosis factor alpha small interfering RNA decreases herpes simplex virus-induced inflammation in a mouse model. J. Dermatol. Sci. 2008; 52, 87–97. https://doi.org/10.1016/j.jdermsci.2008.05.001.

led to an improvement of the lesions and CNV, which suggests that this miRNA could be a promising target to treat stromal keratitis[382].

3.2.3. Gene therapy targeted to corneal inflammatory lymphangiogenesis

Lymphangiogenesis is the growth of novel lymphatic vessels, and it plays an important role in pathological processes, such as tumor metastasis, tumor growth, and inflammatory diseases[383]. Specifically in the cornea, lymphangiogenesis can be induced in chronic inflammation, and it is a primary mediator of corneal transplant rejection[384]. In recent studies, several secreted factors that promote the development of lymphatic vessels have been identified, including the (VEGF)-C, and VEGFR-3[385]. On the other hand, the blockage of these factors has been shown to alleviate inflammation in certain tissues[386]. In a recent study, miRNA-mediated knockdown of neuropilin-2 (NP2), a high-affinity kinase-deficient co-receptor for (VEGF)-C, significantly inhibited the up-regulation of VEGFR-3 in a mouse model of lipopolysaccharide-induced inflammatory CNV. Moreover, NP2 knockdown specifically inhibited the increase in the number of corneal lymphatic vessels, but did not influence the increase in the number of blood vessels[387].

As mentioned above, MMPs play key roles in degrading the ECM, which allows endothelial cells to invade and form vessels. MMP-2 and MMP-9 are produced by lymphatic endothelial cells (LEC), and synthetic MMP inhibitors inhibit LEC tube formation[388]. Recently, it has been demonstrated that the blockade of MMP-2 and MMP-9 inhibits lymphangiogenesis[389]. In

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382 Bhela S, Mulik S, Gimenez F, Reddy PBJ, Richardson RL, Varanasi SK et al. Role of miR-155 in the pathogenesis of herpetic stromal keratitis. Am. J. Pathol. 2015; 185, 1073–1084. https://doi.org/10.1016/j.ajpath.2014.12.021.

383 Liao S, Von Der Weid PY. Inflammation-induced lymphangiogenesis and lymphatic dysfunction. Angiogenesis. 2014; 17, 325–334. https://doi.org/10.1007/s10456-014-9416-7.

384 Dietrich T, Bock F, Yuen D, Hos D, Bachmann BO, Zahn G et al. Cutting edge: lymphatic vessels, not blood vessels, primarily mediate immune rejections after transplantation. J. Immunol. 2010; 184, 535–539. https://doi.org/10.4049/jimmunol.0903180.

385 Mumprecht V, Detmar M. Lymphangiogenesis and cancer metastasis. J. Cell. Mol. Med. 2009; 13, 1405–1416. https://doi.org/10.1111/j.1582-4934.2009.00834.

386 Goyal S, Chauhan SK, Dana R. Blockade of prolymphangiogenic vascular endothelial growth factor C in dry eye disease. Arch. Ophthalmol. 2012; 130, 84–89. https://doi.org/10.1001/archophthalmol.2011.266.

387 Tang X, Sun J, Du L, Du H, Wang L, Mai J et al. Neuropilin-2 contributes to LPS-induced corneal inflammatory lymphangiogenesis. Exp. Eye Res. 2016; 143, 110–119. https://doi.org/10.1016/j.exer.2015.10.017.

388 Bruyère F, Melen-Lamalle L, Blacher S, Roland G, Thiry M, Moons L et al. Modeling lymphangiogenesis in a three-dimensional culture system. Nat. Methods. 2008; 5, 431–437. https://doi.org/10.1038/nmeth.1205.

389 Du HT, Du LL, Tang XL, Ge HY, Liu P. Blockade of MMP-2 and MMP-9 inhibits corneal lymphangiogenesis. Graefe's Arch. Clin. Exp. Ophthalmol. 2017; 255, 1573–1579. https://doi.org/10.1007/s00417-017-3651-8.

another study, a plasmid DNA encoding shRNA against MMP-9 was effective at inhibiting this protein in mice cornea[390].

Naturally occurring sVEGFR-2 (soluble VEGFR-2) is a molecular uncoupler of blood and lymphatic vessels whose modulation might have a therapeutic role in lymphatic vascular malformations, transplantation, and potentially in tumor lymphangiogenesis and lymphedema. In an experimental work, tissue-specific loss of sVEGFR-2 in mice induced, at birth, spontaneous lymphatic invasion of the normally alymphatic cornea and hyperplasia of skin lymphatics without accompanying changes in blood vasculature. The administration of sVEGFR-2 inhibited lymphangiogenesis but not the hemangiogenesis induced by corneal suture injury or transplantation, enhanced corneal allograft survival, and suppressed lymphangioma cellular proliferation. These results enable new therapeutic strategies by selectively modulating aberrant lymphatic proliferation without encountering the potential adverse effects of non-specific anti-angiogenic therapy[391].

3.2.4. Gene therapy targeted to corneal graft survival

The cornea is the most commonly transplanted organ in the Unites States, with almost 50,000 cases every year[392]. However, immunological rejection occurs in a relatively high proportion, even though corneal allografts have the privilege of being among the most successful solid organ transplants. There are two strategies to manage the immune-mediated graft rejection: the inhibition or regression of CNV that frequently occurs by the ingrowth of lymph vessels, and the prevention of reverse immune-mediated graft rejection[393]. Gene therapy is an interesting approach to introduce immunoregulatory molecules and anti-angiogenic factors into the graft or the recipient to prevent rejection. In line with these two strategies, graft survival can be promoted by the overexpression of anti-angiogenesis factors, by modulation the immune response, and by the inhibition of apoptotic pathways.

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390 Azkur A, Kim B, Suvas S, Lee Y, Kumaraguru U, Rouse B. Blocking mouse MMP-9 production in tumor cells and mouse cornea by short hairpin (sh)RNA encoding plasmids. Oligonucleotides. 2005; 15, 72–84. https://doi.org/10.1089/oli.2005.15.72.

391 Albuquerque RJ, Hayashi T, Cho WG, Kleinman ME, Dridi S, Takeda A et al. Alternatively spliced vascular endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth. Nat Med. 2009; 15, 1023-1030. https://doi.org/10.1038/nm.2018.

392 Eye Bank Association of America. 2011–2012 Year in review: focused on restoring sight worldwide. Eye Bank Association of America, 2012, 1-13. http://issuu.com/moiremarketing/docs/ebaa-2011-12yir-final. Accessed 30 March 2020.

393 Qazi Y, Hamrah P. Gene therapy in corneal transplantation. Semin. Ophthalmol. 2013; 28, 287–300. https://doi.org/10.3109/08820538.2013.825297.

The modulation of anti- and proinflammatory cytokines that mediate graft rejection is an interesting approach for increasing the survival of corneal grafts. IL-10 and IL-12 genes incorporated in adenoviral vectors have been assayed in different animal models, with positive results[394,395,396,397]. Gene targets may be subdivided in antiangiogenic molecules, inhibitors of T cell costimulation, immunomodulators or anti-apoptotic factors[393]. Figure XIV outlines different examples of these targets, as strategy to improve corneal graft survival by gene therapy.

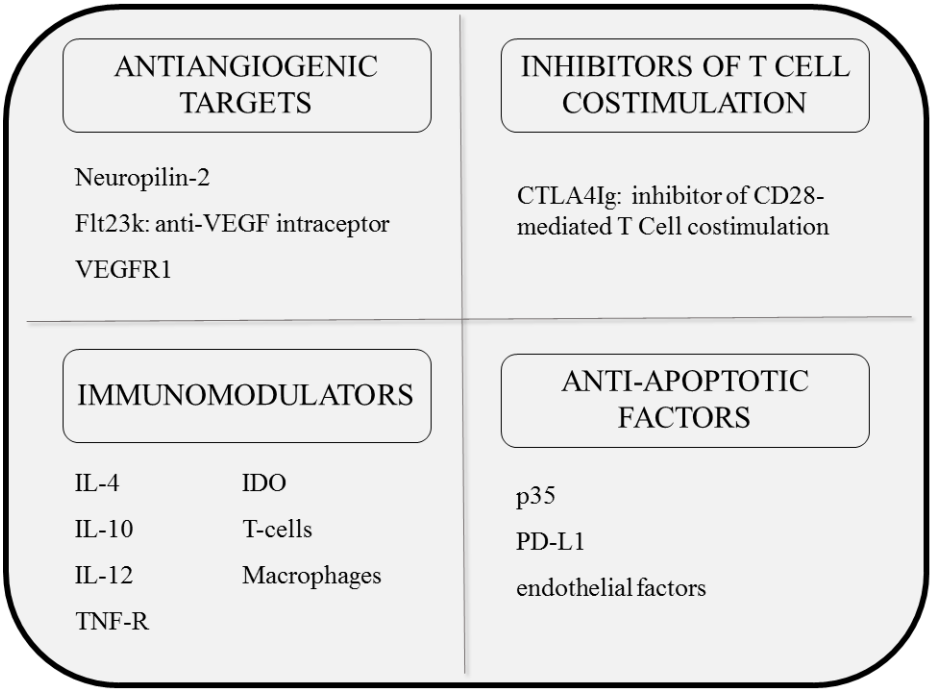

Figure XIV. Targets to modulate by gene therapy, anti- and proinflammatory cytokines that mediate graft rejection. VEGF: vascular endothelial growth factor; VEGFR1: VEGF receptor 1; CTLA cytotoxic T-lymphocyte antigen; IL: interleukin; TNF-R: tumor necrosis factor; IDO: indoleamine dioxygenase; PD-L1: programmed death-ligand 1.

The immunomodulatory effects of adenovirus-mediated gene transfer of a Th1 antagonist, IL-12p40 were evaluated *in vitro*, and on an allogeneic graft survival in a rat experimental

394 Gong N, Pleyer U, Volk H-D, Ritter T. Effects of local and systemic viral interleukin-10 gene transfer on corneal allograft survival. Gene Ther. 2007; 14, 484–490. https://doi.org/10.1038/sj.gt.3302884.

395 Klebe S, Sykes P, Coster D, Krishnan R, Williams K. Prolongation of sheep corneal allograft survival by ex vivo transfer of the gene encoding interleukin-10. Transplantation. 2001; 71, 1214–1220. https://doi.org/10.1097/00007890-200105150-00006.

396 Parker DG, Coster DJ, Brereton HM, Hart PH, Koldej R, Anson DS, Williams KA. Lentivirus-mediated gene transfer of interleukin 10 to the ovine and human cornea. Clin. Exp. Ophthalmol. 2010; 38, 405–413. https://doi.org/10.1111/j.1442-9071.2010.02261.

397 Klebe S, Coster DJ, Sykes PJ, Swinburne S, Hallsworth P, Scheerlinck J-PY et al. Prolongation of sheep corneal allograft survival by transfer of the gene encoding ovine IL-12-p40 but not IL-4 to donor corneal endothelium. J. Immunol. 2005; 175, 2219–26. https://doi.org/175/4/2219.

keratoplasty model. Adenovirus-mediated gene transfer in cultured corneas led to significant IL-12p40 protein expression that inhibited the production of IFN-γ by alloreactive naive T cells. However, no prolongation of allogeneic graft survival of both adenovirus-IL-12p40 modified rat corneas and systemically treated rats was obtained after transplantation[398]. IL-10 gene transfer has also been evaluated as a tool to prolong limbal allograft survival in a rat model. Both adenoviral and lentiviral vectors were able to transfect limbal graft tissue *ex vivo* with biological active IL-10, leading to delayed rejection compared to the control groups[399].

398 Ritter T, Yang J, Dannowski H, Vogt K, Volk HD, Pleyer U. Effects of interleukin-12p40 gene transfer on rat corneal allograft survival. Transpl. Immunol. 2007; 18, 101–107. https://doi.org/10.1016/j.trim.2007.05.004.

399 Kaufmann C, Mortimer LA, Brereton HM, Irani YD, Parker DGA, Anson DS et al. Interleukin-10 gene transfer in rat limbal transplantation. Curr. Eye Res. 2017; 42 (11), 1426–1434. https://doi.org/10.1080/02713683.2017.1344714.

OBJECTIVES

Chronic inflammation of the cornea induces visual disturbance, and often results in tissue destruction that leads to corneal ulceration, scarring and, even, perforation, causing visual impairment and blindness. Current treatment options for corneal inflammation are restricted by limited efficacy, adverse effects, short duration of action and do not tackle properly some of the injury related risks, highlighting the need of new therapeutic strategies. In this sense, interleukin-10 (IL-10) is a potent anti-inflammatory cytokine with an essential role in the immune response associated to ocular surface pathologies; however, its therapeutic use is limited due to biopharmaceutical issues, mainly low ocular bioavailability, caused by the corneal barrier and short half-life.

Gene therapy has shown great potential for the treatment of diseases affecting the ocular surface, and non-viral gene therapy has proven to be a feasible alternative even though there is still room for improvement. In this sense, gene supplementation to overexpress IL-10 in the cornea is a promising strategy to treat chronic inflammation.

Accordingly, the main objective of this thesis was to develop a gene therapy system based on solid lipid nanoparticles (SLNs) for topical instillation to address corneal inflammation by *de novo* IL-10 production. In order to achieve this aim, the following steps were carried out:

1. Design and optimization of highly versatile multi-component nanovectors based on various types of SLNs combined with ligands of different nature, including protamine and polysaccharides. SLNs were prepared either by emulsification/solvent evaporation method or by coacervation technique.

2. Physicochemical characterization of the SLN-based vectors, including particle size, surface charge, morphology, and ability to bind, protect and release the genetic material.

3. *In vitro* evaluation of the SLN-based vectors in cultured human corneal epithelial cells (HCE-2) in terms of cellular uptake, intracellular disposition, transfection efficacy, IL-10 production, and cell viability.

4. *Ex vivo* evaluation in explanted corneas of rabbits, including uptake of the SLN-based vectors and transfection efficacy.

5. Formulation of the SLN-based vectors as eye drops and assessment of the influence of the viscosity modifier polyvinyl alcohol (PVA) on the technological and rheological properties of the formulation.

6. *In vivo* administration of the formulations to wild type and IL-10 deficient Knock Out mice by topical instillation on the ocular surface, in order to evaluate the corneal localization of the vectors, the transfection capacity, the ability to produce IL-10 and the distribution of this therapeutic cytokine in corneal tissues.

EXPERIMENTAL RESEARCH

CHAPTER 1:

Gene delivery in the cornea: *in vitro* & *ex vivo* evaluation of solid lipid nanoparticle-based vectors

The content of this chapter has been published in Nanomedicine (Lond); Journal Impact Factor JCR 2018: 4.717 (Q1):

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1.1. Introduction

Inflammation is the underlying process in severe ocular surface diseases, such as dry eye syndrome, allergic diseases and contact lens-related injuries as well as bacterial and viral infections[1]. The chronic inflammation associated with these conditions alters the corneal epithelial barrier[2], and results in vision loss and an impairment in life quality. The conventional therapy against ocular inflammation is the systemic administration or topical instillation of corticosteroids[3]. However, cataracts and increased intraocular pressure are adverse effects that are frequently caused by the long-term use of corticosteroids. Therefore, the development of new therapeutic strategies for the treatment of corneal inflammation becomes necessary.

One possible approach is the administration of IL-10. This soluble and multifunctional cytokine, which is produced by several types of cells, displays both anti-inflammatory and immunosuppressive effects[4]. Several studies have confirmed the essential role that IL-10 plays in the regulation of bowel inflammation, chronic infections and neuroimmune diseases [5,6], in the avoidance of allograft rejections[7,8], and in the immune response associated with ocular surface pathologies. Specifically in terms of ocular diseases, treatment with IL-10 in animal models has proven to be successful in promoting corneal transplant survival[9], and modulating herpes

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simplex virus-induced stromal keratitis (HSK)[10], which is a significant infectious cause of blindness in developed nations. The blinding illness in ocular herpes simplex virus infections is not the result of viral replication, but rather of the subsequent host immunologic response to the virus. It is here that the properties of IL-10 have been found to play a protective role in mice HSK models. However, the low bioavailability of this protein after topical administration, which is caused by the corneal barrier, and its short half-life, hamper the anti-inflammatory effect, even after frequent topical administration at high doses. Gene supplementation, in which the plasmids that encode for IL-10 (p-IL10) are administered in the cornea, is an alternative that may overcome these drawbacks. An important benefit of this therapy is the sustained synthesis of the protein *de novo* in corneal cells, which provides a long-term anti-inflammatory effect.

The well-known need for suitable delivery systems that can facilitate successful gene therapy must be considered. Ideally, a system for corneal gene delivery must meet certain requirements: manufacture at high concentration and purity using simple and reproducible procedures, corneal cell targeting to provide high therapeutic gene levels in a tissue-selective manner, and a lack of local toxicity, immunological reaction and injury to the extracellular matrix and surrounding tissues[11]. In this sense, cationic solid lipid nanoparticles (SLNs) present several advantages for corneal gene therapy. SLNs consist of a solid lipid core surrounded by a layer of surfactants in an aqueous dispersion and are usually composed of well-tolerated physiological lipids, that have been approved for pharmaceutical preparations for human use[12]. Furthermore, a variety of production methods, which have been successfully implemented in the pharmaceutical and cosmetic industries have been developed to manufacture SLNs and have furnished stable delivery systems that can undergo long-term storage[13]. Regarding delivery to the cornea, their nanometre-range dimensions and lipophilic properties mean that SLNs can enhance corneal penetration and the cellular uptake of active molecules, extend ocular

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13 Sengel-Turk CT, Gumustas M, Uslu B, Ozkan SA. Nanosized drug carriers for oral delivery of anticancer compounds and the importance of the chromatographic techniques. In: Nano- and Microscale Drug Delivery Systems. Grumezescu AM (Ed.), Elsevier, Amsterdam, The Netherlands, 2017; 165-195.

retention time and provide a controlled release profile, improving ocular bioavailability[14,15,16,17]. Concerning their use as gene delivery systems, SLNs have been documented to be one of the most effective lipid-based non-viral vectors, both *in vitro* and *in vivo*[18,19,20,21]. Moreover, these nanoparticles may be functionalized with a number of ligands to overcome barriers for gene transfer, such as, interaction with targeted cells, cellular uptake, appropriate intracellular distribution and entry to the nucleus. Chitosan, dextran and hyaluronic acid[22,23,24], which have

14 Seyfoddin A, Al-Kassas R. Development of solid lipid nanoparticles and nanostructured lipid carriers for improving ocular delivery of acyclovir. Drug Dev. Ind. Pharm. 2013; 39(4):508-519. https://doi.org/10.3109/03639045.2012.665460.

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21 Apaolaza PS, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez JM, Friedrich U, Torrecilla J et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. Biomaterials. 2016; 90:40-49. https://doi.org/10.1016/j.biomaterials.2016.03.004.

22 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Bartkowiak A, Rodríguez-Gascón A. New gene delivery system based on oligochitosan and solid lipid nanoparticles: 'in vitro' and 'in vivo' evaluation. Eur. J. Pharm. Sci. 2013; 50(3-4):484-491. https://doi.org/10.1016/j.ejps.2013.08.013.

23 Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Rodríguez-Gascón AR, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. Int. J. Pharm. 2014; 465(1-2):413-426. https://doi.org/10.1016/j.ijpharm.2014.02.038.

24 Apaolaza PS, del Pozo-Rodríguez A, Torrecilla J, Rodríguez-Gascón A, Rodríguez JM, Friedrich U et al. Solid lipid nanoparticle-based vectors intended for the treatment of X-linked juvenile retinoschisis by gene therapy: in vivo approaches in Rs1h-deficient mouse model. J. Control. Rel. 2015; 217:273-283. https://doi.org/10.1016/j.jconrel.2015.09.033.

received recognition for their biocompatibility, biodegradation and mucoadhesive properties, are such ligands and are commonly used in the design of ocular drug-delivery systems[25,26].

The aim of this study is the development of p-IL10 delivery systems for transfection in the cornea, which may be useful for the topical therapeutic management of corneal inflammation-related diseases. After the physicochemical characterization of the SLN-based vectors, their efficacy and intracellular behaviour in human corneal epithelial cells was studied, and their capacity to transfect corneal tissues was evaluated *ex vivo* in corneas that had been explanted from rabbits.

1.2. Materials and methods

1.2.1. Materials

1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) was obtained from Avanti Polar-lipids, Inc. (AL, USA), Tween 80 and dichloromethane from Panreac (Madrid, Spain), sodium behenate from Nu-Chek Prep (Eleysian, AL, USA) and Precirol® ATO5 was generously provided by Gattefossé (Madrid, Spain). Protamine sulfate salt Grade X (P), dextran (Mw of 3.26 KDa) (DX), ammonium chloride, glycol chitosan (CH), partially hydrolyzed polyvinyl alcohol 9000-10000 Da Mw (PVA9000) and Nile Red were acquired from Sigma-Aldrich (Madrid, Spain). Hyaluronic acid (Mw of 100 KDa) (HA) was purchased from Lifecore Biomedical (MN, USA), and Bemiparin was a kind gift from Rovi® (Madrid, Spain). The plasmid pcDNA3-EGFP (6.1 kb), that encodes GFP, was kindly provided by the laboratory of Professor BHF Weber (University of Regensburg, Germany) and pUNO1-hIL10 (3.7 kb), which encodes human IL-10, was provided by InvivoGen (CA, USA). The promoter in this second plasmid (hEF1/human T-cell leukemia virus [HTLV]) comprises the EF-1α core promoter coupled to the R segment and the U5 sequence (R-U5') of the HTLV Type 1. According to the manufacturer, this combination increases the steady state transcription and significantly increases translation efficiency.

Deoxyribonuclease I (DNase I) and sodium dodecyl sulphate (SDS) were obtained from Sigma-Aldrich, GelRed™ from Biotium (CA, USA) and the materials used in electrophoresis on agarose gel were purchased from Bio-Rad (Madrid, Spain).

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25 Chaiyasan W, Srinivas SP, Tiyaboonchai W. Crosslinked chitosan-dextran sulfate nanoparticle for improved topical ocular drug delivery. Mol. Vis. 2015; 21:1224-1234.

26 Widjaja LK, Bora M, Chan PN, Lipik V, Wong TT, Venkatraman SS. Hyaluronic acid-based nanocomposite hydrogels for ocular drug delivery applications. J. Biomed. Mater. Res. A. 2014; 102(9):3056-3065. https://doi.org/10.1002/jbm.a.34976.

Cell culture reagents, including Dulbecco´s Modified Eagle´s Medium/Nutrient Mixture F-12 with GlutaMAX™ (DMEM/F-12 with GlutaMAX™), fetal bovine serum and penicillin-streptomicin, were acquired from Life Technologies (ThermoFisher Scientific, Madrid, Spain). Human insulin solution was obtained from Sigma-Aldrich, EGF from Myltenyi Biotec, and Trypsin-EDTA from Lonza.

Triton X-100 and DNA from salmon sperm were provided by Sigma-Aldrich, reporter lysis buffer by Promega Biotech Ibérica (Madrid, Spain), and 4',6-diamidine-2'-phenylindole dihydrochloride(DAPI)-fluoromount-G by Southern Biotech (AL, USA). Paraformaldehyde (PFA) was obtained from Panreac, while phosphate buffered saline (PBS) and HEPES buffer were purchased from Gibco (ThermoFisher Scientific, Madrid, Spain). Transfectin® Lipid-Reagent was acquired from Bio-Rad, while ELISA for IL-10 with the DuoSet Ancillary reagent kit was purchased from R & D Systems.

The Tissue-Tek® O.C.T™ compound was obtained from Sakura Finetek Europe (Leiden, The Netherlands). Other chemicals, unless specified, were reagent grade from Sigma Aldrich (Madrid, Spain) and Panreac (Barcelona, Spain).

1.2.2. Preparation of SLNs and vectors

SLNs were prepared using two different techniques: emulsification/solvent evaporation (SLN_1), which has previously been described[27], and coacervation (SLN_2), which was partially modified from Chirio et al.[28] and Clemente et al.[29]; the precipitation of SLN_2 was obtained using 5 M ammonium chloride and 1 M hydrochloric acid, instead of 1 M sodium phosphate and 1 M hydrochloric acid.

SLN_1 were made up of a core of the solid lipid Precirol ATO5 and a cationic lipidic surface based on DOTAP and the surfactant Tween 80. In order to prepare SLN_1-based vectors, the plasmid (pcDNA3-EGFP or pUNO1-hIL10) was first mixed with an aqueous solution of protamine (P) and then with an aqueous solution of either the polysaccharide DX, bemiparin (BE) or HA. The complexes obtained were added to the SLN_1 suspension, and the electrostatic interactions led

27 Apaolaza PS, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez JM, Friedrich U, Torrecilla J et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. Biomaterials. 2016; 90:40-49. https://doi.org/10.1016/j.biomaterials.2016.03.004.

28 Chirio D, Gallarate M, Peira E, Battaglia L, Muntoni E, Riganti C et al. Positive-charged solid lipid nanoparticles as paclitaxel drug delivery system in glioblastoma treatment. Eur. J. Pharm. Biopharm. 2014; 88(3):746-758. https://doi.org/10.1016/j.ejpb.2014.10.017.

29 Clemente N, Ferrara B, Gigliotti CL, Boggio E, Capucchio MT, Biasibetti E et al. Solid lipid nanoparticles carrying temozolomide for melanoma treatment. Preliminary in vitro and in vivo studies. Int. J. Mol. Sci. 2018; 19(2):pii:E255. https://doi.org/10.3390/ijms19020255.

to the binding of the complex by the SLNs, and to the formation of the final vector. The weight ratios of the components are summarized in Table I.

SLN_2 were composed of behenic acid as the lipid matrix, were coated with PVA9000, as the suspending agent, and used CH as the cationizing agent. In order to prepare SLN_2-based vectors, the plasmid was first complexed with P, and then with the SLN_2. Vectors with different P:DNA:SLN_2 ratios were prepared (Table I).

Table I. Weight ratios of the vectors prepared and evaluated.

Name of the vector	Polysaccharide	Weight ratio
DX-SLN_1	DX	DX:P:DNA:SLN_1 1:2:1:5
BE2-SLN_1	BE	BE:P:DNA:SLN_1 0.1:2:1:5
BE3-SLN_1	BE	BE:P:DNA:SLN_1 0.1:3:1:5
HA-SLN_1	HA	HA:P:DNA:SLN_1 0.5:2:1:2
DNA-SLN_2	-	P:DNA:SLN_2 0:1:20
P2-SLN_2	-	P:DNA:SLN_2 2:1:5
P4-SLN_2	-	P:DNA:SLN_2 4:1:10

BE: Bemiparin; DX: Dextran; HA: Hyaluronic acid; P: Protamine; SLN: Solid lipid nanoparticle.

1.2.3. Size and zeta potential measurements

A Zetasizer Nano series-Nano ZS (Malvern Instruments, Worcestershire, UK) was used to measure size, polydispersity index (PDI) and superficial charge of SLNs and the final vectors. The samples were diluted in Milli-Q™ water (EDM Millipore, MA, USA) for the particle size and zeta potential measurements, which were carried out using photon correlation spectroscopy and laser doppler velocimetry, respectively.

1.2.4. Transmission electronic microscopy images

Transmission electronic microscopy (TEM) images of the SLN_1 and SLN_1-based vectors were already present in the literature[30]. Visualization of SLN_2 and the SLN_2-based vectors was performed using electron microscopy negative staining. For that purpose, 10 µl of the sample was adhered onto glow discharged carbon coated grids for 60 s. After removing the remaining liquid, via blotting on filter paper, the staining was carried out with 2% uranyl acetate for 60 s. Samples were visualized using a Philips EM208S TEM and digital images were acquired on an Olympus SIS purple digital camera. Technical and human support for TEM was provided by the

30 Apaolaza PS, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez JM, Friedrich U, Torrecilla J et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. Biomaterials. 2016; 90:40-49. https://doi.org/10.1016/j.biomaterials.2016.03.004.

General Service (SGIker) of Analytical Microscopy and High Resolution in Biomedicine at the University of the Basque Country UPV/EHU.

1.2.5. Agarose gel electrophoresis assay

With the aim of studying the DNA binding efficiency of the vectors, the protection from DNase I digestion and the SDS-induced release of DNA, 0.7% agarose gel electrophoresis containing Gel Red™ was used and analysed using an Uvitec Uvidoc D-55-LCD-20 M Auto transilluminator, as previously described[31]. The capacity of the vectors to bind electrostatically the DNA was evaluated by adding the complexes at a final concentration of 0.03 µg DNA/µl diluted in MilliQ™ water in the gel. For DNAse I protection, the same concentration was exposed to 1 U DNase I/2.5 µg DNA and then incubated at 37ºC for 30 min. A SDS solution (4%) was mixed with the samples, to a final concentration of 1%, to release DNA from the SLNs. The pcDNA3-EGFP and pUNO1-hIL10 plasmids were added, untreated, as controls, as well as the 1 kb DNA ladder from NIPPON Genetics Europe (Dueren, Germany).

1.2.6. In vitro studies

The human corneal epithelium (HCE-2) cell line was used for *in vitro* assays. HCE-2 cells were maintained in medium, which was composed of DMEM/F-12 with GlutaMAX™, fetal bovine serum (15%), insulin (4 mg/ml), EGF (10 ng/ml) and penicillin-streptomycin (1%), incubated at 37ºC with 5% CO_2 and subcultured every 7 days.

1.2.6.1. *In vitro transfection*

Cells were seeded on 24-well plates at a density of 150,000 cells using 1 ml of medium per well, and then allowed to adhere for 24 h. A total of 75 µl of each vector (2.5 µg DNA) was then added to each well, $_{co}$ntaining 0.5 ml of medium, and the plates were incubated for 4 h at 37ºC in 5% CO_2. Thereafter, vectors were removed, cells were refreshed with 1 ml of complete medium, and the cell culture was allowed to grow for 72 h. Naked plasmids and the complexes without SLNs were also tested at the same dose of DNA. Transfection efficacy obtained with the vectors was compared with that obtained with the commercial transfectant TransFectin® Lipid-Reagent (Bio-Rad, Madrid, Spain), which was used according to the manufacturer's protocol.

1.2.6.2. *Quantification of GFP and cell viability*

A fluorometric assay was carried out to quantify intracellular GFP 72 h after treatment. Briefly, cells were washed with 300 µl of PBS, 400 µl of reporter lysis buffer 1x was then added, and the

31 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Bartkowiak A, Rodríguez-Gascón A. New gene delivery system based on oligochitosan and solid lipid nanoparticles: 'in vitro' and 'in vivo' evaluation. Eur. J. Pharm. Sci. 2013; 50(3-4):484-491. https://doi.org/10.1016/j.ejps.2013.08.013.

plate was frozen to complete the lysis of the cell culture. After thawing, each well was scrapped and the lysate was centrifuged at 12,000 g for 2 min at 4ºC. In order to measure the amount of GFP contained in 100 µl of the supernatant at 525 nm, a Glomax Multidetection System (Promega Biotech Iberica, Madrid, Spain) was employed, and GFP amount was expressed as relative fluorescent units (RFU). The mean value of the auto-fluorescence detected in the non-treated cells was subtracted from the fluorescence measured in each well, and it was expressed as RFU.

The percentage of transfected cells was measured using a FACSCalibur flow cytometer (Becton Dickinson Bioscienses, CA, USA). For this purpose, cells were washed with 0.5 ml of PBS 72 h after transfection and then detached using 0.5 ml of trypsin-EDTA and, after incubation for 10 min, centrifuged at 1000 rpm for 5 min. The supernatant was discarded and cells were resuspended in 0.5 ml of PBS. Ten thousand events were collected for each sample. Transfection efficacy was measured at 525 nm (FL1), and cell viability at 650 nm (FL3). Propidium iodide was employed for dead cell exclusion[32].

1.2.6.3. *Quantification of IL-10*

In order to measure the levels of IL-10 expressed by the cells 72 h after addition of the complexes, an ELISA kit was carried out. Secreted and intracellular IL-10 was quantified. In order to quantify intracellular IL-10, cells were detached from the wells and lysed, as described in section 2.7 for intracellular GFP quantification. For secreted IL-10, the medium of each well was retired and centrifuged. A total of 100 µl of each sample was added to a 96-well plate that was covered with the corresponding capture antibody, and the assay was then performed according to the manufacturer's instructions.

1.2.6.4. *Cellular uptake of the vectors*

In order to study the entrance of the complexes into HCE-2 cells, SLNs were labelled with the fluorescent dye Nile Red (λ = 590 nm), and the vectors were prepared as described in section 1.2.2. Vectors were added to each well, and after 2 h of incubation at 37ºC, the culture medium was retired and cells washed with PBS before being detached from the wells, as described in section 1.2.6.2. for the percentage of GFP transfected cells. Vector entry into the cells was analysed using a FACSCalibur flow cytometer at 650 nm (FL3).

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32 Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Rodríguez-Gascón AR, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. Int. J. Pharm. 2014; 465(1-2):413-426. https://doi.org/10.1016/j.ijpharm.2014.02.038.

1.2.6.5. *Intracellular disposition of the vectors*

Cells were seeded and incubated at 37ºC and 5% CO_2 for 24 h in Millicell EZ slides (Millipore) at a density of 150,000 cells and 1 ml per well, and they were then treated with vectors containing the plasmid, which was labelled with ethidium monoazide. After 4, 12 and 24 h, the slides were washed with PBS and fixed with PFA 4%. DAPI-fluoromount-G™ was used as the mounting fluid, to label the nuclei. The slides were then studied using an inverted fluorescence microscopy (Nikon TMS).

1.2.7. Ex vivo studies

Ex vivo studies were performed on rabbit corneas. Eyes were enucleated, within 2 h after animal death, from 12-week albino rabbits killed in a slaughterhouse for food purposes. Corneas were excised and kept in sterile Steinhardt medium[33], according to a protocol currently used for human cornea transplantation. A scleral ring of nearly 4 mm was maintained around the explanted corneas. The internalization of Nile Red-labelled vectors and the transfection efficacy, after treating the corneas with vectors bearing pcDNA3-EGFP, were studied using a previously-documented corneal holder[34]; it is a Plexiglas and glass structure, with donor and receiving compartments (0.65 ml volume). The cornea was placed in the orifice (0.50 cm^2) that divides the two compartments. To minimize the corneal irritation the o-ring holds only the scleral ring around the corneal circumference. A total of 200 µl of SLNs under study were diluted to 600 µl with PBS, vortexed for 5 s, sonicated for 30 s, and then introduced into the donor compartment of the corneal chamber (epithelial side), while the receiving chamber was filled with PBS. In this preliminary study, the effect of tear washing was not considered, being the chamber a static system. Thus, the administered volume was constrained by chamber size and cannot resemble the real volume of tear flow. However, it was considered that an eye drop has a volume of nearly 25-56 µl, in front of a tear flow of about 1 µl/min, and the dilution of SLNs in PBS before administration was designed in order to resemble the ratio between administered nanosuspension and tear volume during the 2 h experiment.

1.2.7.1. *Cell internalization of the vectors*

The corneas were kept at 37ºC in the chamber for 2 h, then removed, rinsed with normal saline buffer and observed using fluorescence microscopy on a DMI4000B fluorescence microscope

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33 Steinhardt RA. US7087369 B2 (2006). http://patft.uspto.gov/netacgi/nph-Parser?Sect1=PTO1&Sect2=HITOFF&d=PALL&p=1&u=%2Fnetahtml%2FPTO%2Fsrchnum.htm&r=1&f=G&l=50&s1=7,087,369.PN.&OS=PN/7,087,369&RS=PN/7,087,369.

34 Battaglia L, D'Addino I, Peira E, Trotta M, Gallarate M. Solid lipid nanoparticles prepared by coacervation method as vehicles for ocular cyclosporine. J. Drug Del. Sci. Technol. 2012; 22(2):125-130. https://doi.org/10.1016/S1773-2247(12)50016-X.

(Leica Microsystems, Barcelona, Spain). The corneas were subsequently still kept in vials with Steinhardt medium at 37ºC for 24 h, prior to further fluorescence microscopy observation. The staining of cell nuclei was performed by incubating the corneas in 1 µM DAPI in PBS for 30 min endothelial side up, prior to observation. Images were acquired and merged using the Leica Application Suite V3 software.

1.2.7.2. *Transfection studies*

The corneas were kept at 37ºC in the chamber for 2 h, then removed from the chamber, rinsed with normal saline and kept in Steinhardt medium at 37ºC for 48 h, allowing GFP protein expression to occur. After incubation, the corneas were fixed with 4% PFA for 30 min and then transferred into a scintillation vial containing 3 ml of PBS 1x. After 5 min, the PBS was replaced with 3 ml of a 30% sucrose in PBS 1x solution and the sample was incubated overnight at 4ºC. After the incubation period, the volume was replaced with 3 ml of Tissue-Tek OCT (Optimum cutting temperature formulation). The cornea was then quick-frozen in liquid nitrogen, and later sectioned (14 µm) on a cryostat (Cryocut 3000, Leica).

GFP detection was performed by immunofluorescence. Sections were fixed with 4% paraformaldehyde for 10 min at room temperature. Next, they were washed in PBS, blocked and permeabilized in PBS 0.1 M, 0.1% Triton X-100 and 2% normal goat serum for 1 h at room temperature. Subsequently, samples were incubated in primary antibody (polyclonal anti-GFP, IgG fraction) for 2 h at room temperature, then washed again in PBS, and incubated in secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG). Lastly, sections were washed in PBS and mounted with Fluoromount G. The fluorescence in each sample was analysed using inverted fluorescence microscopy (Nikon TMS).

Sections of the cornea were also analysed using Masson´s trichrome staining technique. All samples were histologically evaluated and examined with an optical microscope (Olympus BX50).

1.2.8. Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics 23 (IBM, NY, USA). The normal distribution of the samples was assessed using the Shapiro-Wilk test, and homogeneity of variance, using the Levene test. Comparisons were performed using either an ANOVA or Student's t-test. Differences were considered statistically significant at $p < 0.05$. Results are expressed as mean ± standard deviation.

1.3. Results

1.3.1. Size, polydispersity index and zeta potential

Table II summarizes the particle size, PDI and zeta potential of plain SLNs. As can be seen, SLN_1 had a slightly smaller size than SLN_2 (257 vs 341 nm), while the PDIs were similar and under 0.35 in both cases. Zeta potential was positive, but lower for SLN_2 (+21 vs +42 mV).

Table II. Physicochemical characterization of SLN_1 and SLN_2.

SLNs	Size (nm)	PDI	Zeta potential (mV)
SLN_1	257.7 ± 6.3	0.32 ± 0.04	+41.8 ± 1.2
SLN_2	341.0 ± 0.9*	0.33 ± 0.00	+21.0 ± 0.8*

** p<0.05 with respect to SLN_1. n = 3; data are expressed as mean ± standard deviation. PDI: Polydispersity index; SLN: Solid lipid nanoparticle.*

The size of the SLN_1-based vectors bearing the plasmid pcDNA3-EGFP (Table III) ranged from 143.2 (BE3-SLN_1) to 218.9 nm (HA-SLN_1), while they displayed PDI values that were always lower than 0.35. The surface charge varied from +28.1, in the case of HA-SLN_1, to +39 mV.

Table III. Physicochemical characterization of SLN_1-based vectors bearing the plasmid pcDNA3-EGFP.

Vectors with pcDNA3-EGFP	Size (nm)	PDI	Zeta potential (mV)
DX-SLN_1	199.8 ± 0.4	0.26 ± 0.01	+38.4 ± 1.6
BE2-SLN_1	198.2 ± 1.6	0.29 ± 0.04	+38.9 ± 2.6
BE3-SLN_1	143.2 ± 7.2*	0.31 ± 0.05	+38.6 ± 1.1
HA-SLN_1	218.9 ± 1.2*	0.32 ± 0.04	+28.1 ± 1.6*

** p<0.05 with respect to the other formulations. n = 3; data are expressed as mean ± standard deviation. BE: Bemiparin; DX: Dextran; HA: Hyaluronic acid; PDI: Polydispersity index; SLN: Solid lipid nanoparticle.*

Regarding SLN_2-based vectors (Table IV), three formulations were characterized. P4-SLN_2 was the smallest in term of size (278 nm) and its zeta potential was the highest (+26 mV). The vector that was prepared without protamine (DNA-SLN_2) was slightly bigger (325 nm; $p < 0.05$) and its surface charge was lower (+7.5 mV; $p < 0.05$). By contrast, the formulation P2-SLN_2 was the largest (434 nm; $p < 0.05$) and its surface charge was the lowest ($p < 0.05$), at +4.2 mV. All the PDI measurements were under 0.3.

Table IV. Physicochemical characterization of SLN_2-based vectors bearing the plasmid pcDNA3-EGFP.

Vectors with pcDNA3-EGFP	Size (nm)	PDI	Zeta potential (mV)
DNA-SLN_2	325.7 ± 1.2*	0.24 ± 0.01	+7.5 ± 0.2*
P2-SLN_2	434.2 ± 25.4*	0.30 ± 0.02	+4.2 ± 0.2*
P4-SLN_2	278.0 ± 5.7*	0.29 ± 0.01	+26.2 ± 1.3*

** p<0.05 with respect to the other formulations. n = 3; data are expressed as mean ± standard deviation. PDI: Polydispersity index; SLN: Solid lipid nanoparticle.*

No changes (p > 0.05) in particle size, PDI or zeta potential were observed (data not shown) when SLNs were labelled with Nile Red.

1.3.2. TEM images

TEM photographs of the SLN_2 (Figure IA) and P4-SLN_2 vectors (Figure IB) show the spherical shape of the nanoparticles, as well as of the final vectors.

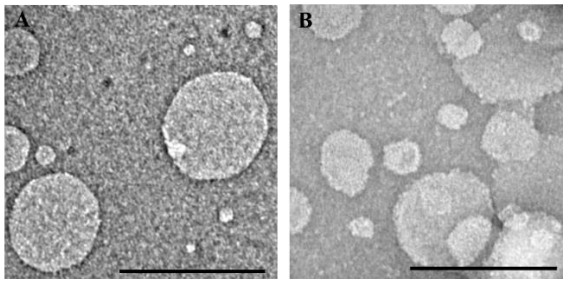

Figure I. Images of plain SLN_2 and P4-SLN_2 vectors acquired by TEM. (A) Photograph of SLN_2. (B) Photograph of P4-SLN_2 vectors. Scale bar: 200 nm. SLN: Solid lipid nanoparticle; TEM: Transmission electronic microscopy.

1.3.3. Binding, resistance to the DNase I and SDS-induced release of pcDNA3-EGFP from vectors

The capacity to bind, protect and release the plasmid pcDNA3-EGFP from BE2-SLN_1 and BE3-SLN_1 vectors (Figure IIA), as well as from SLN_2-based complexes (Figure IIB) was evaluated using agarose gel electrophoresis. DX-SLN_1 and HA-SLN_1 have shown to adequately bind, protect and release DNA in previous studies[35,36].

35 Torrecilla J, del Pozo-Rodríguez A, Solinís MÁ, Apaolaza PS, Berzal-Herranz B, Romero-López C et al. Silencing of hepatitis C virus replication by a non-viral vector based on solid lipid nanoparticles containing a shRNA targeted to the internal ribosome entry site (IRES). Colloids Surf. B Biointerfaces. 2016; 146:808-817. https://doi.org/10.1016/j.colsurfb.2016.07.026.

36 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Bartkowiak A, Rodríguez-Gascón A. New gene delivery system based on oligochitosan and solid lipid nanoparticles: 'in vitro' and 'in vivo' evaluation. Eur. J. Pharm. Sci. 2013; 50(3-4):484-491. https://doi.org/10.1016/j.ejps.2013.08.013.

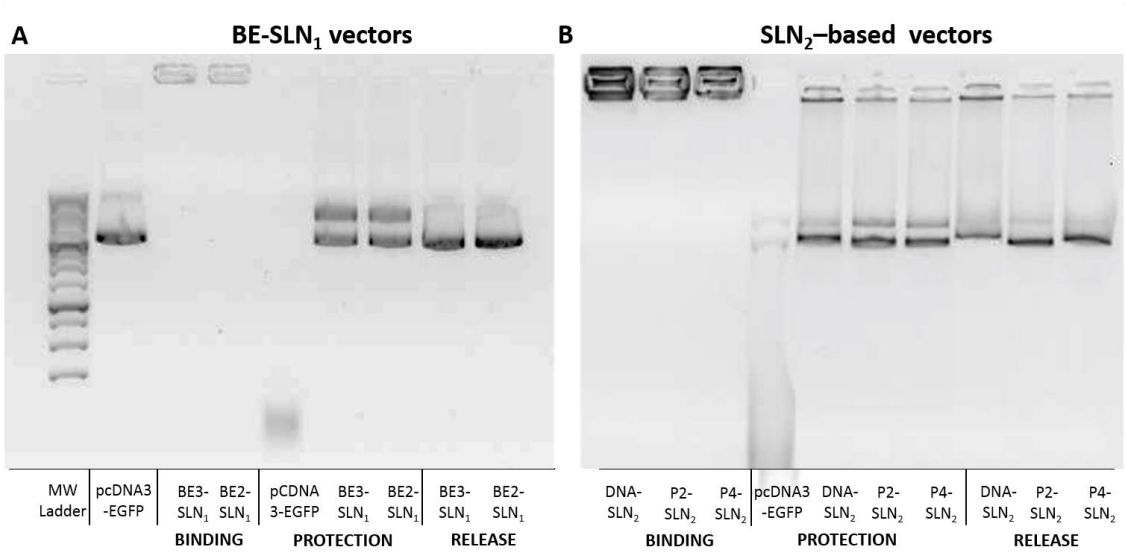

Figure II. Study of the binding, protection and release of vectors formed with pcDNA3-EGFP. (A) BE-SLN$_1$ vectors. (B) SLN$_2$-based vectors. MW ladder corresponds to the 1 kb DNA ladder from NIPPON Genetics Europe. BE: Bemiparin; MW: molecular weight; SLN: Solid lipid nanoparticle.

In the binding studies, the vectors were placed in the wells at a final concentration of 0.03 µg DNA/µl, and the absence of bands in the corresponding lanes demonstrated that DNA was fully bound to the vectors in all cases. The capacity of the vectors to release the plasmid was studied treating the vectors with SDS for 5 min, prior to placement in the wells. SDS is able to break the interaction between SLNs and DNA without disruption of the structure of lipid nanoparticles. Nevertheless, when the plasmid is too highly condensed by SLNs the release does not occur. After the SDS treatment of the SLN$_1$-based vectors, DNA was able to migrate from the loading wells, which demonstrates its ability to be completely released. However, the plasmid was partially detected in the loading wells that correspond to the SLN$_2$-based vectors (lanes 8-10), which indicates that complete release was not achieved.

In order to evaluate the protection capacity, before addition of the vectors to the gel, they were first incubated with DNase I for 30 min, and later SDS was added to the mixture. All the formulations were able to protect the plasmid, while free DNA was totally degraded (lane 5 in gel A and lane 4 in gel B). After treatment with DNase I, the plasmid released from the formulations showed two bands, while the control plasmid (lane 2 in Figure IIA) only showed one band. The lower band (which shows high intensity) corresponds to the supercoiled form and the upper band to the open circular form. The change detected in the bands indicates that DNase I turned the supercoiled form, which is the DNA topology with the most transfection capacity, into open circular by cutting one of the DNA double strands.

1.3.4. GFP transfection and cell viability in vitro

Transfection efficacy of pcDNA3-EGFP bearing vectors was determined 72 h after treatment with the vectors. The percentage of HCE-2 transfected cells and the amount of GFP, that was expressed as relative fluorescence units, were measured. Cell viability was also evaluated at that time. In the case of the SLN_2-based vectors, only the results of the vector P4-SLN_2 are represented, as the other vectors were not able to transfect. The transfection efficacy and cell viability of the BE2-SLN_1 and BE3-SLN_1 vectors were similar, and the former was used in the following studies. The naked plasmid and the complexes without SLNs were also added to the cells in the same conditions, resulting in no transfection.

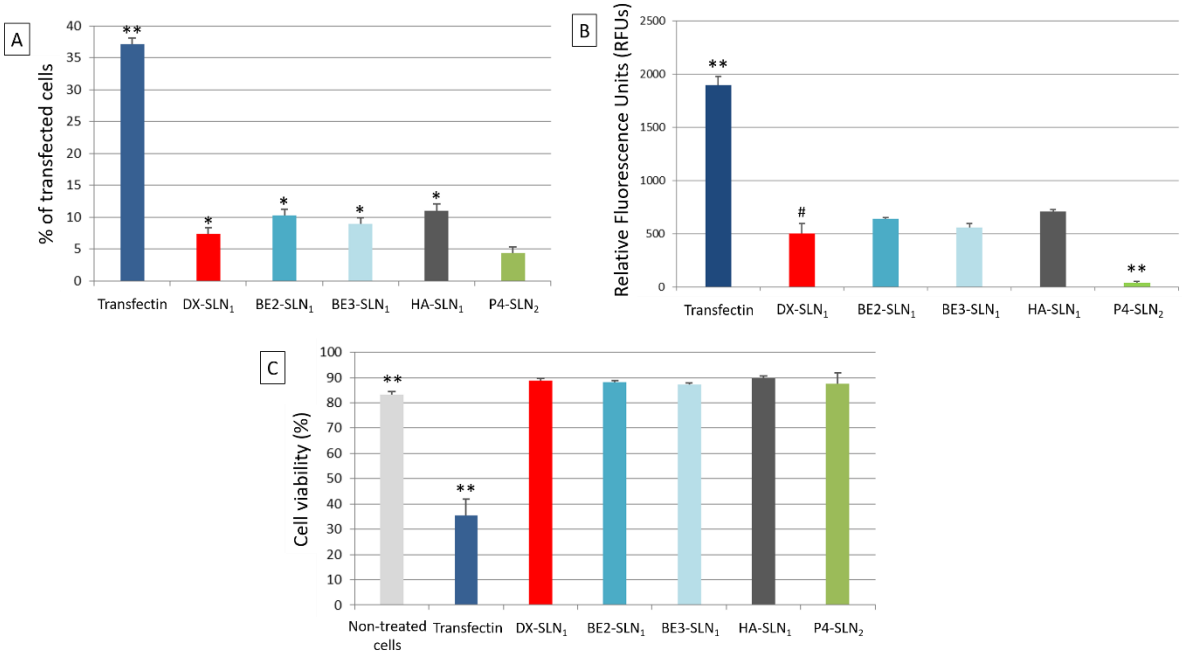

*Figure III. Transfection and cell viability 72 h after treatment of HCE-2 cells with the pcDNA3-EGFP vectors. (A) Percentage of transfected HCE-2 cells. (B) RFUs of transfected HCE-2 cells. (C) Cell viability. (n = 3; data are expressed as mean ± standard deviation). * p<0.05 with respect to P4-SLN_2; ** p<0.01 with respect to the other formulations; # p<0.05 with respect to BE2-SLN_1 and HA-SLN_1. BE: Bemiparin; DX: Dextran; HA: Hyaluronic acid; SLN: Solid lipid nanoparticle; HCE: Human corneal epithelium; RFU: Relative fluorescence unit.*

The SLN_2-based vectors were found to be less effective than the SLN_1-based vectors in terms of the percentage of transfected cells ($p < 0.05$) and relative fluorescence units ($p < 0.01$). While commercial TransFectin was found to be more effective than SLN-based vectors ($p < 0.01$), cell viability (Figure IIIC) decreased drastically (35% of viable cells). Cell viability, after the treatment of HCE-2 cells with the SLN-based vectors, was over 85%, and did not show differences compared with the non-treated cells and to the cells treated with the naked plasmid and the complexes without SLNs (data not shown).

1.3.5. Uptake of Nile Red-labelled SLNs in HCE-2 cells

Nile Red-labelled vectors were added to HCE-2 culture cells, and after 2 h of incubation cells were washed with PBS and detached from the wells. Vector entry into the cells was analysed using flow cytometry; the results are represented in the histograms in Figure IV. The displacement to the right of the histograms that correspond to the cells treated with the vectors, compared with the histogram that belongs to the non-treated cells (filled gray), indicates that Nile Red-labelled vectors entered all the cells in all cases. The variations in the displacements describe the different fluorescence intensities in the cells (expressed as the X mean in the graph). Those changes are related to the SLN to plasmid ratios; 2 to 1 in HA-SLN_1, which showed less intensity, 5 to 1 in DX-SLN_1 and BE-SLN_1, which were in the middle, and 10 to 1 in the P4-SLN_2 vectors, which gave the highest intensity.

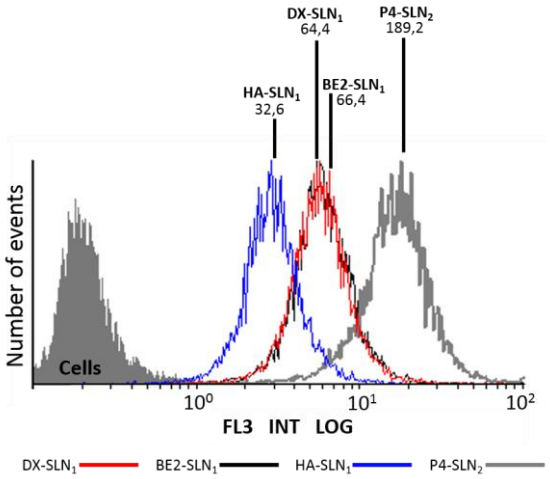

Figure IV. Flow cytometry analysis of cellular uptake of vectors using Nile Red-labelled SLNs in HCE-2 cells. The values indicated over the lines correspond to the X mean intensity of fluorescence. HCE: Human corneal epithelium; BE: Bemiparin; DX: Dextran; HA: Hyaluronic acid; SLN: Solid lipid nanoparticle.

1.3.6. Intracellular disposition

In order to study vector distribution into HCE-2 cells, DX-SLN_1, BE2-SLN_1, HA-SLN_1 and P4-SLN_2 vectors containing the plasmid labelled with ethidium monoazide (red fluorescence) were added to the culture cells, and at different times (4, 12 and 24 h), cells were washed, fixed and mounted with DAPI-fluoromount-G, to label nuclei in blue. Figure V shows the images acquired by inverted fluorescence, where differences in terms of intracellular DNA condensation and distribution were observed. When the plasmid is highly condensed, due to the electrostatic interactions with the components of the vectors, red fluorescence appears dotted (arrow heads). Over time, and due to the interaction of the vectors with intracellular components, plasmid decondensation occurs, which results in a more diffused fluorescence (asterisks).

In the case of the BE2-SLN_1 and DX-SLN_1 vectors, the plasmid appeared condensed 4 h after transfection, but at 12 and 24 h, the diffused red fluorescence indicates plasmid decondensation. The plasmid in the P4-SLN_2 vectors remained highly condensed even 24 h after the treatment of the cells, while DNA in the HA-SLN_1 vectors was seen to be poorly condensed in the cytoplasm from 4 h post-transfection.

Regarding the intracellular disposition, the plasmid appeared to be dispersed all over the cytoplasm and in the perinuclear area at 4 h, while, after 12 h, the plasmid was also located in the nucleus of some cells, which is necessary for gene expression. However, in the case of the P4-SLN_2 vectors, the plasmid was hardly detected in the nuclei.

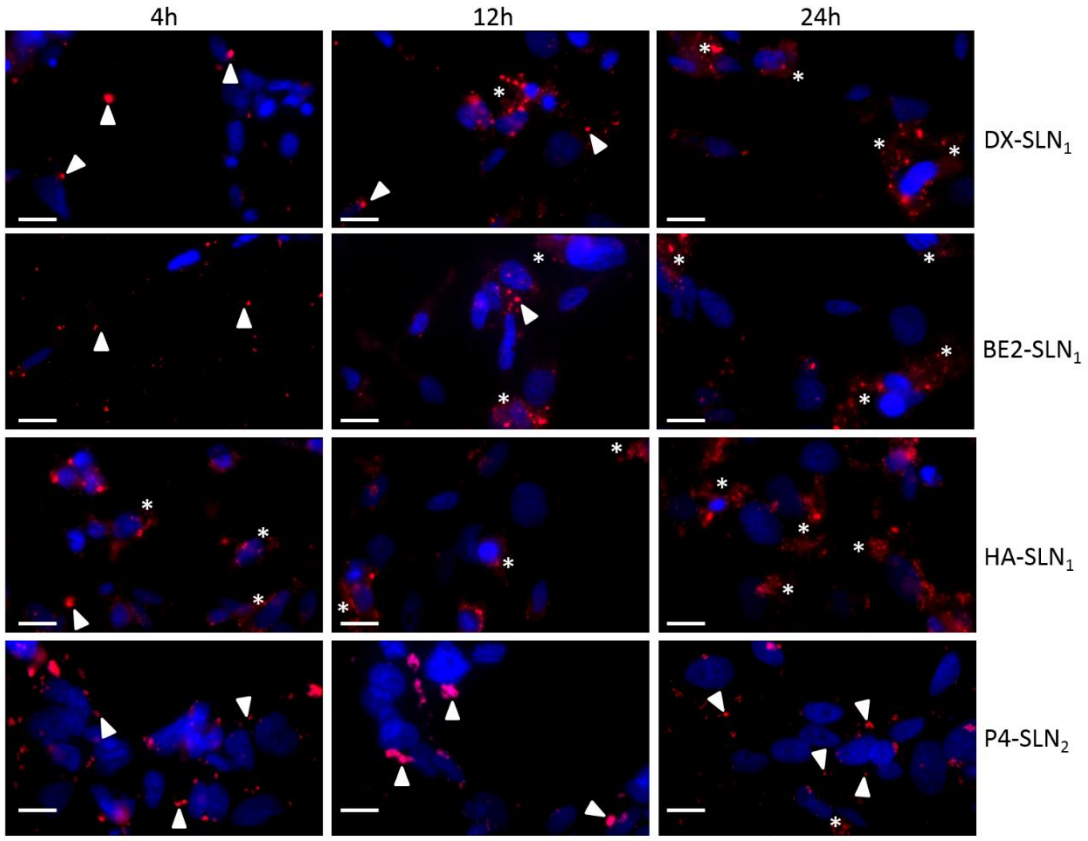

Figure V. Fluorescence microscopy images 4, 12 and 24 h after the addition of vectors containing the EMA-labelled plasmid (red) in HCE-2 cells. Nuclei were labelled with DAPI (blue). Arrow heads indicate areas where condensed plasmid was detected; asterisks indicate areas where decondensed plasmid was detected. Magnification 60x. Scale bar: 20 µm. BE: Bemiparin; DX: Dextran; HA: Hyaluronic acid; SLN: Solid lipid nanoparticle; DAPI: 4',6-diamidine-2'-phenylindole dihydrochloride; EMA: Ethidium monoazide; HCE: Human corneal epithelium.

1.3.7. Ex vivo studies

1.3.7.1. *Internalization in cornea explants*

Internalization in cornea tissue was qualitatively analysed using fluorescence microscopy with Nile Red-labelled SLNs. In optical microscopy, the endothelial layer can be observed clearly, after addition of alizarin red dye to the endothelial side, in order to increase the contrast of cell margins for observation in normal light mode. Since the dye can interfere in the fluorescence study, alternative nuclei staining was obtained with DAPI, added to the endothelial side, and only fluorescence images are provided. A total of 2 h after incubation with Nile Red-labelled vectors, red fluorescence was detected around the DAPI stained nuclei, while 24 h later it was colocalized with the DAPI-stained nuclei, thus resulting in violet merging of cells. Figure VI shows a representative distribution of Nile Red-labelled DX-SLN_1 vectors in cornea tissue. Images of untreated corneas were included as reference. Uptake of topically administered nanoparticles is mainly limited by the corneal barriers, among which the epithelial layer; thus, in this preliminary internalization study the demonstrated uptake in the underlying endothelial cells assesses the overcoming of corneal barriers.

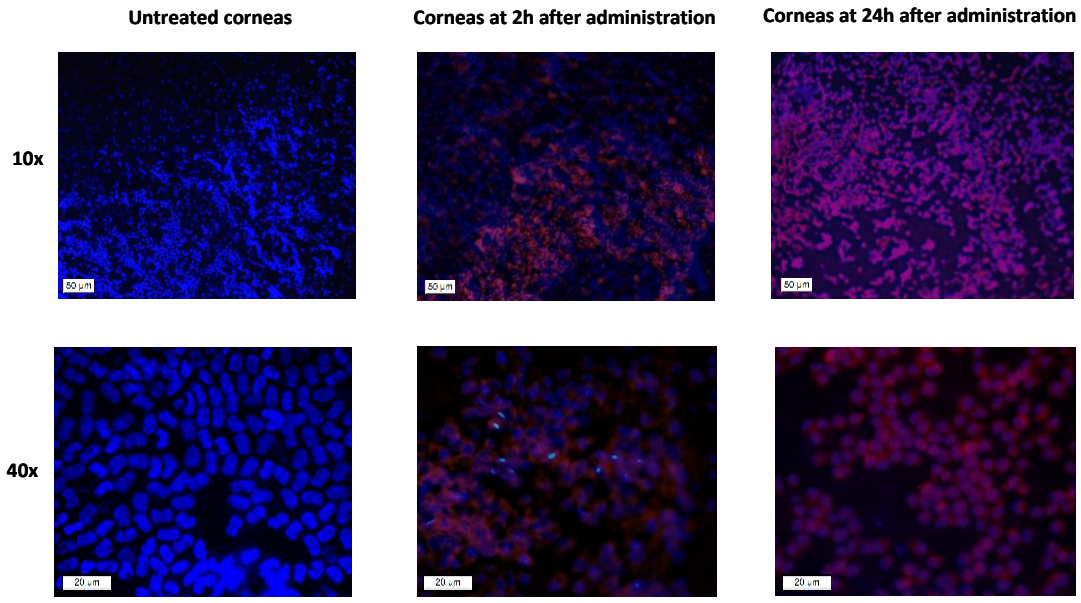

Figure VI. Fluorescence microscopy images (above: 10x; below: 40x) of Nile Red-labelled DX-SLN_1 internalization in cornea tissue. Left: untreated corneas; middle: 2 h after incubation; right: 2 h after incubation and 24 h in Steinhardt medium at 37°C. DX: Dextran; SLN: Solid lipid nanoparticle.

1.3.7.2. *Transfection in cornea explants*

Figure VII shows GFP detection by immunofluorescence, 48 h after treating cornea tissue with the vectors containing pcDNA3-EGFP plasmid. Three rabbit corneas were treated with each vector, and for protein detection, six cryosections from each eye were immunolabelled and observed using inverted fluorescence microscopy. As control, the immunofluorescence

procedure was also carried out in non-treated corneas, and no green fluorescence was detected. Cryosections were obtained from the periphery and from the center of the cornea. In all the sections obtained from the treated corneas GFP was detected. However, the distribution of transfected cells differed according to composition of the vectors. DX-SLN_1 induced GFP expression in the epithelium, stroma and endothelium, HA-SLN_1 in the epithelium and stroma, while the BE2-SLN_1 and P4-SLN_2 vectors were only able to transfect epithelial cells.

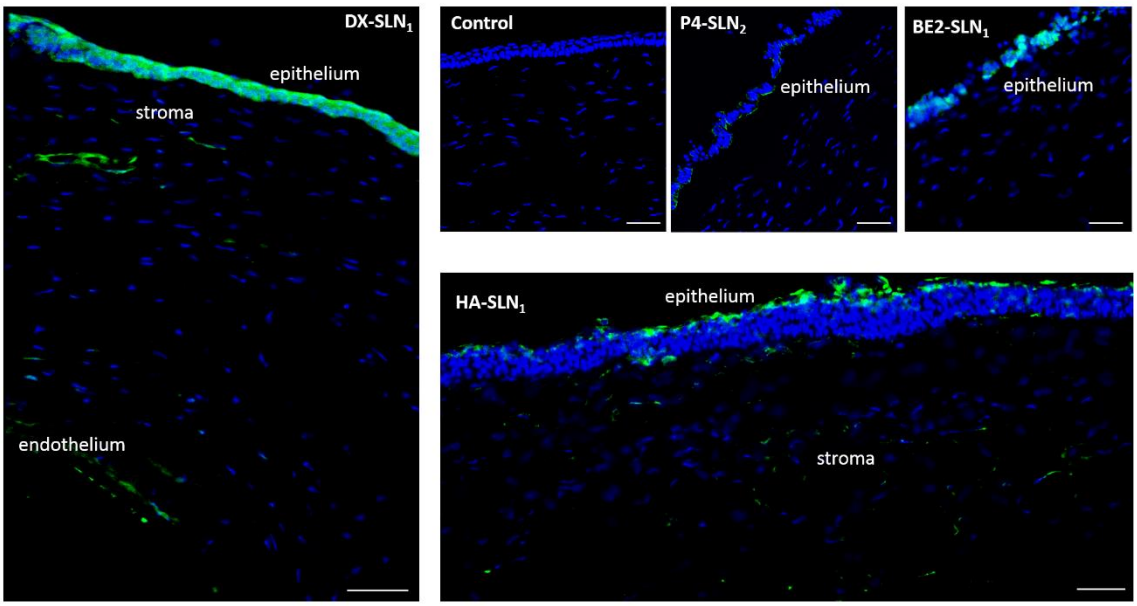

Figure VII. GFP transfection in explanted rabbit corneas 48 h after treatment with DX-SLN_1 (left), BE2-SLN_1 (top right), P4-SLN_2 (top middle) and HA-SLN_1 (below). As a control (top left), a non-treated cornea immunolabelled with primary and secondary antibodies has been included. Scale bar: 50 µm. BE: Bemiparin; DX: Dextran; GFP: Green fluorescent protein; HA: Hyaluronic acid; SLN: Solid lipid nanoparticle.

1.3.7.3. *Trichromic study*

Masson's trichrome staining was carried out in six histological sections from each eye, which were obtained from the center and from the periphery of each cornea. The microscopic images (Figure VIII) revealed that no difference was present in the structures of non-treated corneas (Figure VIIIA) and transfected tissues. Both treated and non-treated corneas were kept in Steinhardt medium at 37ºC for 48 h in order to compare the effect of the vectors and avoid any interference that may be caused by the cornea handling procedure. The images show that the non-viral SLN-based vectors did not alter the corneal structure, since the treated corneas (B, C, D, E) showed an architecture close to the non-treated rabbit corneas (A).

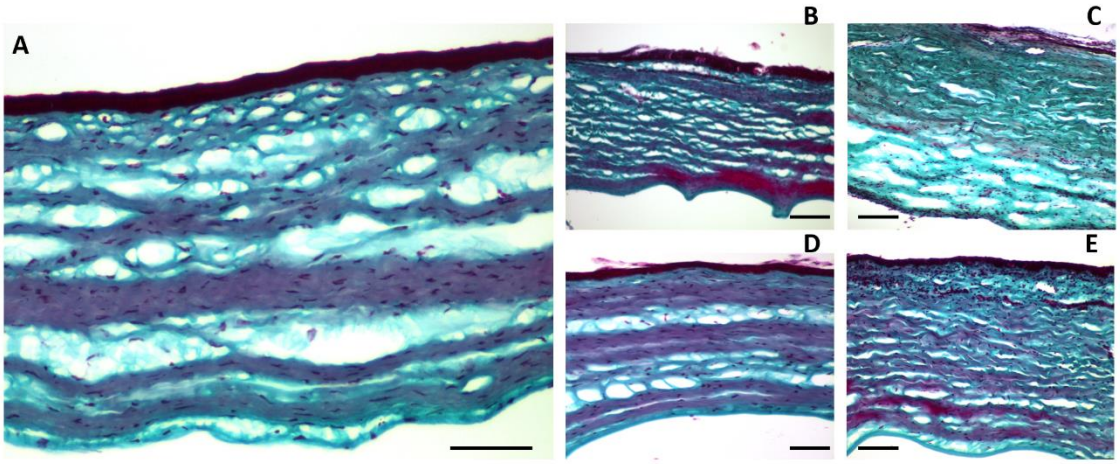

Figure VIII. Microscope image of cornea tissues stained using Masson´s trichrome technique. (A) Non-treated cornea; (B) cornea treated with DX-SLN$_1$; (C) cornea treated with BE2-SLN$_1$; (D) cornea treated with HA-SLN$_1$; (E) cornea treated with P4-SLN$_2$. Scale bar: 50 µm. BE: Bemiparin; DX: Dextran; HA: Hyaluronic acid; SLN: Solid lipid nanoparticle.

1.3.8. Studies with pUNO1-hIL10

1.3.8.1. Vector characterization

Differences in the plasmid size may affect the final features of the SLN-based vectors. Therefore, the delivery systems containing pUNO1-hIL10 were also characterized in terms of size, PDI and zeta potential (Table V). The vector P4-SLN$_2$ had a larger size (510.7 nm) than the SLN$_1$-based vectors. Surface charge was positive, at around +36 mV, in the case of BE2-SLN$_1$ and DX-SLN$_1$, and lower in the case of HA-SLN$_1$ and P4-SLN$_2$, at around +29 mV. PDI was under 0.45 in all cases and did not show statistical differences.

Table V. Physicochemical characterization of SLN-based vectors bearing the plasmid pUNO1-hIL10.

Vectors with pUNO1-hIL10	Size (nm)	PDI	Zeta potential (mV)
DX-SLN$_1$	233.1 ± 81.7	0.36 ± 0.10	+36.1 ± 2.2
BE2-SLN$_1$	290.1 ± 20.8	0.34 ± 0.03	+36.6 ± 4.6
HA-SLN$_1$	242.1 ± 38.0	0.35 ± 0.04	+29.6 ± 0.8$^{\#}$
P4-SLN$_2$	510.7 ± 81.2*	0.44 ± 0.03	+29.5 ± 2.2$^{\#}$

** p<0.05 with respect to the other formulations. # p<0.05 with respect to BE2-SLN$_1$ and DX- SLN$_1$. n = 3; data are expressed as mean ± standard deviation. BE: Bemiparin; DX: Dextran; HA: Hyaluronic acid; PDI: Polydispersity index; SLN: Solid lipid nanoparticle.*

1.3.8.2. Agarose gel electrophoresis

The binding, protection and release capacity of the vectors are also influenced by the size of the plasmid. Figure IX shows the gel electrophoresis for the study of these characteristics in the vectors that bare the plasmid pUNO1-hIL10, that was performed as previously explained for pcDNA3-GFP vectors.

SLN_1-based vectors were able to bind, protect and release the plasmid properly. However, in the case of SLN_2-based vectors, the plasmid hardly migrated through the gel (lanes 11 and 15), which indicates that it was scarcely released from the vectors.

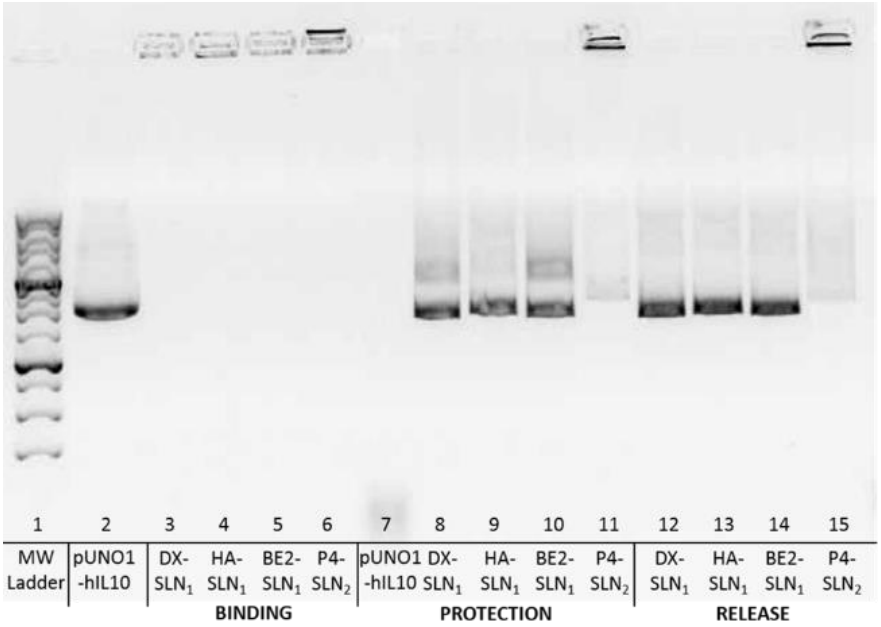

Figure IX. Study of the binding, protection and release of vectors formed with pUNO1-hIL10. Protection samples were treated with DNase I and SDS, and samples of release lanes, only with SDS. MW ladder corresponds to the 1 kb DNA ladder from NIPPON Genetics Europe. BE: Bemiparin; DX: Dextran; HA: Hyaluronic acid; SLN: Solid lipid nanoparticle; MW: Molecular weight; SDS: Sodium dodecyl sulphate.

1.3.8.3. Transfection efficacy with pUNO1-hIL10

IL-10 levels secreted by HCE-2 cells 72 h after treatment with the vectors were analysed by ELISA in the culture medium of the cells. The basal production of non-treated cells was not detectable. In addition, in order to analyse the effect of the SLNs in the IL-10 production by HCE-2 cells, vectors bearing the plasmid pcDNA3-EGFP instead of pUNO1-hIL10 were used for transfection, and IL-10 levels were also undetectable. SLN-based vectors induced IL-10 expression and, as can be seen in Figure X, transfection with SLN_1-based vectors resulted in higher extracellular IL-10 levels (over 10,000 pg/ml) than were caused by SLN_2-based vectors (100 pg/ml). Intracellular levels of the cytokine were under 200 pg/ml in all cases.

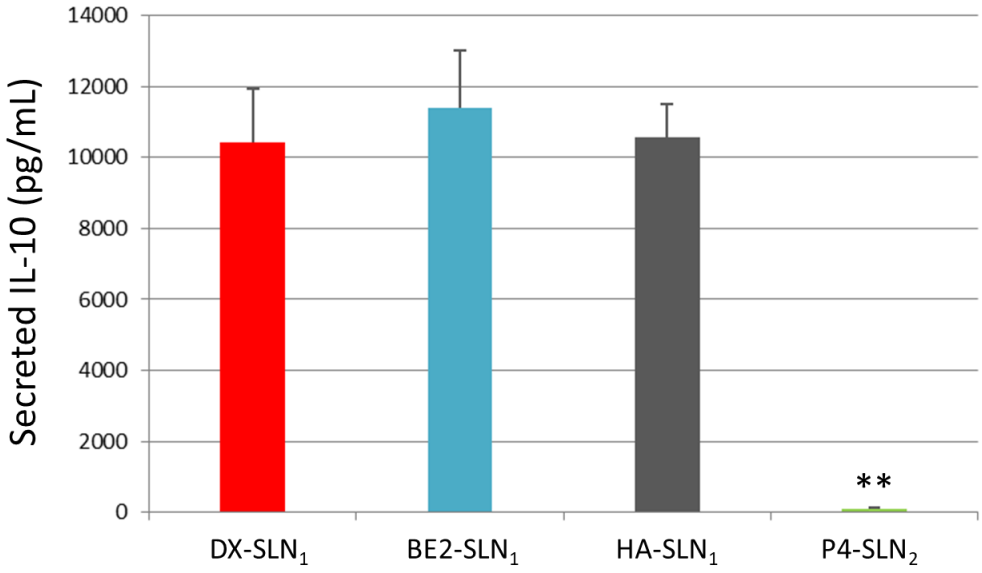

*Figure X. Levels of IL-10 secreted by HCE-2 cells 72 h after treatment with SLN-based vectors bearing the plasmid pUNO1-hIL10. **p < 0.01 with respect to the other formulations. BE: Bemiparin; DX: Dextran; HA: Hyaluronic acid; SLN: Solid lipid nanoparticle; HCE: Human corneal epithelium.*

1.4. Discussion

Gene therapy is a promising treatment for corneal inflammation, as it is a strategy to delivery potent anti-inflammatory genes, such as the one that encodes IL-10, in order to induce a long-term anti-inflammatory response through the *de novo* synthesis of cytokines. Additionally, the fact that it can be administered topically on the corneal surface, and over repeated administrations, makes this therapeutic approach very advantageous. However, limitations to the capacity of delivery systems to overcome the physiological obstacles to transfection, such as precorneal (tear turnover, nasolachrymal drainage) and corneal (tight junctions and hydrophobicity of epithelium) barriers, tissue-selective targeting, cell internalization, escape from endo-lysosomal vesicles, movement through the cytoplasm and entry into the nucleus[37], are still partially unresolved.

This work evaluates two types of cationic SLNs, which differ both in preparation method and composition, as non-viral vectors for corneal gene therapy. SLN_1, which have demonstrated a good capacity to act as non-viral vectors, were prepared using the classical emulsification/solvent evaporation technique[38], while SLN_2 were prepared by means of the

37 Rodríguez-Gascón A, del Pozo-Rodríguez A, Isla A, Solinís MÁ. Gene therapy in the cornea. In: eLS. John Wiley & Sons Ltd, Chichester, UK. 2016. https://doi.org/10.1002/9780470015902.a0024274.

38 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Bartkowiak A, Rodríguez-Gascón A. New gene delivery system based on oligochitosan and solid lipid nanoparticles: 'in vitro' and 'in vivo' evaluation. Eur. J. Pharm. Sci. 2013; 50(3-4):484-491. https://doi.org/10.1016/j.ejps.2013.08.013.

newer solvent-free method, named coacervation[39,40], as an alternative for comparison. The avoidance of solvents during SLN production is an advantageous feature of this second type of lipid nanoparticles. SLN_1 showed higher zeta potential than SLN_2, mainly due to the presence of DOTAP, which is more cationic in character than CH, the cationic agent included in SLN_2. It is important to note that the superficial charge of cationic SLNs designed for gene therapy determines DNA condensation capacity, and superficial charge value of the final complexes[41].

SLNs were electrostatically combined with the peptide protamine and a variety of polysaccharides to form the final complexes with plasmids. The vectors were characterized in terms of size, superficial charge and shape, since these physicochemical properties have significant effects on bio-distribution and cellular internalization[42,43]. All the vectors presented particle sizes in the nanometre range, making them suitable for retention and corneal permeation after topical administration[44], and positive superficial charge. This cationic surface facilitates the cellular uptake of the nanoparticulate systems[45], thanks to interactions with the negatively charged cell membrane. Furthermore, after topical administration onto the surface of the eye, cationic vectors interact with the negatively charged mucus, thus favouring retention at the corneal surface and improving corneal permeation via endocytic uptake by epithelial cells[46]. In SLN_2-vectors, the proportion of protamine and SLNs with respect to DNA had to be

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39 Chirio D, Gallarate M, Peira E, Battaglia L, Muntoni E, Riganti C et al. Positive-charged solid lipid nanoparticles as paclitaxel drug delivery system in glioblastoma treatment. Eur. J. Pharm. Biopharm. 2014; 88(3):746-758. https://doi.org/10.1016/j.ejpb.2014.10.017.

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43 Shang L, Nienhaus K, Nienhaus GU. Engineered nanoparticles interacting with cells: size matters. J. Nanobiotechnol. 2014; 12, 5. https://doi.org/10.1186/1477-3155-12-5.

44 Seyfoddin A, Al-Kassas R. Development of solid lipid nanoparticles and nanostructured lipid carriers for improving ocular delivery of acyclovir. Drug Dev. Ind. Pharm. 2013; 39(4):508-519. https://doi.org/10.3109/03639045.2012.665460.

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increased (up to protamine:DNA 4:1 and DNA:SLN 1:10) to ensure a cationic surface (at +26 mV). Either the absence of protamine or the use of lower ratios (DNA-SLN_2 and P2-SLN_2 formulations), resulted in almost neutral vectors in terms of superficial charge, and they were not able to transfect culture cells.

HA-SLN_1 and DX-SLN_1 vectors have previously proven themselves able to transfect a variety of cells *in vitro* and *in vivo*[47,48,49], and their capacity to transfect corneal cells both *in vitro* and *ex vivo* has also been demonstrated in this work. One of the other two new vectors designed to transfect the cornea was prepared with BE. This is a second-generation low molecular weight heparin with a mean MW of 3.6 KDa. Low molecular weight heparin are anticoagulant drugs that also possess anti-inflammatory effects[50], and the BE in the vectors may contribute to this effect. The plasmid in BE2-SLN_1 was adequately condensed, protected and released, due, in part, to the protamine, which is an excellent aid for transfection that is mediated by lipid-based vectors (DNA condenser, it has nuclear localization signals that translocate DNA from the cytoplasm to the nucleus, and it improves transcriptional activity)[51]. This peptide was also used to prepare complexes with SLN_2. TEM images showed that these SLN_2-vectors had a spherical shape. Previous studies have shown that spherical SLN-based vectors are well taken up by different types of cells[52,53]. The shape of nanoparticulate systems is important since it influences their

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47 Ruiz de Garibay AP, Solinís MÁ, del Pozo-Rodríguez A, Apaolaza PS, Shen JS, Rodríguez-Gascón A. Solid lipid nanoparticles as non-viral vectors for gene transfection in a cell model of Fabry disease. J. Biomed. Nanotechnol. 2015; 11:500-511. https://doi.org/10.1166/jbn.2015.1968.

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cellular internalization; it has been documented that the higher surface area of elongated nanoparticles facilitates their interaction with cell surfaces, and provides higher adhesion to cells compared with spherical nanoparticles[54], whereas the spherical shape favours cellular uptake better than an ellipsoidal shape[55].

In order to test the usefulness of the SLN-based vectors as gene delivery systems for the cornea, transfection studies were carried out in HCE-2 cells with the reporter plasmid pcDNA3-EGFP, and with the therapeutic plasmid pUNO1-IL10, which encodes the anti-inflammatory cytokine IL-10. The levels of this cytokine secreted by HCE-2 cells treated with the most effective formulations were over 1 ng/ml, which are expected to exert an anti-inflammatory effect. In this regard, in a recent work published by Wang et al.[56], IL-10 levels at 0.8 ng/ml in a 3D inflammation model resulted in reduction of proinflammatory cytokines, such as TNF-α, and successful inhibition of inflammation. Moreover, the presence of the hybrid promoter EF-1α/HTLV in the plasmid that contains the IL-10 gene, will be likely useful to yield persistent expression of the anti-inflammatory cytokine *in vivo*[57].

Regardless of the plasmid used, the P4-SLN_2 formulation was found to be less effective than SLN_1-based vectors. Since entry into cells was not a limitation for any of the vectors (Figure IV), the difference in transfection levels may be related to the intracellular behaviour of the formulations. Plasmids must be protected if they are to avoid degradation by intracellular components, but they must also be released in the cytoplasm if they are to enter the nucleus. The intracellular disposition of the DNA (Figure V), together with the only partial release of the plasmid from SLN_2-based vectors in electrophoresis gel (Figure IIB), indicates that the plasmid was more condensed, even after 24 h, than was the case with SLN_1-based vectors. The degree of DNA condensation conditions the capacity to bind, release and protect the plasmid, and it depends on the electrostatic interactions with the cationic components of the formulations. CH, the cationic polysaccharide included in SLN_2, electrostatically binds the DNA strongly and

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54 Agarwal R, Singh V, Jurney P, Shi L, Sreenivasan S, Roy K. Mammalian cells preferentially internalize hydrogel nanodiscs over nanorods and use shape-specific uptake mechanisms. Proc. Natl Acad. Sci. USA. 2013; 110:17247-17252. https://doi.org/10.1073/pnas.1305000110.

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efficiently, and protects it from nuclease degradation, but the release of the DNA is compromised. In a previous work[58], in line with these results, non-viral vectors composed of cationic SLNs and oligochitosans showed a high DNA condensation degree, which resulted in poor transfection of cultured cells *in vitro*. However, after intravenous administration, transfection was detected in a number of organs. This lack of correlation highlights the necessity to develop models for the evaluation of new delivery systems, at earliest phases of the development process, which better match *in vivo* conditions.

In this sense, cornea explants best mimic *in vivo* behaviour, as the various layers of the cornea are intact. The outermost layer is the epithelium, which is composed of several layers of cells that constantly undergo mitosis. Behind the epithelial cells, there is a transparent film called Bowman's layer, which is composed of collagen. This protein is also the main component of the next layer, the stroma. This is the thickest layer of the cornea and contains fibroblasts (keratocytes), which are plane cells aligned in parallel to the ocular surface and produce the collagen. The layer behind the stroma is Descemet's membrane, which is made up of collagen fibers synthesized by the cells that form the corneal endothelium, the innermost layer. Endothelial cells form a monolayer and are amitotic in humans. Unlike other animal models, such as rodents, corneal endothelial cells in rabbits have limited replicative ability and thus resemble human corneal endothelia[59]. All the vectors were able to transfect corneal tissue (Figure VII) in explants from rabbits, although the distribution of transfected corneal cells varied according to the ligands in the formulations. Vectors formulated with CH and BE were only able to transfect the epithelium, while HA also allowed the transfection of stromal cells to occur. The vector that was prepared with DX was observed to be the most effective, since the green protein was detected abundantly in the epithelium, and also in the stroma and in endothelium. The transfection of the endothelium, formed of non-mitotic cells, is an appropriate target for corneal diseases, as gene expression duration, which is a common limitation of non-viral vectors, can be maintained for longer in cells that do not undergo cell division. The *ex vivo* transfection obtained using DX-SLN_1 vectors matches an *in vivo* study in rats[60], in which GFP expression was detected in the corneal epithelium, stroma and endothelium after topical administration of a similar

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formulation in the eye. These results confirm that the explanted corneas from rabbits are a good model to evaluate new gene therapy-based formulations.

1.5. Conclusion

SLN-based vectors are promising gene delivery systems for topical administration to the eye, where they can facilitate IL-10 synthesis by corneal cells, making them useful for treating inflammation-related eye surface diseases. Vectors were able to transfect the epithelium, the stroma, and even the endothelium in varying degrees according to SLN composition and polysaccharide surface coating. SLN-based non-viral vectors could be designed to modulate biodistribution and therefore transfection within the cell layers of the cornea, according to expected therapeutic effect and duration of action.

CHAPTER 2:

Topical administration of SLN-based gene therapy for the treatment of corneal inflammation by *de novo* IL-10 production

The content of this chapter is under revision for publication in Pharmaceutics; Journal Impact Factor JCR 2018: 4.773 (Q1):

Vicente-Pascual M, Gómez-Aguado I, Rodríguez-Castejón J, Rodríguez-Gascón A, Muntoni E, Battaglia L, del Pozo-Rodríguez A, Solinís MÁ. **Topical administration of SLN-based gene therapy for the treatment of corneal inflammation by *de novo* IL-10 production.** Pharmaceutics.

2.1. Introduction

The cornea, a transparent anatomical structure in the anterior segment of the eye, plays a significant role in sight by refracting light to focus a visual image. This tissue can be injured by several factors (infections, dry eye, disorders of the eyelids, physical and chemical damage, and a wide variety of underlying diseases) causing corneal inflammation or keratitis[1]. Common symptoms and signs of keratitis include eye pain, blurred vision, photophobia, tearing, and eye redness, reaching to visual impairment and blindness when chronic inflammation results in tissue destruction[2]. Current therapeutic management of keratitis shows limited efficacy, adverse effects and short duration effect[3]. The advanced therapies, including gene therapy, are new rising approaches under evaluation.

Gene therapy medicinal products generally consist of a vector or delivery formulation/system containing a genetic construct engineered to express a specific transgene ('therapeutic sequence') for the regulation, repair, replacement, addition or deletion of a genetic sequence[4]. These products are expected to have a significant impact on the biopharmaceutical market shortly[5]. A significant number of studies have evaluated the potential of gene therapy to deliver specific anti-inflammatory factors to treat keratitis. Interleukin-10 (IL-10) is a potent immunomodulatory cytokine that interacts with antigen presenting cells inhibiting the production of proinflammatory cytokines such as IL-1, IL-6, IL-8 and tumor necrosis factor (TNF)-

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alpha[6,7,8,9,10,11]. IL-10 gene delivery has been proposed to induce a sustained synthesis of the protein *de novo* in corneal cells, providing a long-term anti-inflammatory effect[12].

For the management of diseases in the anterior segment of the eye, topical administration is by far the most common route, although the low bioavailability of active molecules is an important limitation. Formulations need to face lacrimal turnover, nasolacrimal drainage, blinking reflex, corneal barrier and absorption of drugs by the conjunctiva[13,14]. An ideal delivery system for topical ocular administration should be easily administered, well tolerated with minimal side effects and with high retention time on the ocular surface to improve the penetration on the corneal tissue[13,15]. The design of delivery systems specifically adapted to the kind of the genetic material, the route and way of administration and the target cell, is a key challenge for the clinical translation of non-viral gene therapy. Solid Lipid Nanoparticles (SLNs) are non-viral vectors composed of well-tolerated physiological lipids, which have previously demonstrated the capacity to transfect the epithelium, the stroma and the endothelium of rabbit explanted

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6 Torrecilla J, del Pozo-Rodríguez A, Vicente-Pascual M, Solinís MÁ, Rodríguez-Gascón A. Targeting corneal inflammation by gene therapy: Emerging strategies for keratitis. Exp. Eye Res. J. 2018; 176, 130-140. https://doi.org/10.1016/j.exer.2018.07.006.

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corneas[12]. Due to their nanometre-range size, lipophilic properties and usually cationic surface, SLNs can improve the ocular bioavailability of nucleic acids after topical administration, by means of enhancing the corneal penetration and the cellular uptake, extending ocular retention time and providing a controlled release profile[16,17,18,19]. Additionally, the physicochemical stability of SLNs makes possible their inclusion in solutions of viscosity modifiers widely used in ophthalmic eye drops, such as polyvinyl alcohol (PVA), in order to increase the residence time of the formulation in contact with the cornea and reduce drainage from lachrymal fluid[20,21].

The aim of the present work was the development, *in vitro* and *in vivo* evaluation in mice of four different gene therapy topical medicinal products to treat corneal inflammation. The formulations, based on two different types of SLNs, contained the plasmid encoding IL-10 to provide the *de novo* expression of this protein into the cornea. After optimization, the formulations were administered as eye drops to wild type mice and to IL-10 Knock Out (KO) mice, to evaluate the *in vivo* biodistribution and transfection capacity, and the ability to produce IL-10 in corneal tissues.

2.2. Materials and methods

2.2.1. Materials

DOTAP (1.2-Dioleoyl-3- trimethylammonium-propane chloride salt) was purchased from Avanti Polar-lipids Inc. (AL, USA), Tween 80 and dichloromethane from Panreac (Madrid, Spain) and sodium behenate from Nu-Chek Prep (Eleysian, AL, USA). Precirol® ATO5 was kindly provided by

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Gattefossé (Madrid, Spain), Natrosol™ 250 M pharma MW 720,000, 4,500–6,500 cps, and Oramix CG110 from Safic-Alcan (Barcelona, Spain).

Protamine sulfate salt Grade X (P), dextran (Mn of 3.26 KDa) (DX), DEAE-dextran, partially hydrolyzed PVA 9000-10000 Da Mw (PVA9000), PVA average Mw 85000-124000, 87-89% hydrolysed, Cell Counting Kit-8 (CCK-8) and IR-780 iodide were obtained from Sigma-Aldrich (Madrid, Spain). Hyaluronic acid (Mw of 100 KDa) (HA) was acquired from Lifecore Biomedical and sodium hyaluronate cosmetic grade from Disproquima DSM (Barcelona, Spain). Plasmid pcDNA3-EGFP (6.1 kb) encoding the green fluorescent protein (GFP) was generously provided by the laboratory of Professor B.H.F. Weber (University of Regensburg, Germany). Plasmid pUNO1-hIL10 (3.7 kb), that encodes human IL-10, was acquired from InvivoGen (CA, USA).

Human Corneal Epithelial (HCE-2) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and reagents employed in HCE-2 cells culture, Dulbecco´s Modified Eagle´s Medium/Nutrient Mixture F-12 with GlutaMAX™ (DMEM/F-12 with GlutaMAX™), fetal bovine serum (FBS), attachment factor, trypsin-EDTA and penicillin-streptomicin, were obtained from Life Technologies (ThermoFisher Scientific, Madrid, Spain). EGF was acquired from Myltenyi Biotec (Madrid, Spain). ELISA for IL-10 and the DuoSet Ancillary reagent kit were obtained from R&D Systems (Minneapolis, MN, USA).

Triton X-100 and DNA from salmon sperm were purchased in Sigma-Aldrich (Madrid, Spain), DAPI-Fluoromount-G by Southern Biotech (Birmingham, USA), paraformaldehyde (PFA) from Panreac, PBS and ProLong™ Diamond Antifade Mountant with DAPI were acquired from Gibco (ThermoFisher Scientific, Madrid, Spain).

GFP polyclonal antibody and goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody Alexa Fluor 488 were purchased from Life Technologies (ThermoFisher Scientific, Madrid, Spain), rabbit Anti-IL-10 antibody and rat monoclonal CD44 antibody from Abcam (Cambridge, UK). Tissue-Tek® O.C.T™ compound was obtained from Sakura Finetek Europe (Leiden, The Netherlands). Other chemicals, if not specified, were reagent grade from Sigma Aldrich (Madrid, Spain) and Panreac (Barcelona, Spain).

2.2.2. Preparation of SLNs and vectors

Two kinds of SLNs were prepared by different methods: emulsification/solvent evaporation (SLN_{EE}) and by coacervation (SLN_C).

SLN_{EE} consisted of a solid lipid core of Precirol® ATO5, a cationic lipidic surface based on DOTAP together with the surfactant Tween 80, as previously published[22]. Briefly, DOTAP and Tween 80 were dissolved in water, then, this aqueous solution was mixed with Precirol® ATO5 dissolved in dichloromethane, and the mixture was sonicated. Later, dichloromethane was evaporated.

SLN_C were constituted by a lipid matrix of behenic acid, coated by PVA9000, as suspending agent, and DEAE-dextran as cationizing agent. For their preparation, behenic acid and PVA9000 were dissolved in water at 80ºC under stirring, and when the solution became translucent, NaOH was added, turning then transparent. DEAE-dextran was incorporated dropwise, and the mixture became turbid. Then, HCl was quickly added turning the suspension into white, and, finally, it was cooled in a water bath under stirring.

When necessary, IR780 iodide was incorporated in the preparation of both SLNs to label them. In the case of SLN_{EE}, IR780 iodide was added together with Precirol® ATO5, whereas in the case of SLN_C it was mixed at the end of the formation of the nanosuspension.

The vectors were formed at different weight to weight ratios (Table I) as previously documented[23,24]. Briefly, the plasmid DNA (pcDNA3-EGFP or pUNO1-hIL10) was mixed with an aqueous solution of protamine (P) for 5 min; then, an aqueous solution of polysaccharide, dextran (DX) or hyaluronic acid (HA) was added and mixed for 15 min; finally, the suspension of SLNs was incorporated to the complexes previously obtained.

Table I. Weight ratios of the complexes.

Name of the complex	Weight ratio
DX-SLN_{EE}	DX:P:DNA:SLN_{EE} 1:2:1:5
HA-SLN_{EE}	HA:P:DNA:SLN_{EE} 0.5:2:1:2
DNA-SLN_C	DNA:SLN_C 1:10
HA-SLN_C	HA:P:DNA:SLN_C 0.5:1:1:10

DX: Dextran; HA: Hyaluronic acid; P: Protamine; SLN: Solid lipid nanoparticle.

22 Apaolaza PS, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez JM, Friedrich U, Torrecilla J et al. Structural recovery of the retina in a retinoschisin-deficient mouse after gene replacement therapy by solid lipid nanoparticles. Biomaterials 2016, 90, 40–9. https://doi.org/10.1016/j.biomaterials.2016.03.004.

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In order to formulate the vectors with PVA, once the vectors were prepared, they were mixed with an aqueous solution of PVA (85000-124000 MW) to a final concentration of 1% PVA.

2.2.3. Size and zeta potential of SLNs and vectors

SLNs and vectors were examined by dynamic light scattering, to determine size and polydispersity index, and by laser doppler velocimetry, to measure zeta potential. Samples were appropriately diluted in Milli-Q™ water (EDM Millipore, Billerica, MA) and analysed using a Zetasizer Nano series-Nano ZS (Malvern Instruments, Worcestershire, UK). Each measurement was carried out in triplicate.

2.2.4. Transmission electronic microscopy (TEM) images

Visualization of SLN_C was performed using electron microscopy negative staining. For that purpose, 10 µl of the sample was adhered onto glow discharged carbon coated grids for 60 s. Then the remaining liquid was removed by blotting on filter paper, and the samples were stained with 2% uranyl acetate for 60 s. SLN_C were visualized using a Philips EM208S TEM and digital images were acquired on an Olympus SIS purple digital camera. Technical and human support for TEM was provided by the General Service (SGIker) of Analytical Microscopy and High Resolution in Biomedicine at the University of the Basque Country UPV/EHU. TEM images of the SLN_{EE} were previously published[25].

2.2.5. Ability of the vectors to bind, protect and release the plasmid DNA

In order to evaluate DNA binding efficacy of the SLN_C, as well as their protection and release capacity, a 0.7% agarose gel electrophoresis containing Gel Red™ was employed. Assessment of the ability of the vectors to bind electrostatically pcDNA3-EGFP and pUNO1-hIL10 plasmids was performed by preparing the complexes at a final concentration of 0.03 µg/µl of DNA in $MilliQ_{TM}$ water. This concentration of DNA was also subjected to 1 U DNase I/2.5 µg DNA during 30 minutes at 37ºC to study the protection capacity of the complexes. Finally, the release of DNA from the vectors was performed with a SDS solution (4%) to a final concentration of 1%. As control, naked pcDNA3-EGFP or pUNO1-hIL10 plasmid and 1 kb DNA ladder from NIPPON Genetics Europe (Dueren, Germany) were added. For the analysis of the gel, an Uvitec Uvidoc

25 Apaolaza PS, del Pozo-Rodríguez A, Torrecilla J, Rodríguez-Gascón A, Rodríguez JM, Friedrich U et al. Solid lipid nanoparticle-based vectors intended for the treatment of X-linked juvenile retinoschisis by gene therapy: In vivo approaches in Rs1h-deficient mouse model. J. Controlled Release. 2015; 217, 273–283. https://doi.org/10.1016/j.jconrel.2015.09.033.

D-55-LCD-20M Auto transilluminator was used as previously reported[26]. DX-SLN_{EE} and HA-SLN_{EE} were assessed in previous studies[26,27].

2.2.6. Adhesion test

An *in vitro* model developed by Gallarate et al.[28] was followed to simulate the flow rate of the formulations on the corneal surface, employing a gel with the same surface tension of tear fluid (28 dyne/cm). The gel was composed by 5% hydroxyethyl cellulose (Natrosol™ 250 M), 1% decylpolyglucoside (Oramix CG110), 3% HA cosmetic grade and water q.s. 100 %. The gel was spread on a glass support until the appropriate hardness was achieved, then it was sloped 18°. One-hundred µL of each sample were added on the top and the time needed for each drop to flow over a distance of 15 cm was measured. The assay was repeated three times with each formulation and the flow rate values were expressed as distance covered/time (cm/s).

2.2.7. Rheology studies

Rheological behaviour of the formulations was assessed employing an Advanced Rheometer AR1000 (TA Instruments). The cone angle was 2° and the plate diameter was 40 mm. Measurements were carried out at room temperature. Rheology Advantage™ software (TA Instruments) was used to collect results. The shear stress and viscosity data were collected at shear rates from 5 to 1000 s^{-1} with 10 points per decade and fitted to the power law model:

$$\tau = k\,(\gamma)n, \qquad (1)$$

where τ is the shear stress (Pa), γ·is the shear rate (s^{-1}), k is the consistency coefficient ($Pa \cdot s^n$) and n is the flow behaviour index. In order to study the overall flow characteristics, the logarithm of shear stress versus logarithm of shear rate was plotted:

$$\log \tau = \log k + n \log \gamma. \qquad (2)$$

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26 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Avilés-Triqueros M, Weber BHF, Fernández E, Rodríguez-Gascón A. Dextran and Protamine-Based Solid Lipid Nanoparticles as Potential Vectors for the Treatment of X-Linked Juvenile Retinoschisis. Hum. Gene Ther. 2012; 23, 345–55. https://doi.org/10.1089/hum.2011.115.

27 Vicente-Pascual M, Albano A, Solinís MÁ, Serpe L, Rodríguez-Gascón A, Foglietta F et al. Gene delivery in the cornea: In vitro & ex vivo evaluation of solid lipid nanoparticle-based vectors. Nanomed. J. 2018; 13, 1847–64. https://doi.org/10.2217/nnm-2018-0112.

28 Gallarate M, Chirio D, Bussano R, Peira E, Battaglia L, Baratta F, Trotta M. Development of O/W nanoemulsions for ophthalmic administration of timolol. Int. J. Pharm. 2013; 440, 126–34. https://doi.org/10.1016/j.ijpharm.2012.10.015.

The value of n indicates the rheological behaviour of the fluid: n value lower than 1 indicates a shear-thinning behaviour, and it is classified as pseudoplastic; if n is 1, the rheological behaviour is Newtonian; and when n is greater than 1, it is dilatant. The value of k helps to figure out the viscosity when fluids have similar flow behaviour index[29,30,31].

Viscosity values were plotted to evaluate the behaviour at the different shear rates, and then compared at 10 and 500 s^{-1}.

2.2.8. pH measurement

pH of the vectors and plasmid solutions (with or without PVA 1%) were determined in triplicate employing a Crison Basic 20 pH meter (Crison Instruments, Barcelona, Spain), which was calibrated daily.

2.2.9. In vitro studies

For *in vitro* studies HCE-2 cells were cultured in DMEM/F-12 with GlutaMAX™ supplemented with 15% of fetal bovine serum, 4 mg/mL of insulin, 10 ng/mL of EGF and 1% of penicillin-streptomycin. Cells were incubated at 37ºC with 5% CO_2 and sub-cultured every 7 days (at 80% of confluence) in flasks previously treated with 4 mL of Attachment Factor.

2.2.9.1. *Transfection efficacy of the vectors containing the plasmid pUNO1-hIL10*

Cells were seeded on 24-well plates previously incubated with Attachment Factor at a density of 150,000 cells/well, and allowed to adhere and create a monolayer for 72 hours. Then, cells were treated with 75 µL of each vector (2.5 µg of pUNO1-hIL10 plasmid) for 4 hours. After the incubation period, the medium with the vectors was removed and cells were allowed to grow 72 hours more.

In order to quantify IL-10 levels, an enzyme-linked immunosorbent assay (ELISA) was used. The medium of the wells was removed and centrifuged, then 100 µL of the supernatant was added to a 96-well plate and secreted IL-10 was measured following the manufacturer´s instructions of the ELISA kit.

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29 Coffey MJ, Decory HH, Lane SS. Development of a non-settling gel formulation of 0.5% loteprednol etabonate for anti-inflammatory use as an ophthalmic drop. Clin. Ophthalmol. 2013; 7, 299–312. https://doi.org/10.2147/OPTH.S40588.

30 Nwosu OU, Ewulon CM. Rheological Behaviour of Eco-friendly Drilling Fluids from Biopolymers. J. Polym. Biopolym. Phys. Chem. 2014; 2, 50–4. https://doi.org/10.12691/jpbpc-2-3-2.

31 Maftoonazad N, Shahamirian M, John D, Ramaswamy H. Development and evaluation of antibacterial electrospun pea protein isolate-polyvinyl alcohol nanocomposite mats incorporated with cinnamaldehyde. Mater. Sci. Eng. C. 2019; 94, 393–402. https://doi.org/10.1016/j.msec.2018.09.033.

2.2.9.2. *In vitro cell viability*

Cells were seeded in a 96-well plate at 5 x 10^3 cells per well, after incubation of the plate with Attachment Factor, and allowed to grow for 24 hours. Then, HCE-2 cells were exposed to 10 µL of the formulations and to a positive control (10% Triton X-100 in PBS solution). Four hours later, vectors were removed and fresh medium was added to the wells. Cell cytotoxicity was evaluated 72 hours later, after 4 hours of incubation with 10 µL of CCK-8 (water soluble tetrazolium salt, WST-8), employing a microplate reader with a wavelength of 450 nm (Glomax Multi Detection System (Promega)), following manufacturer´s instructions. The percentage of viable cells was expressed as percentage respect to untreated cells.

2.2.10. In vivo studies

Six-week-old male C57BL/6 mice and IL-10 KO mice (JAX stock #002251)[32] acquired from The Jackson Laboratory were employed for the *in vivo* studies with a weight between 20-25 g. These experiments were approved by the Animal Experimentation Ethics Committee of the University of the Basque Country UPV/EHU (license M20/2018/142) following the Spanish and European Union (EU) laws and all the procedures were followed in accordance. Before the experiments started, mice were allowed to acclimatize. Animals possessed food and water *ad libitum* and they were maintained under controlled temperature, humidity and day-night cycles.

In order to avoid distress during experimental manipulation, mice were anesthetized with 1-2% isoflurane (IsoFlo, Abbott, Spain) in air, at a flow rate of 0.5-1 L/min.

Animals were humanely euthanatized by cervical dislocation. Eyeballs were removed, washed in physiological saline solution and fixed with 4% PFA during 30 minutes. Later on, they were washed with PBS for 5 minutes and incubated at 4ºC with 30% sucrose in PBS until the eye was sunk. Next day, half of the volume was replaced with Tissue-Tek® O.C.T™ compound and rocked at room temperature for 2 hours. Finally, eyeballs were embedded in a mould with 100% Tissue-Tek® O.C.T™ compound, frozen at -80ºC and histological sections of 14 µm were made on a cryostat (Cryocut 3000, Leica) for further studies.

2.2.10.1. *Detection of CD44 receptor in cornea from wild type and KO mice*

The presence of the CD44 (receptor for HA) in wild type and IL-10 KO mice corneas was studied by immunofluorescence. Cryosections were washed with PB buffer, blocked and permeabilized with a blocking solution (20% PB buffer, 0.3% Triton X-100, 10% goat serum and water q.s. 100%)

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32 Kühn R, Löhler J, Rennick D, Rajewsky K, Müller W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell. 1993; 75, 263–74.

during 30 minutes. Later, cryosections were incubated for 24 hours at 4ºC with the rat monoclonal CD44 antibody. Then, after washing the samples with PB, they were incubated with Alexa Fluor 488-conjugated goat anti-rat IgG for 30 minutes protected from the light. Finally, after a washing period, samples were dried and mounted with DAPI-Fluoromount-G. Tissue sections were examined under a Zeiss LSM800 confocal microscope. Sequential acquisition was used to avoid overlapping of fluorescent emission spectra. From each cornea, six sections representing the whole tissue were analysed. Technical and human support for confocal microscopy was provided by the General Service (SGIker) of Analytical Microscopy and High Resolution in Biomedicine at the University of the Basque Country UPV/EHU.

2.2.10.2. *Corneal localization of the vectors*

In order to follow the presence of the vectors in the cornea, the following formulations were topically administered on the ocular surface of wild type mice; DX-SLN_{EE}, HA-SLN_{EE}, and HA-SLN_{C}, all of them with and without PVA. For this purpose, we prepared the vectors with IR780 iodide dyed SLNs and the plasmid pcDNA3-EGFP.

Three instillations of 2.5 µL of the formulations were administered employing a micropipette to one eye in each animal (the other eye was kept as control) at 3 minutes intervals, twice separated by 12 hours. Mice were sacrificed 2 hours after last dose and eyeballs were removed and treated as explained above. Three animals per formulation were evaluated.

Sections were mounted with ProLong™ Diamond Antifade Mountant with DAPI, and were examined under a Zeiss LSM800 confocal microscope. Sequential acquisition was used to avoid overlapping of fluorescent emission spectra. From each cornea, six sections representing the whole tissue were analysed.

2.2.10.3. *In vivo transfection studies*

2.2.10.3.1. Topical administration

To evaluate the capacity of the vectors to transfect the cornea, we administered topically on the ocular surface of wild type mice the following formulations with the plasmid pcDNA3-EGFP: DX-SLN_{EE}, HA-SLN_{EE}, and HA-SLN_{C}, with and without PVA. Additionally, we also administered naked plasmid with and without PVA. A total of 4.5 µg of DNA per day were administered, separated in two doses during 3 days. Each dose consisted on 3 instillations of 2.5 µL at 3 minutes intervals.

We also evaluated the vectors containing the plasmid pUNO1-hIL10 in both wild type and in IL-10 KO mice. The animals were treated following the same dosing protocol described above with the following formulations: DX-SLN_{EE} with PVA, HA-SLN_{EE} with PVA and HA-SLN_{C} with PVA. Naked plasmid with PVA was also studied.

Three animals per formulation were evaluated, and in each animal, one eye was treated and the other one was kept as control.

2.2.10.3.2. Evaluation of gene expression

Forty-eight hours after last dose, mice were sacrificed and eyeballs were removed, fixed and sectioned as explained above. Immunofluorescence staining was performed to evaluate GFP or IL-10 expression. Slides containing the sections were washed with PB buffer, blocked and permeabilized employing a solution of 20% PB, 0.3% Triton X-100, 10% goat serum and water q.s. 100% during 30 minutes. Later, primary antibody anti-GFP or anti-IL-10 was incubated 24 hours at 4ºC. After that, samples were washed and secondary antibody goat anti-rabbit IgG Alexa Fluor 488 was added for a 30 minutes incubation protected from light. After a washing period, samples were dried and mounted with DAPI-Fluoromount-G. Tissue sections were examined under a Zeiss LSM800 confocal microscope. Sequential acquisition was used to avoid overlapping of fluorescent emission spectra. From each cornea, six sections representing the whole tissue were analysed.

2.2.10.3.3. Structural analysis of the cornea

The structure of the corneal sections of wild type and IL-10 KO mice was analysed by the Masson's trichrome staining technique, before and after treatment with the formulations. Samples were observed in a Leica DM IL LED Fluo inverted microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany).

2.2.11. Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics 23 (IBM) software, employing Saphiro-Wilk test for evaluation of normal distribution of samples and Levene test for homogeneity and variance. ANOVA or Student´s t test were used for comparisons and $p<0.05$ was consider statistically significant.

2.3. Results

2.3.1. Size and zeta potential of SLNs and vectors

Table II shows the mean diameter, PDI and zeta potential of the two kind of SLNs and the vectors bearing the plasmid pcDNA3-EGFP or the plasmid pUNO1-hIL10. Particle size of SLN_C resulted to be significantly higher than that of SLN_{EE} (453.9 ± 13.6 nm vs 202.2 ± 28.2 nm), and both presented positive charge and PDI lower than 0.3, which indicates homogeneity in the particle size.

The size of the vectors ranged from 159.4 to 447.9 nm, and zeta potential from +14.7 to +39.3 mV. DX-SLN_{EE} vectors, regardless of the plasmid used, presented smaller size and higher zeta

potential than the other vectors. The highest size corresponded to DNA-SLN_C vectors. Moreover, when the plasmid pUNO1-hIL10 was used, the zeta potential significantly decreased in the vectors DNA-SLN_C and HA-SLN_C (+14.7 ± 0.7 and +15.8 ± 2.4, respectively), respect to the same vectors containing the plasmid pcDNA3-EGFP.

Table II. Physical characterization of nanoparticles and SLN_{EE}- and SLN_C-based vectors bearing the plasmid pcDNA3-EGFP or the plasmid pUNO1-hIL10.

Sample	Size (nm)	PDI	Zeta potential (mV)
SLN_{EE}	202.2 ± 28.2*	0.25 ± 0.01	+51.3 ± 2.2
HA-SLN_{EE}			
pcDNA3-EGFP	204.9 ± 15.0	0.18 ± 0.07	+29.2 ± 3.1
pUNO1-hIL10	266.1 ± 11.4	0.34 ± 0.03	+29.7 ± 1.2
DX-SLN_{EE}			
pcDNA3-EGFP	177.3 ± 23.2#	0.33 ± 0.06	+39.3 ± 1.5#
pUNO1-hIL10	159.4 ± 4.9‡	0.27 ± 0.02	+34.1 ± 0.6‡
SLN_C	453.9 ± 13.6	0.26 ± 0.03	+33.8 ± 2.5
DNA-SLN_C			
pcDNA3-EGFP	404.1 ± 7.2#	0.27 ± 0.02	+20.8 ± 1.5
pUNO1-hIL10	447.9 ± 17.0‡	0.29 ± 0.04	+14.7 ± 0.7&
HA-SLN_C			
pcDNA3-EGFP	368.5 ± 7.4	0.24 ± 0.02	+21.9 ± 0.9
pUNO1-hIL10	374.7 ± 14.5	0.29 ± 0.02	+15.8 ± 2.4&

***** *p<0.05 respect to SLN_C;* **#** *p<0.05 respect to the other vectors bearing the plasmid pcDNA3-EGFP;* **‡** *p<0.05 respect to the other vectors bearing the plasmid pUNO1-hIL10;* **&** *p<0.05 respect to the same vector bearing the plasmid pcDNA3-EGFP. PDI: polidispersity index; DX: Dextran; HA: Hyaluronic acid; SLN: Solid lipid nanoparticle. n = 3; data are expressed as mean ± standard deviation.*

There was not statistically significant change in particle size, PDI and zeta potential of SLNs and vectors labelled with IR 780 iodide.

2.3.2. TEM images

TEM photograph of the SLN_C (Figure I) showed the spherical shape of the nanoparticles.

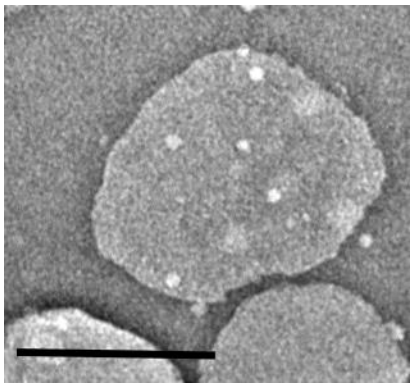

Figure I. Image of SLN_C acquired by transmission electronic microscopy (TEM). Scale bar: 500 nm. SLN: Solid lipid nanoparticle.

2.3.3. Ability of the vectors to bind, protect and release the plasmid DNA

An agarose gel electrophoresis was employed in order to evaluate the ability of DNA-SLN_C and HA-SLN_C vectors to bind, protect and release the pcDNA3-EGFP and pUNO1-hIL10 plasmids (Figure IIA and IIB, respectively).

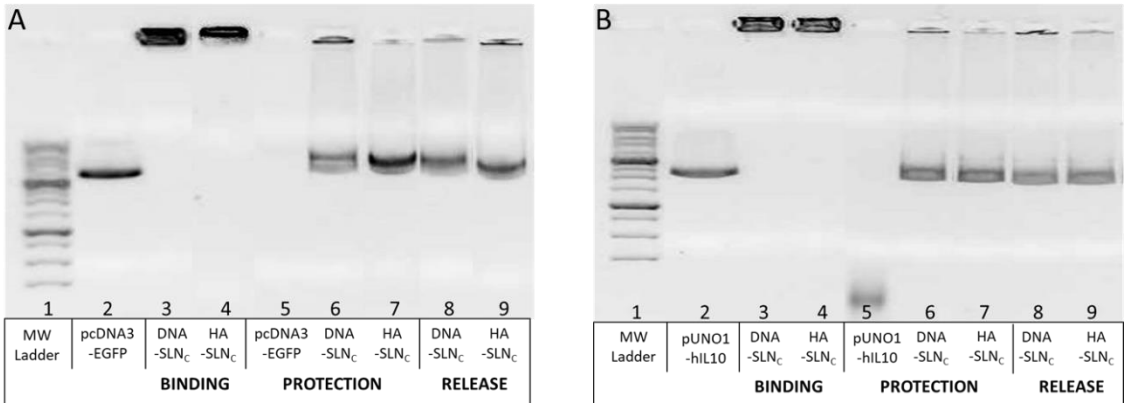

Figure II. Capacity of the vectors to bind, protect and release the pcDNA3-EGFP plasmid (A) and the pUNO1-hIL10 plasmid (B). To study the release of the plasmid, the samples were treated with SDS, and for the protection assay, the samples were mixed first with DNase I and later with SDS to release the plasmid DNA. MW: molecular weight; HA: hyaluronic acid; SLN: Solid lipid nanoparticle; SDS: sodium dodecyl sulphate; DNase I: deoxyribonuclease I.

As can be seen in lanes 3 and 4, DNA was completely bound to the vectors, since no band was observed on the correspondent lanes, and the plasmid was detected on the loading wells.

The release of the plasmid was evaluated after treating the complexes with SDS for 5 minutes. The DNA bands in lanes 8 and 9 indicate that both vectors were able to release the plasmids, although not completely, since the plasmids were also partially detected in the loading wells, especially in the case of the vectors prepared with the IL-10 plasmid (Figure IIB).

2.3.4. Adhesion test: flow rates

An adhesion test was performed in order to study the behaviour of the formulations in terms of flow rates; the higher the flow rate the lower the adhesiveness. As it can be observed in Figure III, the solution of plasmid pcDNA3-EGFP, and HA-SLN_{EE} and DX-SLN_{EE} vectors showed a flow rate similar to that of water. The addition of PVA decreased the flow rate, which means that it increased the adhesiveness of the plasmid solution and all the formulations, except for HA-SLN_C vector, which did not show significant differences in the flow rate with or without PVA. The flow rate values obtained with the plasmid pUNO1-hIL10 did not show significant differences respect to these presented in Figure III.

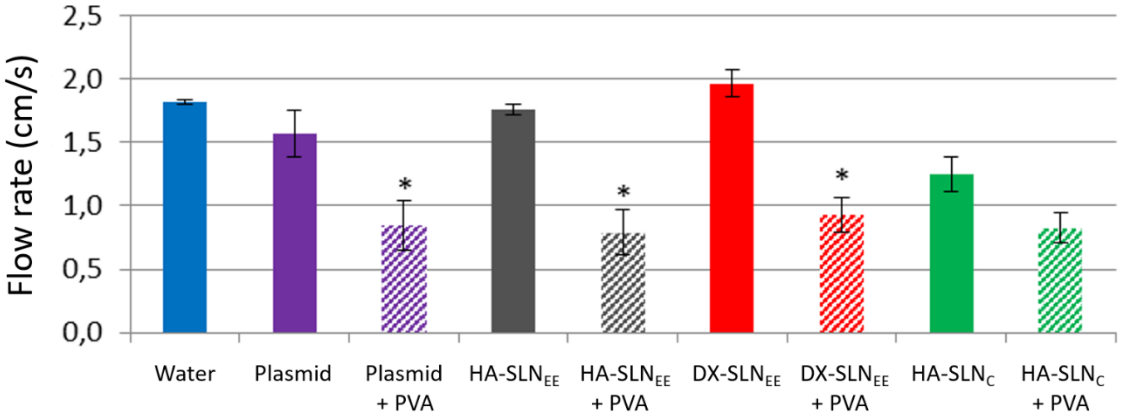

*Figure III. Flow rates of water, plasmid solution, SLNs and vectors with and without PVA (expressed as cm/s). *p<0.05 respect to the same formulation without PVA. PVA: polyvinyl alcohol; HA: Hyaluronic acid; DX: Dextran; SLN: Solid lipid nanoparticle.*

2.3.5. Rheology studies

Table III summarizes the results obtained in the rheological studies. In all cases, when plotting the logarithm of shear stress versus logarithm of shear rate, high coefficient of determination values (R^2) were obtained.

Table III. High coefficient of determination (R^2), viscosity at shear rate of 10 and 500 s^{-1} (mPa.s), consistency coefficient (k) and flow behaviour index (n) values of vectors and plasmid pcDNA3-EGFP solution with and without PVA.

Sample	R^2	Viscosity 10 s^{-1}	Viscosity 500 s^{-1}	k	n
Water	0.9947	0.73	0.87	0.001	1.065
Plasmid solution	0.9847	0.78	0.98	0.001	1.11
Plasmid solution + PVA	0.9995	2.14	1.91	0.002	0.976
HA-SLN$_{EE}$	0.9588	18.40	2.92	0.067	0.519
HA-SLN$_{EE}$ + PVA	0.9592	8.26	2.38	0.015	0.681
DX-SLN$_{EE}$	0.9921	1.60	1.00	0.002	0.876
DX-SLN$_{EE}$ + PVA	0.9969	3.31	2.16	0.004	0.879
HA-SLN$_{C}$	0.8642	10.70	1.75	0.028	0.525
HA-SLN$_{C}$ + PVA	0.9995	3.29	3.08	0.003	0.989

PVA: polyvinyl alcohol; HA: Hyaluronic acid; DX: Dextran; SLN: Solid lipid nanoparticle

pcDNA3-EGFP plasmid solution, with and without PVA, and HA-SLN$_C$ with PVA showed a flow behaviour similar to water, with a n index near 1, indicating Newtonian behaviour, where viscosity is constant independently of the shear rate applied. By contrast, all the SLN$_{EE}$-based vectors, with and without PVA, and the vector HA-SLN$_C$ (without PVA) showed lower values of n, which indicates a pseudoplastic (shear-thinning) behaviour. In Figure IV the decrease in the apparent viscosity of these vectors as shear rate increased can be observed.

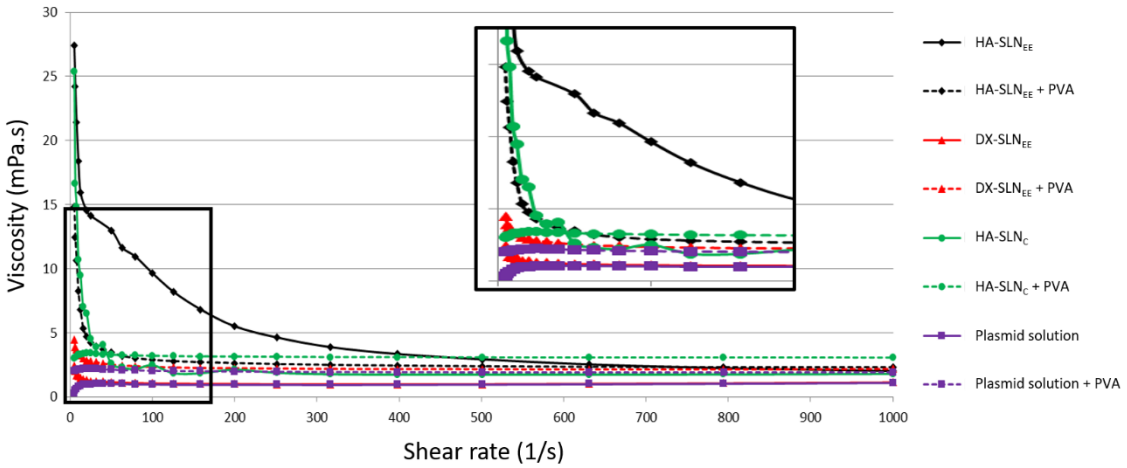

Figure IV. Viscosity curve at shear rates from 5 to 1000 s^{-1} of water, the solution of plasmid pcDNA3-EGFP and vectors, with and without PVA. PVA: polyvinyl alcohol; HA: Hyaluronic acid; DX: Dextran; SLN: Solid lipid nanoparticle.

As it is observed in Table III and Figure IV, at low shear rate values, PVA increased the apparent viscosity of plasmid solution and DX-SLN$_{EE}$ vectors, whereas in the case of the vectors containing HA the viscosity was much higher without PVA. At high shear rates, PVA increased or maintained the viscosity of HA-containing formulations.

Table IV and Figure V summarizes the results obtained in the rheological studies with the plasmid pUNO1-hIL10 and the vectors prepared with it. The results are consistent with those obtained with the plasmid pcDNA3-EGFP.

Table IV. High coefficient of determination (R^2), viscosity at shear rate of 10 and 500 s^{-1} (mPa.s), consistency coefficient (k) and flow behaviour index (n) values of vectors and plasmid pUNO1-hIL10 solution with and without PVA.

Sample	R^2	Viscosity 10 s^{-1}	Viscosity 500 s^{-1}	k	n
Water	0.9921	0.75	0.90	0.001	1.070
Plasmid solution	0.9924	1.21	0.93	0.001	1.053
Plasmid solution + PVA	0.9960	2.05	1.49	0.002	0.954
HA-SLN$_{EE}$	0.9697	22.41	2.77	0.086	0.465
HA-SLN$_{EE}$ + PVA	0.9980	6.26	2.36	0.012	0.742
DX-SLN$_{EE}$	0.9981	1.73	1.08	0.002	0.878
DX-SLN$_{EE}$ + PVA	0.9952	4.00	2.82	0.006	0.883
HA-SLN$_{C}$	0.9550	12.29	1.64	0.029	0.523
HA-SLN$_{C}$ + PVA	0.9987	4.59	3.04	0.006	0.909

PVA: polyvinyl alcohol; HA: Hyaluronic acid; DX: Dextran; SLN: Solid lipid nanoparticle

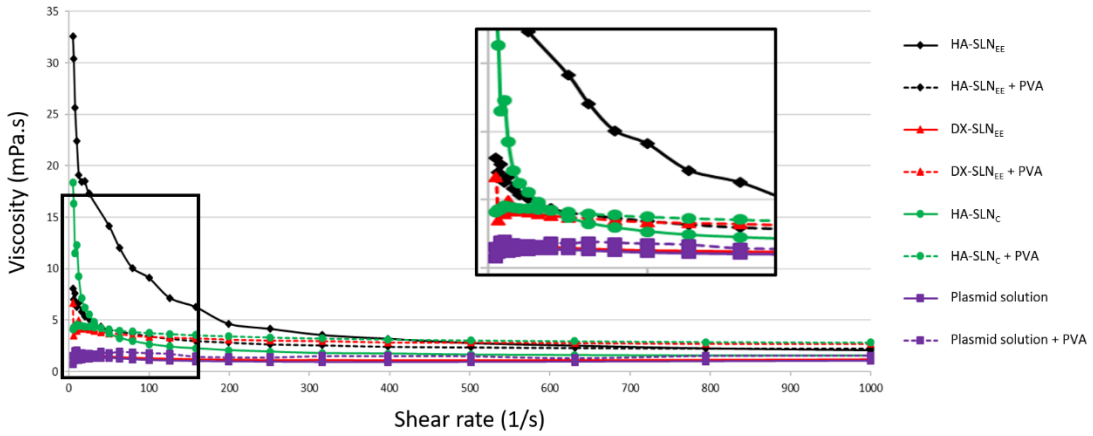

Figure V. Viscosity curve at shear rates from 5 to 1000 s^{-1} of water, the solution of plasmid pUNO1-hIL10 solution and vectors, with and without PVA.PVA: polyvinyl alcohol; HA: Hyaluronic acid; DX: Dextran; SLN: Solid lipid nanoparticle.

The plasmid solutions, with and without PVA, and HA-SLN$_C$ with PVA showed a flow behaviour similar to water (n index near 1), indicating Newtonian behaviour, whereas the values of n were lower in all the SLN$_{EE}$-based vectors, with and without PVA, and the vector HA-SLN$_C$ without PVA. This is indicative of a pseudoplastic behaviour.

2.3.6. pH values

Table V features the pH values of the plasmid solutions and the vector suspension, with and without PVA. The results correspond to the plasmid pcDNA3-GFP; the pH values obtained with the plasmid pUNO1-hIL10 did not show significant differences respect to these presented in Table V. The pH of the plasmid and the vectors prepared with SLN$_{EE}$ ranged from 7.5 to 7.0; however, the vectors prepared with SLN$_C$ presented a lower pH, around 4.0. In all cases, the PVA hardly modified the pH.

Table V. pH measurements of plasmid solutions and vectors with and without PVA.

Sample	pH
Plasmid solution	7.4 ± 0.13
Plasmid solution + PVA	7.3 ± 0.13
HA-SLN$_{EE}$	7.3 ± 0.03
HA-SLN$_{EE}$ + PVA	7.2 ± 0.01
DX-SLN$_{EE}$	7.5 ± 0.10
DX-SLN$_{EE}$ + PVA	7.0 ± 0.18
HA-SLN$_C$	4.0 ± 0.07
HA-SLN$_C$ + PVA	4.3 ± 0.11

PVA: polyvinyl alcohol; HA: Hyaluronic acid; DX: Dextran; SLN: solid lipid nanoparticle. n = 3; data are expressed as mean ± standard deviation.

2.3.7. In vitro studies

2.3.7.1. *Transfection efficacy of the vectors containing the plasmid pUNO1-hIL10*

In order to study the capacity of HA-SLN_C bearing the pUNO1-hIL10 plasmid to induce the expression of IL-10, HCE-2 cells were treated with the vector. Seventy-two hours later the concentration of IL-10 in the culture medium was 9.1 ± 0.8 ng/mL. This level was similar to that of DX-SLN_{EE} and HA-SLN_{EE}, which were assessed in a previous study[33]. In the culture medium of untreated cells and the cells treated with the free plasmid and with the plasmid condensed with protamine and the HA or DX (without SLNs), IL-10 was not detectable.

2.3.7.2. *In vitro cell viability*

The viability of the HCE-2 cells after the treatment with the formulations was assessed by using the CCK-8 assay. As shown in Figure VI, in all cases cell viability of the HCE-2 cells was higher than 80%, except for the positive control Triton X-10, which reduced the cell viability to 30.51 ± 3.30%.

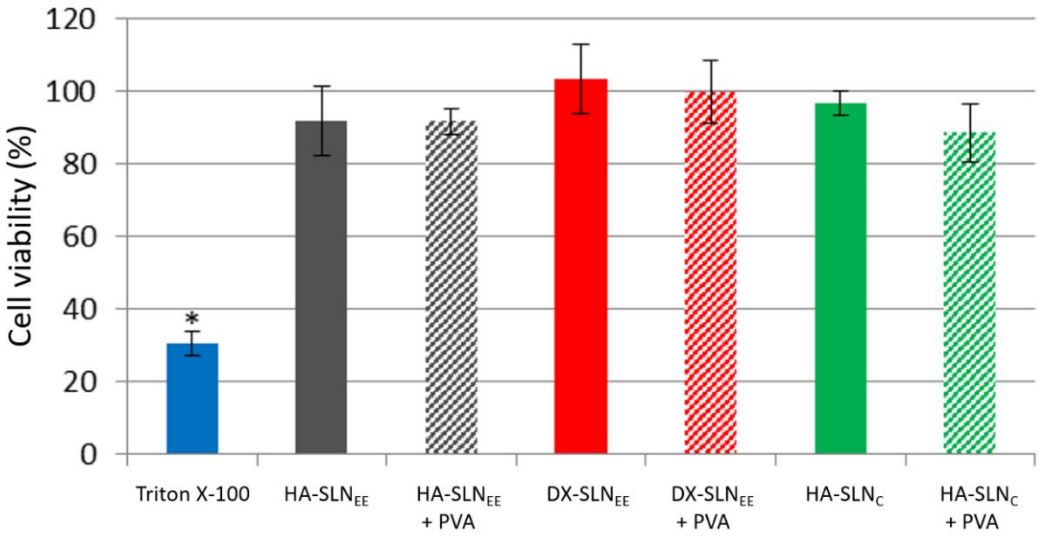

*Figure VI. Cell viability after treatment of HCE-2 cells with Triton X-100 as positive control, and with the following formulations HA-SLN_C, HA-SLN_C with PVA, DX-SLN_{EE}, DX-SLN_{EE} with PVA, HA-SLN_{EE} and HA-SLN_{EE} with PVA. The percentage of viable cells was expressed as percentage respect to untreated cells. *p<0.01 respect to the formulations. HA: Hyaluronic acid; PVA: polyvinyl alcohol; DX: Dextran; SLN: solid lipid nanoparticle; HCE-2 cells: human corneal epithelial cells. n = 3; data are expressed as mean ± standard deviation.*

33 Vicente-Pascual M, Albano A, Solinís MÁ, Serpe L, Rodríguez-Gascón A, Foglietta F et al. Gene delivery in the cornea: In vitro & ex vivo evaluation of solid lipid nanoparticle-based vectors. Nanomed. J. 2018; 13, 1847–64. https://doi.org/10.2217/nnm-2018-0112.

2.3.8. In vivo studies

2.3.8.1. *Detection of CD44*

Figure VII shows the expression of the CD44 receptor (green colour) in the corneal tissue of wild type and IL-10 KO mice. As can be seen, in the cornea of wild type animals, CD44 was detected only in the epithelial layers, whereas in the corneas of IL-10 KO mice, CD44 was detected in epithelium and also in the stroma (arrows).

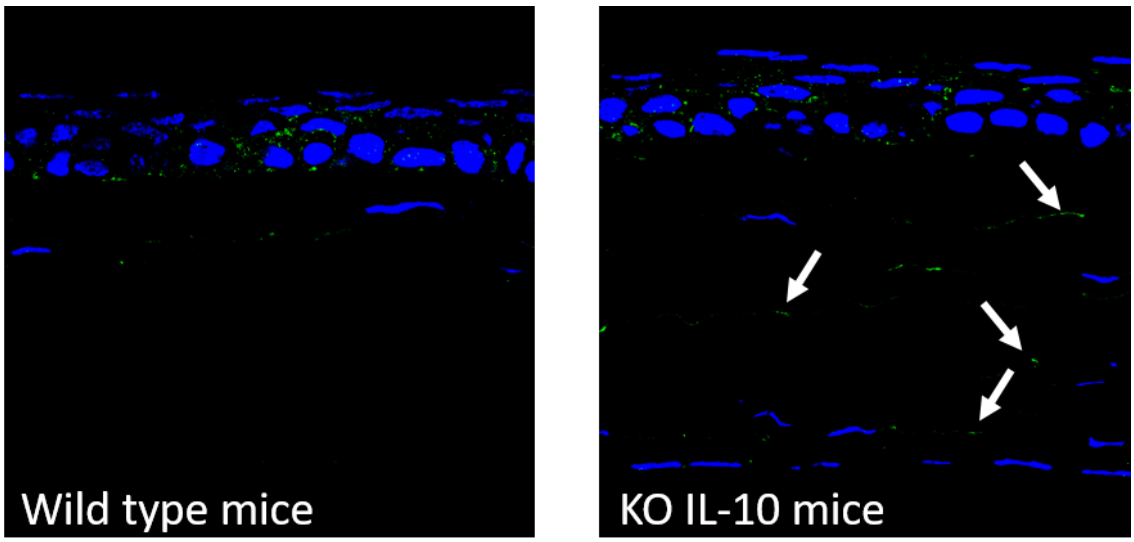

Figure VII. CD44 detection by immunofluorescence in corneal tissue from wild type and IL-10 KO mice (20x). Blue: nuclei stained with DAPI. Green: CD44. KO: knock out.

2.3.8.2. *Corneal localization of the vectors*

In order to study the corneal localization of the formulations (with and without PVA), they were prepared with IR780 iodide labelled SLNs (red) and administered topically on the ocular surface of wild type mice. Figure VIII features images of the corneas 2 h after the instillation of the last dose. All the vectors were localized on the corneal epithelium.

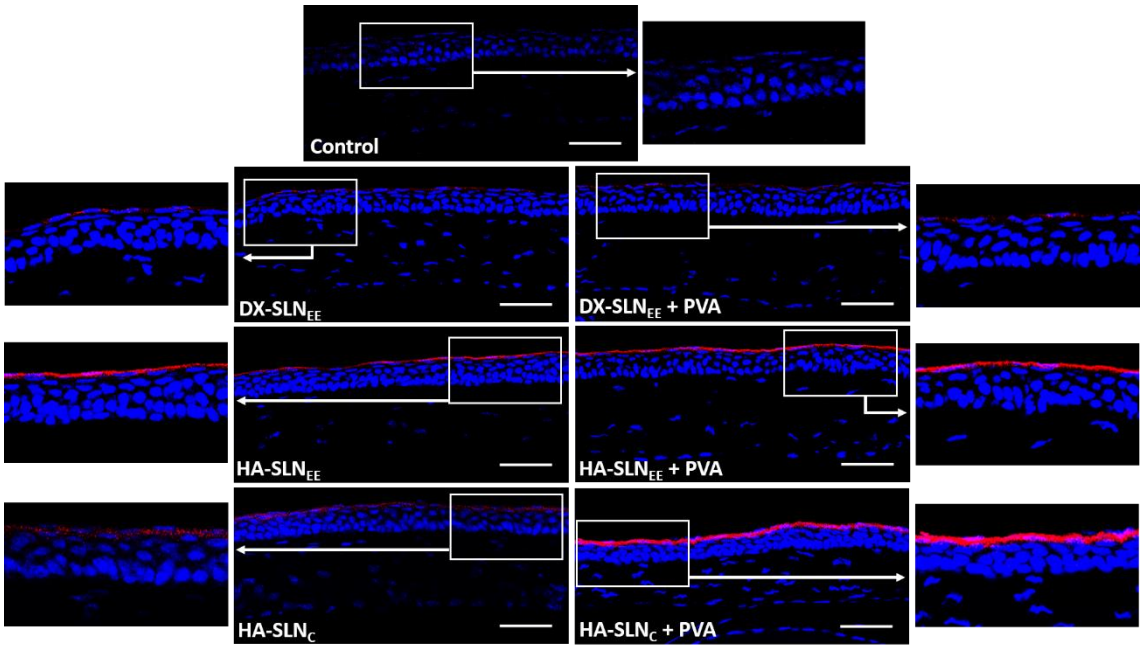

Figure VIII. Localization of the formulations on the cornea of wild type mice (20x), 2h after the instillation of the last dose. Blue: nuclei stained with DAPI. Red: vectors with IR780 iodide labelled SLNs. Scale bar: 50 µm. DX: Dextran; SLN: Solid lipid nanoparticle; PVA: polyvinyl alcohol; HA: Hyaluronic acid.

When HA-SLN$_{EE}$, prepared with 2.5 times less of particles than DX formulations for the same DNA dose, was administered, higher fluorescence intensity was observed, regardless of the presence of PVA. This higher fluorescence intensity indicates a higher amount of HA-SLN$_{EE}$ vectors in the corneal epithelium than in the case of DX-SLN$_{EE}$. On the contrary, HA-SLN$_{C}$ vectors, although prepared with a higher SLN$_{C}$:DNA ratio (10:1) than in SLN$_{EE}$-based vectors, presented a lower fluorescence intensity, which is indicative of a lower amount of vectors in the cornea. The incorporation of PVA increased the fluorescence intensity on the cornea of the SLN$_{EE}$- and SLN$_{C}$-based vectors formulated with HA as polysaccharide. However, the combination of DX-SLN$_{EE}$ with PVA did not modify the corneal localization and fluorescence intensity observed.

2.3.8.3. *In vivo transfection with the vectors containing the plasmid pcDNA3-EGFP*

In order to assess the location of the transfected corneal cells, the vectors bearing the plasmid pcDNA3-EGFP were topically administered to wild type mice. This plasmid encodes the reporter GFP, which is an intracellular protein. Transfection results are summarized in Figure IX.

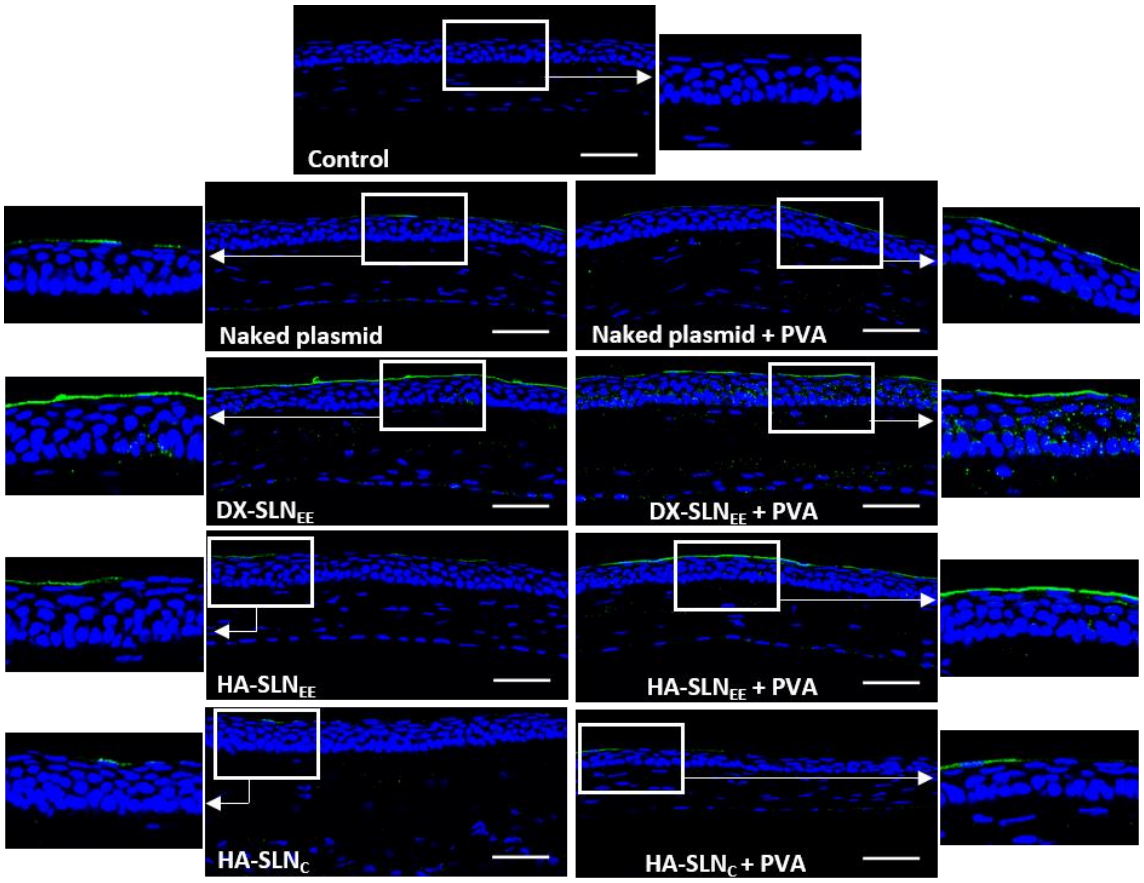

Figure IX. Corneal transfection in vivo in wild type mice treated with naked plasmid and vectors bearing the plasmid pcDNA3-EGFP with and without PVA (20x). Blue: nuclei stained with DAPI. Green: green fluorescent protein. Scale bar: 50 µm. DX: Dextran; SLN: Solid lipid nanoparticle; PVA: polyvinyl alcohol; HA: Hyaluronic acid.

GFP was detected in the 100% of the sections analysed. All the formulations were able to transfect the epithelial cells, although in the corneas of mice treated with naked DNA and with HA-SLN$_C$ (with or without PVA), GFP was localized in a discontinuous way.

GFP produced by the DX-SLN$_{EE}$ was detected mainly in the surface of the epithelium, but the protein was also observed in inner epithelial layers, whereas HA-SLN$_{EE}$ and HA-SLN$_C$ transfected only the outermost layer of the corneal epithelium.

No difference was observed in transfection between the naked plasmid with and without PVA. However, PVA notably affected the transfection capacity of the vectors. On the one hand, DX-SLN$_{EE}$ with PVA induced higher expression in deeper layers of the epithelium; on the other hand, HA-SLN$_{EE}$ and HA-SLN$_C$ combined with PVA resulted in higher fluorescence intensity, which is associated to a higher protein expression.

2.3.8.4. *In vivo transfection with the vectors containing the pUNO1-hIL10 plasmid*

The ability of the vectors combined with PVA to express IL-10 was assessed in wild type mice (Figure X) and IL-10 KO mice (Figure XI) after topical administration. Both wild type and IL-10 KO

treated mice showed IL-10 transfection in the 100% of the sections analysed, representative of the whole cornea.

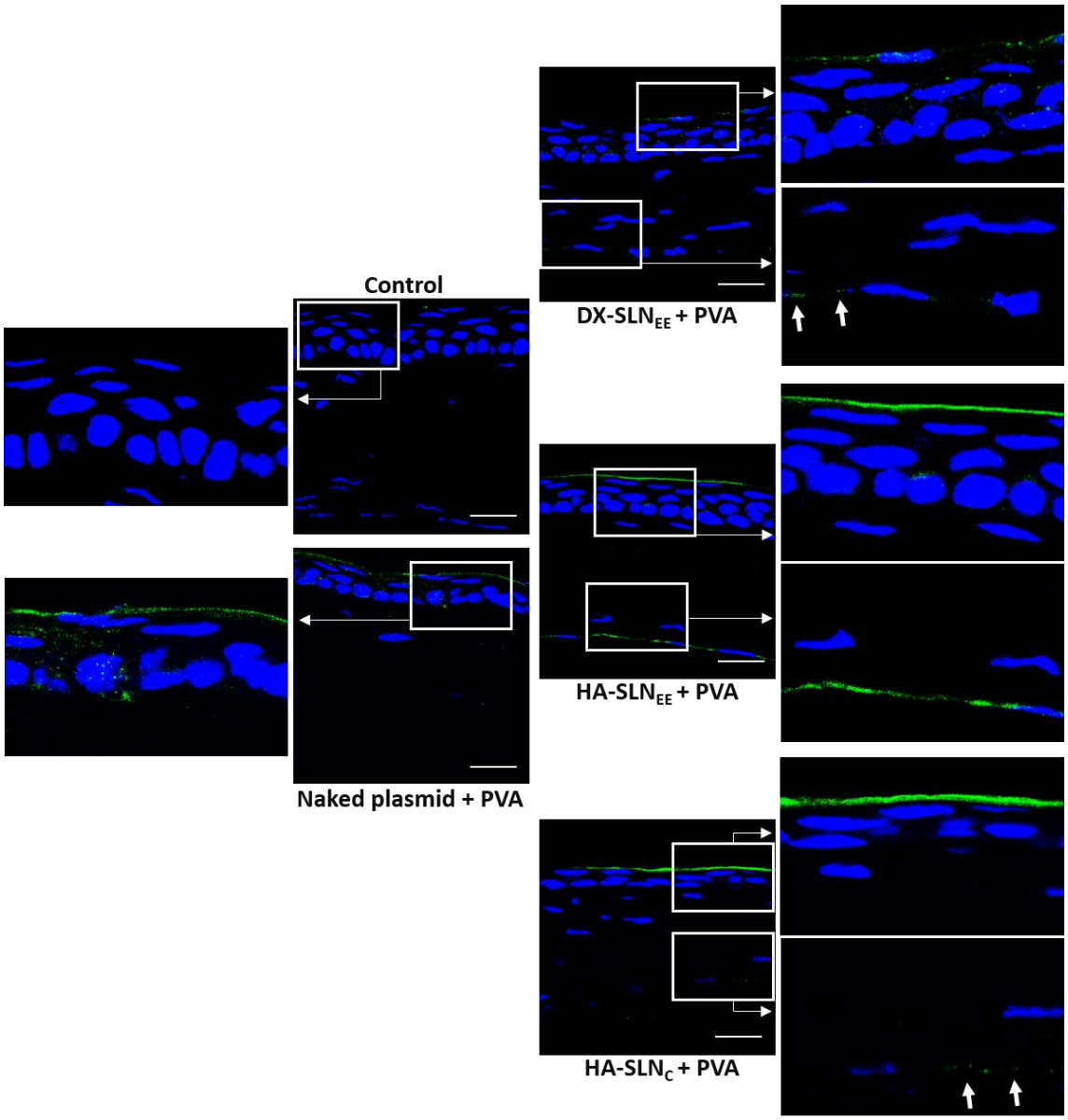

Figure X. Corneal transfection in vivo in wild type mice treated with naked plasmid and vectors bearing the plasmid pUNO1-hIL10 with PVA (60x). Blue: nuclei stained with DAPI. Green: IL-10. Scale bar: 20 µm. DX: Dextran; SLN: Solid lipid nanoparticle; PVA: polyvinyl alcohol; HA: Hyaluronic acid.

In the corneas of wild type mice (Figure X) treated with naked plasmid and DX-SLN$_{EE}$, IL-10 was detected in a discontinuous way (as dots), whereas in the corneas treated with HA-SLN$_{EE}$ and HA-SLN$_{C}$, the presence of the protein was continuous along the cornea. A higher intensity of fluorescence, indicative of higher protein synthesis, was observed in the corneas treated with HA-SLN$_{EE}$ and HA-SLN$_{C}$ formulations. In addition, the secreted IL-10 was even detected in the endothelial layer of the corneas treated with SLN$_{EE}$-based vectors.

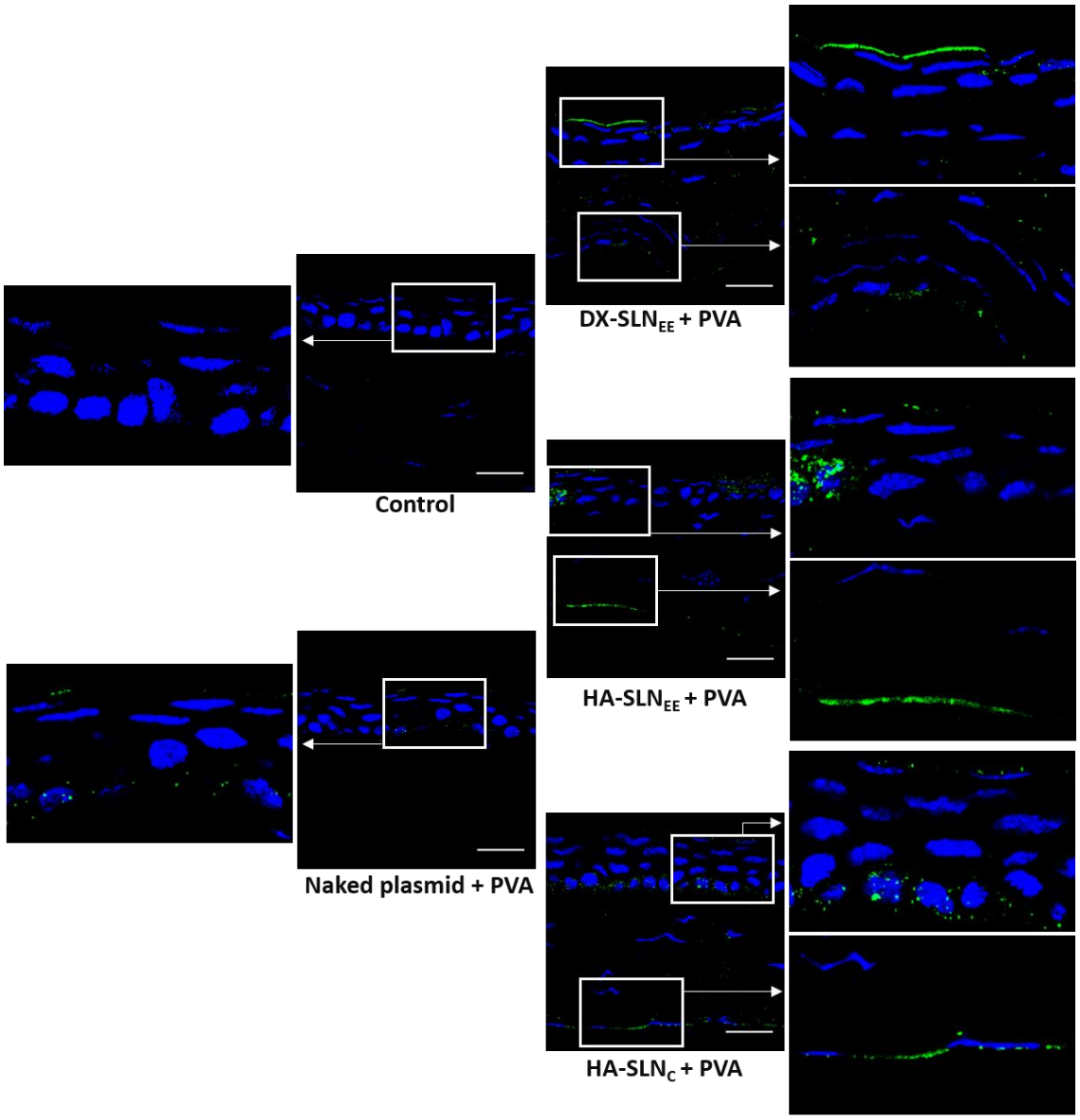

Figure XI. Corneal transfection in vivo in IL-10 KO mice treated with naked plasmid and vectors bearing the plasmid pUNO1-hIL10 with PVA (60x). Blue: nuclei stained with DAPI. Green: IL-10. Scale bar: 20 µm. DX: Dextran; SLN: Solid lipid nanoparticle; PVA: polyvinyl alcohol; HA: Hyaluronic acid.

In IL-10 KO mice (Figure XI) the location profile of the secreted IL-10 was quite similar with the three vectors. IL-10 was detected in both the epithelium and the endothelium, and as in wild type mice, the intensity of fluorescence was higher in the corneas treated with the formulations containing HA as polysaccharide.

2.3.8.5. *Structural analysis of the cornea*

Figure XII depicts histological sections of the corneas from wild type and IL-10 KO mice treated with the naked pUNO1-hIL10 plasmid and with the vectors containing the plasmid and combined with PVA, as well as untreated corneas (control).

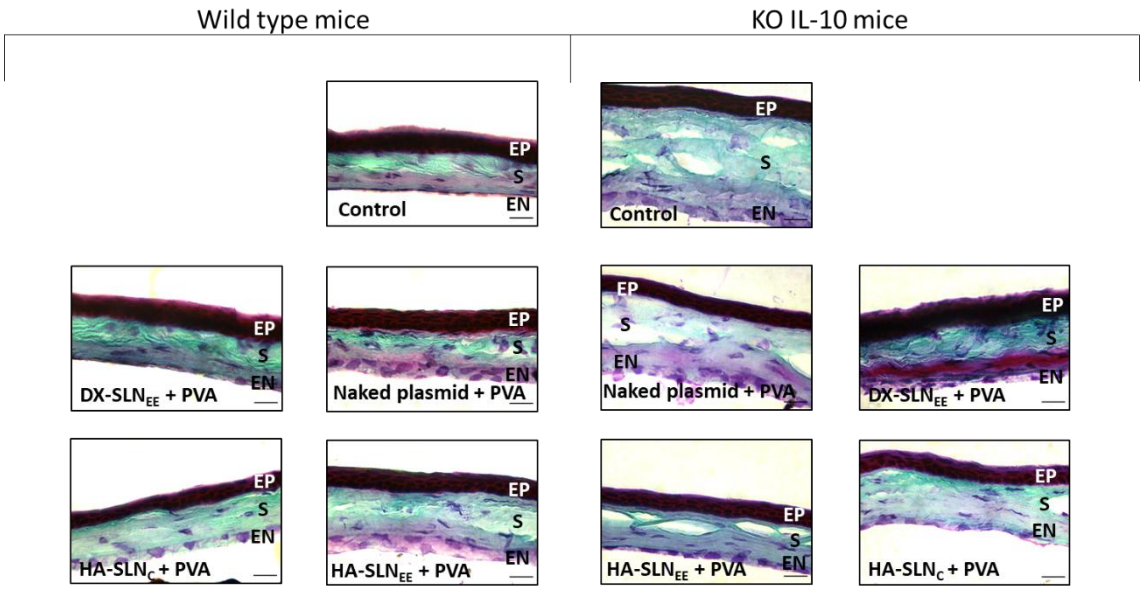

Figure XII. Microscopic images of wild type and IL-10 KO mice corneas stained by Masson's trichrome technique. (60x) Scale bar: 20 µm. Green: collagen. Red: cell cytoplasm. Dark brown: cell nuclei. EP: Epithelium, S: Stroma, EN: Endothelium; DX: Dextran; SLN: Solid lipid nanoparticle; PVA: polyvinyl alcohol; HA: Hyaluronic acid.

The histological structure of the corneas from IL-10 KO mice showed differences respect to those from wild type mice. The stroma of the untreated eyes of IL-10 KO mice showed gaps that were not present in the wild type corneas.

The corneas of wild type mice treated during 3 days with the formulations did not show changes in the histological structure respect to the non-treated corneas. Therefore, formulations seems to be well tolerated after repeated topical administration.

2.4. Discussion

The clinical application of nucleic acid medicinal products is closely dependent on the development of effective and safe delivery systems, which must be specifically adapted to the characteristics of the genetic material and to the target tissue. In fact, the issue of delivery is considered as the main challenge in gene therapy and it is especially relevant for the success of gene therapy in the cornea.

The most efficient methods for nucleic acid-based therapies are viral vectors. Retroviruses, lentiviruses, adenoviruses and adeno-associated viruses have been used for transfecting the cornea. However, the induction of the immune response and inflammation limits the application of viral vectors to inflammatory diseases, including cornea inflammation, even considering its

relative immune-privilege[34]. Moreover, they have been frequently administered by invasive methods such as intrastromal, intralimbal, intracameral or after removing the corneal epithelium[35,36,37]. Non-viral gene therapy has proven to be a feasible alternative for corneal gene therapy even after topical administration[38,39,40,41], although there is still room for improvement. Likewise, corneal inflammation is usually associated to pathologies that occur in outbreaks and it does not require the long-term expression of anti-inflammatory mediators. In this sense, topical instillations of non-viral vectors could be self-administered by the patients themselves when symptoms appear. In the present work, we have formulated eye drops containing biocompatible non-viral vectors based on SLNs capable of increasing ocular bioavailability and inducing the expression of IL-10 in different corneal layers after topical administration, to address the treatment of corneal inflammation.

The nanovectors were prepared with two different kinds of cationic SLNs: SLN_{EE}, prepared by emulsification/solvent evaporation, and SLN_C, prepared by coacervation; this second method avoids the use of solvents. The final vectors also contained ligands on their composition, a cationic peptide, protamine, and a polysaccharide, DX or HA, which confers to the vectors a high versatility. On the one hand, the positive charge of SLN facilitates the interaction with the polyanionic corneal surface, increasing the retention time and improving corneal permeation

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34 Torrecilla J, del Pozo-Rodríguez A, Vicente-Pascual M, Solinís MÁ, Rodríguez-Gascón A. Targeting corneal inflammation by gene therapy: Emerging strategies for keratitis. Exp. Eye Res. J. 2018; 176, 130-140. https://doi.org/10.1016/j.exer.2018.07.006.

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39 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Avilés-Triqueros M, Weber BHF, Fernández E, Rodríguez-Gascón A. Dextran and Protamine-Based Solid Lipid Nanoparticles as Potential Vectors for the Treatment of X-Linked Juvenile Retinoschisis. Hum. Gene Ther. 2012; 23, 345–55. https://doi.org/10.1089/hum.2011.115.

40 Bauer D, Lu M, Wasmuth S, Li H, Yang Y, Roggendorf M et al. Immunomodulation by topical particle-mediated administration of cytokine plasmid DNA suppresses herpetic stromal keratitis without impairment of antiviral defense. Graefe's Arch. Clin. Exp. Ophthalmol. 2006; 244, 216–25. https://doi.org/10.1007/s00417-005-0070-z.

41 Gupta S, Fink MK, Ghosh A, Tripathi R, Sinha PR, Sharma A et al. Novel combination BMP7 and HGF gene therapy instigates selective myofibroblast apoptosis and reduces corneal haze in vivo. Investig. Ophthalmol. Vis. Sci. 2018; 59, 1045–57. https://doi.org/10.1167/iovs.17-23308.

through endocytic uptake by corneal epithelial cells[42,43]. On the other hand, polysaccharides determine the interaction with targeted cells, and as a consequence, the internalization process and the intracellular behaviour of the genetic material[44,45]. Finally, protamine, apart from favouring the transcription process and nuclear entry, also contributes binding and protecting the genetic material at intra and extracellular level, thanks to its cationic nature[46].

The particle size of SLN_C (Figure I and Table II), was more than double than that of SLN_{EE} (453.9 ± 13.6 vs 202.2 ± 28 nm, respectively); accordingly, SLN_C-based vectors presented higher particle sizes. Particle size of all the vectors ranged from 159.4 to 447.9 nm and PDI were lower than 0.4, indicating homogeneity in the particle size. Nanoparticles smaller than 800 nm avoid ocular irritation or discomfort and favour uptake by corneal cells[47,48,49]. In addition, nanoparticles with an average diameter ranging from 50 to 400 nm have the ability to overcome physiological barriers, and they lead to higher bioadhesion and corneal penetration when they are topically administered[50,51]. In fact, patients tolerate better smaller particles than larger ones, because the

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42 Battaglia L, Serpe L, Foglietta F, Muntoni E, Gallarate M, del Pozo-Rodríguez A, Solinís MÁ. Application of lipid nanoparticles to ocular drug delivery. Expert Opin. Drug Deliv. 2016; 13, 1743–57. https://doi.org/10.1080/17425247.2016.1201059.

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45 Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Rodríguez-Gascón A, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. Int. J. Pharm. 2014; 65, 413-26. https://doi.org/10.1016/j.ijpharm.2014.02.038.

46 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: The importance of the entry pathway. Eur. J. Pharm. Biopharm. 2011; 79, 495–502. https://doi.org/10.1016/j.ejpb.2011.06.005.

47 Calvo P, Alonso MJ, Vila-Jato JL, Robinson JR. Improved Ocular Bioavailability of Indomethacin by Novel Ocular Drug Carriers. J. Pharm. Pharmacol. 1996; 48, 1147–52.

48 Qaddoumi MG, Ueda H, Yang J, Davda J, Labhasetwar V, Lee VHL. The characteristics and mechanisms of uptake of PLGA nanoparticles in rabbit conjunctival epithelial cell layers. Pharm. Res. 2004; 21, 641–8. https://doi.org/10.1023/b:pham.0000022411.47059.76.

49 Huang HY, Wang MC, Chen ZY, Chiu WY, Chen KH, Lin IC et al. Gelatin–epigallocatechin gallate nanoparticles with hyaluronic acid decoration as eye drops can treat rabbit dry-eye syndrome effectively via inflammatory relief. Int. J. Nanomedicine. 2018; 13, 7251–73. https://doi.org/10.2147/IJN.S173198.

50 Tatke A, Dudhipala N, Janga KY, Balguri SP, Avula B, Jablonski MM, Majumdar S. In situ gel of triamcinolone acetonide-loaded solid lipid nanoparticles for improved topical ocular delivery: Tear kinetics and ocular disposition studies. Nanomaterials. 2019; 9, 1–17. https://doi.org/10.3390/nano9010033.

51 Sánchez-López E, Espina M, Doktorovova S, Souto EB, García ML. Lipid nanoparticles (SLN, NLC): Overcoming the anatomical and physiological barriers of the eye – Part I – Barriers and determining factors in ocular delivery. Eur. J. Pharm. Biopharm. 2017; 110, 70–5. https://doi.org/10.1016/j.ejpb.2016.10.009.

former are more able to penetrate across the corneal barrier[52]. In the present work, DNA-SLN_C vector was the only one with size above 400 nm. Contrary to the others, this vector was prepared without protamine, a peptide known by their ability to condense DNA, which has been directly related with a reduction in size[53]. Hence, the DNA-SLN_C vector was discarded for the following studies, despite all the SLN_C-based vectors were able to fully bind and protect the genetic material, and to release the DNA although not completely (Figure II).

Electrostatic interactions between the components of the formulation determine the final structure and the physicochemical characteristics of the vectors. All the SLN-based products had a positive surface charge (ranging from +14.7 to +39.3 mV) which promotes their interaction and retention in the cornea. The inner layer of the tear film is the mucous network. This layer is in contact with the corneal epithelium and presents sialic acid and sulfate residues responsible of the negative charge of mucin at physiological pH, which favours the uptake of cationic molecules[54,55].

Once the vectors were obtained, PVA, a viscosity-enhancing agent, was added to a final concentration of 1% with the aim of improving corneal retention and ocular bioavailability. PVA is a non-ionic synthetic linear and hydrophilic polymer, widely employed in ophthalmic preparations because of its biocompatibility, the lack of interaction with many active compounds and its capacity to improve the ocular absorption[56]. It is even a component of

52 Almeida H, Amaral MH, Lobão P, Silva AC, Lobo JMS. Applications of polymeric and lipid nanoparticles in ophthalmic pharmaceutical formulations: Present and future considerations. J. Pharm. Pharm. Sci. 2014; 17, 278–93. https://doi.org/10.18433/j3dp43.

53 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: The importance of the entry pathway. Eur. J. Pharm. Biopharm. 2011; 79, 495–502. https://doi.org/10.1016/j.ejpb.2011.06.005.

54 Irimia T, Ghica MV, Popa L, Anuţa V, Arsene AL, Dinu-Pîrvu CE. Strategies for improving ocular drug bioavailability and corneal wound healing with chitosan-based delivery systems. Polymers-Basel. 2018; 10, 1221. https://doi.org/10.3390/polym10111221.

55 Misra A, Shahiwala A. Applications of Polymers in Drug Delivery. Smithers Rapra, United Kingdom. 2014; 546.

56 Mundada AS. Update on Polymers for Ocular Drug Delivery. Smithers Rapra, United Kingdom. 2011; 198.

contact lenses and artificial corneas[57,58,59]. In artificial tears, ocular lubricants and drug-containing eye drops PVA is normally used at a concentration of 0.5-1.4%[60].

Corneal adhesion of the formulations was evaluated with a previously reported *in vitro* model[61]. While the flow rates of a free solution of the plasmid and the SLN_{EE}-based vectors (DX-SLN_{EE} and HA-SLN_{EE}) were similar to that of water, a statistically significant decrease was observed in the flow rate values after the addition of PVA to these formulations (Figure III). Therefore, this polymer provides an enhancement in the adhesiveness properties that would raise their residence time on the corneal surface. PVA did not modify the flow rate values of HA-SLN_C, which were similar to that obtained for SLN_{EE} formulations combined with PVA.

Rheological studies were conducted in order to obtain complementary information about viscosity of the formulations as well as their flow behaviour. A high viscosity can result in discomfort due to blurred vision and foreign body sensation, leading to a faster elimination for reflex tears and blinks[62,63]. All the SLN formulations without PVA, as well as those with PVA, showed a pseudoplastic behaviour, except for HA-SLN_C. For DX-SLN_{EE} the pseudoplastic behaviour was less evident, and the viscosity effect of PVA could be better appreciated. Indeed, a slight increase in the viscosity values was observed in DX-SLN_{EE} vectors when they were combined with PVA. On the contrary, the viscosity of the formulations prepared with HA was much higher without PVA (Table III and IV), maybe related to the inherent viscous and

57 Maftoonazad N, Shahamirian M, John D, Ramaswamy H. Development and evaluation of antibacterial electrospun pea protein isolate-polyvinyl alcohol nanocomposite mats incorporated with cinnamaldehyde. Mater. Sci. Eng. C. 2019; 94, 393–402. https://doi.org/10.1016/j.msec.2018.09.033.

58 Tummala L, Mihranyan F. Biocompatibility of Nanocellulose-Reinforced PVA Hydrogel with Human Corneal Epithelial Cells for Ophthalmic Applications. J. Funct. Biomater. 2019; 10, 35. https://doi.org/10.3390/jfb10030035.

59 Hou Y, Chen C, Liu K, Tu Y, Zhang L, Li Y. Preparation of PVA hydrogel with high-transparence and investigations on its transparent mechanism. RSC Adv. 2015; 5, 24023–30. https://doi.org/10.1039/C5RA01280E.

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61 Gallarate M, Chirio D, Bussano R, Peira E, Battaglia L, Baratta F, Trotta M. Development of O/W nanoemulsions for ophthalmic administration of timolol. Int. J. Pharm. 2013; 440, 126–34. https://doi.org/10.1016/j.ijpharm.2012.10.015.

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mucoadhesive properties of the HA[64,65,66]. The HA formulations without PVA were markedly pseudoplastic, but the rheological behaviour in the presence of PVA was different depending on the type of SLN. PVA solutions have a netwonian behaviour by themselves[67]. When PVA was added to the HA-SLN_C vector the rheological behaviour became Newtonian, whereas the combination of PVA with the HA-SLN_{EE} vector resulted in pseudoplastic behaviour, although n increased and the change on viscosity from low to high shear rates was less marked.

Since tear fluid possesses pseudoplastic properties, topical administered solutions with the same rheological behaviour will be more advantageous[68]. Indeed, at low shear rate, the high viscosity improves retention time, avoiding drainage[69,70,71]. During blinking, which involves high shear rate, instead, decrease of viscosity allows the formulations to spread over the corneal surface, offering less resistance to blinking, making them well accepted[72,73,74]. However, PVA confers a certain viscosity at high shear rate, improving the corneal retention also during the blinking phase.

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64 Irimia T, Ghica MV, Popa L, Anuţa V, Arsene AL, Dinu-Pîrvu CE. Strategies for improving ocular drug bioavailability and corneal wound healing with chitosan-based delivery systems. Polymers-Basel. 2018; 10, 1221. https://doi.org/10.3390/polym10111221.

65 Huang HY, Wang MC, Chen ZY, Chiu WY, Chen KH, Lin IC et al. Gelatin–epigallocatechin gallate nanoparticles with hyaluronic acid decoration as eye drops can treat rabbit dry-eye syndrome effectively via inflammatory relief. Int. J. Nanomedicine. 2018; 13, 7251–73. https://doi.org/10.2147/IJN.S173198.

66 Salzillo R, Schiraldi C, Corsuto L, D'Agostino A, Filosa R, De Rosa M, La Gatta A. Optimization of hyaluronan-based eye drop formulations. Carbohydr. Polym. 2016; 153, 275–83. https://doi.org/10.1016/j.carbpol.2016.07.106.

67 Mucha M. Rheological properties of chitosan blends with poly(ethylene oxide) and poly(vinyl alcohol) in solution. React. Funct. Polym. 1998; 38, 19–25. https://doi.org/10.1016/S1381-5148(98)00028-5.

68 Dubashynskaya NV, Poshina DN, Raik SV, Urtti A. Polysaccharides in Ocular Drug Delivery. Pharmaceutics. 2019; 11, 1–32. https://doi.org/10.3390/pharmaceutics12010022.

69 Coffey MJ, Decory HH, Lane SS. Development of a non-settling gel formulation of 0.5% loteprednol etabonate for anti-inflammatory use as an ophthalmic drop. Clin. Ophthalmol. 2013; 7, 299–312. https://doi.org/10.2147/OPTH.S40588.

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71 Wang X, Zhang Y, Huang J, Xia M, Liu L, Tian C et al. Self-assembled hexagonal liquid crystalline gels as novel ocular formulation with enhanced topical delivery of pilocarpine nitrate. Int. J. Pharm. 2019; 562, 31–41. https://doi.org/10.1016/j.ijpharm.2019.02.033.

72 Dubashynskaya NV, Poshina DN, Raik SV, Urtti A. Polysaccharides in Ocular Drug Delivery. Pharmaceutics. 2019; 11, 1–32. https://doi.org/10.3390/pharmaceutics12010022.

73 Mundada AS. Update on Polymers for Ocular Drug Delivery. Smithers Rapra, United Kingdom. 2011; 198.

74 Achouri D, Alhanout K, Piccerelle P, Andrieu V. Recent advances in ocular drug delivery. Drug Dev. Ind. Pharm. 2013; 39, 1599-617. https://doi.org/10.3109/03639045.2012.736515.

Another requirement for ophthalmic preparations is an appropriate pH. The ideal pH is as close as possible to tears (7.0 – 7.5) to avoid discomfort[75,76]. However, pH values between 4 and 8 are well tolerated by the eye thanks to the buffering capacity of the tears, which prevents eye irritation[77,78,79]. SLN_{EE}-based formulations showed pH values from 7.3 to 7.5, while the pH of SLN_C-based vectors was 4.0, due to the preparation method which is pH-dependent[80]. The formulation of the vectors with PVA hardly modified the pH values (Table V). The administration of the formulations on the ocular surface of the mice showed no sign of irritation on the external ocular tissues. Moreover, Masson´s trichrome staining of the corneal sections did not show histological changes after three days of treatment in both, wild type and IL-10 KO mice, respect to the non-treated corneas (Figure XII).

Apart from the technological properties of the formulations relevant for a certain administration route, gene medicinal products need to demonstrate efficacy in terms of transfection capacity. Once inside the cell, the genetic material has to overcome different barriers for a successful transfection, including escape from endocytic vesicles, diffusion through the cytoplasm and transport into the nucleus for transcription process[81,82,83]. We firstly evaluated *in vitro* the capacity of the vectors to induce the production of the therapeutic protein IL-10 in HCE-2 cells.

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75 Abelson MB, Udell IJ, Weston JH. Normal human tear pH by direct measurement. Arch. Ophthalmol. 1981; 99, 301. https://doi.org/10.1001/archopht.1981.03930010303017.

76 Yamada M, Mochizuki H, Kawai M, Yoshino M, Mashima Y. Fluorophotometric measurement of pH of human tears in vivo. Curr. Eye Res. 1997; 16, 482–6. https://doi.org/10.1076/ceyr.16.5.482.7050.

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79 Stein HA, Stein RM, Freeman MI. The Ophthalmic Assistant: A Text for Allied and Associated Ophthalmic Personnel. Elsevier Health Sciences. 2012; 894.

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82 Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Rodríguez-Gascón A, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. Int. J. Pharm. 2014; 65, 413-26. https://doi.org/10.1016/j.ijpharm.2014.02.038.

83 del Pozo-Rodríguez A, Delgado D, Solinís MÁ, Rodríguez-Gascón A, Pedraz JL. Solid lipid nanoparticles for retinal gene therapy: Transfection and intracellular trafficking in RPE cells. Int. J. Pharm. 2008; 360, 177–83. https://doi.org/10.1016/j.ijpharm.2008.04.023.

HA-SLN_C formulation was able to induce the production IL-10, achieving similar concentrations to that previously obtained with SLN_{EE} vectors[84].

In addition to the ability to overpass the limiting barriers at intracellular level, the vectors must be able to overcome all the accessibility issues, providing an adequate disposition of the genetic material in the target cells. The multi-component nanosystem developed here, when administered topically to mice as eye drops, remained on the corneal epithelium at least 2 hours after the last dose (Figure VIII). Differences in the distribution were detected depending on the composition of the vector, but also depending on the presence of PVA. The HA-SLN_{EE} vector seems to be the most effective to overcome corneal barriers, since, despite it contains a lower amount of SLNs, higher fluorescence intensity was observed into the corneal epithelial cells. CD44 may be involved in the internalization process of the formulations containing HA[85,86]. CD44 is a receptor able to interact with the HA; it has been found under normal conditions in basal and apical layers of the cornea, and its expression is increased during injury[87]. In fact, Figure VII shows CD44 expression in the epithelial layer of wild type mice. The incorporation of PVA to the vectors formulated with HA increased the fluorescence signal, indicative of a higher retention on the cornea. When both HA and PVA are included in the final formulation, the adhesive properties and the decrease in viscosity seem to facilitate the ability of the vectors to penetrate the cornea. The distribution of the vectors matches with the distribution of GFP (Figure IX), indicative of the cells that were transfected, since GFP is an intracellular protein. Both HA-SLN_{EE} and HA-SLN_C with PVA showed higher GFP expression in corneal epithelium. Biopharmaceutics plays a significant role in the design and evaluation of gene therapy medicinal products. In our study, gene delivery systems able to induce the production of the protein in the stratified and renewable epithelial layer present as advantage the high number of cells that can be transfected for the production of the protein. By contrast, gene expression could be maintained during

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84 Vicente-Pascual M, Albano A, Solinís MÁ, Serpe L, Rodríguez-Gascón A, Foglietta F et al. Gene delivery in the cornea: In vitro & ex vivo evaluation of solid lipid nanoparticle-based vectors. Nanomed. J. 2018; 13, 1847–64. https://doi.org/10.2217/nnm-2018-0112.

85 Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Rodríguez-Gascón A, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. Int. J. Pharm. 2014; 65, 413-26. https://doi.org/10.1016/j.ijpharm.2014.02.038.

86 Ruponen M, Rönkkö S, Honkakoski P, Pelkonen J, Tammi M, Urtti A. Extracellular Glycosaminoglycans Modify Cellular Trafficking of Lipoplexes and Polyplexes. J. Biol. Chem. 2001; 276, 33875–80. https://doi.org/10.1074/jbc.M011553200.

87 Fernandes-Cunha GM, Na KS, Putra I, Lee HJ, Hull S, Cheng YC et al. Corneal Wound Healing Effects of Mesenchymal Stem Cell Secretome Delivered Within a Viscoelastic Gel Carrier. Stem Cells Transl. Med. 2019; 8, 478–89. https://doi.org/10.1002/sctm.18-0178.

longer times in cells that do not undergo cell division, such as the endothelium, but this layer contains a low number of cells and a complicated accessibility, being the last of the five layers of the cornea.

The incorporation of PVA did not influence the transfection capacity of the naked plasmid, but it increased the efficacy of all the vectors; hence, PVA-formulations were selected for the following studies with the vectors bearing the plasmid pUNO1-hIL10. IL-10 is a potent anti-inflammatory cytokine, but its therapeutic use is limited due to biopharmaceutical issues, mainly low ocular bioavailability and short half-life. The beneficial effect of IL-10 in ocular diseases have been showed in promoting corneal transplant survival[88], and in herpetic keratitis models[89] including IL-10 deficient mice[90]. We evaluated the ability of the vectors containing PVA to induce the expression of the IL-10 *de novo* into the cornea in wild type and in IL-10 KO mice. After three days of topical treatment, transfection was detected in the 100% of the sections analysed.

In wild type mice (Figure X), HA-SLN_{EE} and HA-SLN_{C} provided the highest intensity of fluorescence indicative of a raised IL-10 synthesis; these results are consistent with the localization and transfection pattern with vectors bearing the plasmid pcDNA3-EGFP. With all the vectors, IL-10 was detected not only in the epithelium but also in the endothelial layer, being the intensity of fluorescence significant higher in the endothelium of corneas transfected with the HA-SLN_{EE}. Since IL-10 is a secreted protein its presence in a certain layer does not necessarily mean that it was expressed there.

In IL-10 KO mice (Figure XI), the location profile of the secreted IL-10 was similar to that obtained in wild type mice, being present in the epithelium and in the endothelium, although in general, low intensity of fluorescence was observed. HA-SLN_{EE} also showed the highest intensity of fluorescence in IL-10 KO mice, although the differences among formulations were less marked than in wild type mice. It should be considered that the capacity of the cells to be transfected and to produce the transgene protein could be affected by the altered condition derived of the deficiency in IL-10. In fact, the histological structure of the corneas showed differences between IL-10 KO and wild type mice, with the presence of gaps in the former (Figure XII). In this sense,

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88 Tahvildari M, Emami-Naeini P, Omoto M, Mashaghi A, Chauhan SK, Dana R. Treatment of donor corneal tissue with immunomodulatory cytokines: A novel strategy to promote graft survival in high-risk corneal transplantation. Sci. Rep. 2017; 7, 1–8. https://doi.org/10.1038/s41598-017-01065-z.

89 Azher TN, Yin XT, Stuart PM. Understanding the role of chemokines and cytokines in experimental models of herpes simplex keratitis. J. Immunol. Res. 2017; 2017, 2–6. https://doi.org/10.1155/2017/7261980.

90 Keadle TL, Stuart PM. Interleukin-10 (IL-10) ameliorates corneal disease in a mouse model of recurrent herpetic keratitis. Microb. Pathog. 2005; 38, 13–21. https://doi.org/10.1016/j.micpath.2004.09.003.

the disorganization of the corneal layers in IL-10 KO mice probably results in a reduced barrier for diffusion of the secreted protein.

2.5. Conclusions

Topical administration of eye drops containing SLN-based gene delivery systems have shown to be a feasible strategy to address corneal inflammation by *de novo* IL-10 production. The formulation of SLN-based vectors with PVA as viscosity modifier provided the system with the adequate versatility needed to overcome all the extra and intracellular barriers for a successful transfection. After three days of treatment by topical instillation, the multi-component nanosystem mainly transfected corneal epithelial cells in both wild type and IL-10 KO mice, being HA-formulations combined with PVA the most effective ones. IL-10 was even capable of reaching the endothelial layer. These promising results highlight the possible contribution of non-viral gene augmentation therapy to the future clinical approach of corneal gene therapy, although additional studies are necessary to improve and follow up long term-expression of IL-10 and its impact on corneal inflammation.

DISCUSSION

Ocular gene therapy has been at the forefront of translational gene therapy, despite only 1,2% of gene therapy clinical trials address ocular diseases[1]. In 1998 the FDA approved the first therapeutic antisense oligonucleotide, formiversen (Vitravene®), indicated for the treatment of cytomegalovirus retinitis. Later on, in 2004, Pegaptanib (Macugen®) was the first therapeutic aptamer approved by the FDA, for the treatment of age-related macular degeneration. More recently, the FDA and the EMA approved Luxturna®, the first ocular drug based on gene therapy[2], for the treatment of Leber Congenital Amaurosis and retinitis pigmentosa. However, the clinical trials related to ocular diseases registered in the database of Gene Therapy Clinical Trials Worldwide are limited to 37, including those in progress or completed, and, surprisingly, only two of them are targeted to the anterior segment of the eye. This situation may be related to the difficulties in designing safe and effective delivery systems; actually, delivery is considered the main challenge in gene therapy, and it is especially relevant for the success of gene therapy in the cornea. In this sense, the approaches for the treatment of corneal diseases by gene therapy should include self-administered delivery systems able to overcome the corneal barriers that limit the penetration of the genetic material to the cornea, and to provide an adequate availability to the target cells, which is a challenging proposition for the pharmaceutical scientist.

Corneal inflammation is a condition that is not properly tackled with available treatments, and it could lead to severe consequences such as visual impairment and blindness. Advanced therapies, including gene therapy, are new rising approaches under evaluation for the therapeutic management of keratitis. In this sense, a novel option to address corneal inflammation is the administration of interleukin-10 (IL-10), a potent immunomodulatory cytokine. However, its therapeutic use is limited due to biopharmaceutical concerns, mainly the low ocular bioavailability caused by the corneal barrier and its short half-life[3,4]. Therapy based on gene supplementation is a promising alternative consisting in the administration of nucleic

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1 Gene Therapy Clinical Trials Worldwide. Provided by the Journal of Gene Medicine. Jon Wiley and Sons Ltd, 2017. http://www.abedia.com/wiley/indications.php. Accessed 11/05/2020.

2 del Pozo-Rodríguez A, Rodríguez-Gascón A, Rodríguez-Castejón J, Vicente-Pascual M, Gómez-Aguado I, Battaglia LS, Solinís MÁ. Gene Therapy. In: Current Applications of Pharmaceutical Biotechnology. Advances in Biochemical Engineering/Biotechnology. Silva A, Moreira J, Lobo J, Almeida H, (Ed.), Springer International Publishing, Switzerland. 2020; Volume 171, 321–368. https://doi.org/10.1007/10_2019_109.

3 Azher TN, Yin XT, Stuart PM. Understanding the role of chemokines and cytokines in experimental models of herpes simplex keratitis. J. Immunol. Res. 2017, 7261980. https://doi.org/10.1155/2017/7261980.

4 Keadle TL, Stuart PM. Interleukin-10 (IL-10) ameliorates corneal disease in a mouse model of recurrent herpetic keratitis. Microb. Pathog. 2005; 38, 13–21. https://doi.org/10.1016/j.micpath.2004.09.003.

acids, able to provide a sustained synthesis of the IL-10 cytokine in corneal cells, leading to the anti-inflammatory effect.

The most efficient systems for nucleic acid-based therapies are viral vectors. Retroviruses, lentiviruses, adenoviruses and adeno-associated viruses have been used for transfection of the cornea. However, they have been frequently administered by invasive methods, such as intrastromal, intralimbal, intracameral or after removing the corneal epithelium,[5,6,7]. Moreover, the induction of the immune response and inflammation associated to viral vectors also limits their application to inflammatory diseases, including those related to the cornea, even considering its relative immune-privilege[8]. As an alternative, non-viral gene therapy has proven to be a feasible option for corneal gene therapy even after topical administration[9,10,11,12], although efforts to improve its efficacy are required. A known limitation of non-viral vectors respect to viral vectors is that the formers usually do not induce long-term protein expression and they require frequent administration. However, corneal inflammation is usually associated to pathologies that occur in outbreaks and it does not require the long-term expression of anti-inflammatory mediators, in addition, non-viral systems would allow the self-administration of topical instillations when symptoms appear.

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6 Solinís MÁ, del Pozo-Rodríguez A, Apaolaza PS, Rodríguez-Gascón A. Treatment of ocular disorders by gene therapy. Eur. J. Pharm. Biopharm. 2015; 95(Pt B):331-42. doi: https://doi.org/10.1016/j.ejpb.2014.12.022.

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10 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Avilés-Triqueros M, Weber BHF, Fernández E, Rodríguez-Gascón A. Dextran and protamine-based solid lipid nanoparticles as potential vectors for the treatment of X-linked juvenile retinoschisis. Hum. Gene Ther. 2012; 23, 345–355. https://doi.org/10.1089/hum.2011.115.

11 Bauer D, Lu M, Wasmuth S, Li H, Yang Y, Roggendorf M et al. Immunomodulation by topical particle-mediated administration of cytokine plasmid DNA suppresses herpetic stromal keratitis without impairment of antiviral defense. Graefe's Arch. Clin. Exp. Ophthalmol. 2006; 244, 216–25. https://doi.org/10.1007/s00417-005-0070-z.

12 Gupta S, Fink MK, Ghosh A, Tripathi R, Sinha PR, Sharma A et al. Novel combination BMP7 and HGF gene therapy instigates selective myofibroblast apoptosis and reduces corneal haze in vivo. Investig. Ophthalmol. Vis. Sci. 2018; 59, 1045–57. https://doi.org/10.1167/iovs.17-23308.

Among non-viral vectors, solid lipid nanoparticles (SLNs) have been regarded as one of the most effective[13], showing promising results in infectious diseases[14], in lysosomal storage disorders[15] and, especially, in ocular pathologies[16,17].

In the present work, we have formulated nanovectors prepared with two kinds of SLNs: SLN_{EE}, prepared by emulsification/solvent evaporation, and SLN_C, prepared by coacervation. The latter is a newer method, which avoids the use of solvents. Cationic SLN_{EE} were obtained by incorporating the surfactant DOTAP, whereas the cationic agents glycol chitosan or DEAE-dextran were used to confer positive charge to SLN_C. When the nanoparticles were prepared with glycol chitosan, the nanoparticles were called $SLN_{C\text{-}CH}$, and when DEAE-dextran was used, they were called $SLN_{C\text{-}DX}$. The vectors were formed by electrostatic interactions of the different components: SLNs, the plasmid DNA (pcDNA3-EGFP or pUNO1-hIL10), the cationic peptide protamine, and a polysaccharide (bemiparine (BE), dextran (DX) or hyaluronic acid (HA)). The multi-component nature of the nanovectors confers to the system a high versatility. On the one hand, the type of polysaccharide determines the interaction with the targeted cells, and, as a consequence, the internalization process and the intracellular disposition of the genetic material[18,19]. On the other hand, protamine, apart from favouring the transcription process and

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16 Apaolaza PS, del Pozo-Rodríguez A, Torrecilla J, Rodríguez-Gascón A, Rodríguez JM, Friedrich U et al. Solid lipid nanoparticle-based vectors intended for the treatment of X-linked juvenile retinoschisis by gene therapy: In vivo approaches in Rs1h-deficient mouse model. J. Control. Release. 2015; 217:273-83. https://doi.org/10.1016/j.jconrel.2015.09.033.

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19 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Avilés-Triqueros M, Weber BHF, Fernández E, Rodríguez-Gascón A. Dextran and protamine-based solid lipid nanoparticles as potential vectors for the treatment of X-linked juvenile retinoschisis. Hum. Gene Ther. 2012; 23, 345–355. https://doi.org/10.1089/hum.2011.115.

nuclear internalization, also contributes to bind and protect the genetic material at intra and extracellular level, thanks to its cationic nature[20].

The final vectors presented a particle size in the nanometre range, from 150 to 500 nm, and a positive superficial charge, from +15 to +40 mV. These properties facilitate corneal penetration and cellular uptake, improving the ocular bioavailability[21,22]. Nanoparticle size lower than 800 nm avoids ocular irritation or discomfort and favours uptake by corneal cells; in fact, smaller particles are better tolerated than larger ones, and the former penetrate better across the corneal barrier[23,24,25,26]. In addition, nanoparticles with an average diameter ranging from 50 to 400 nm have the ability to overcome physiological barriers, leading to a higher bioadhesion when they are topically administered[27,28].

The efficacy of the SLN-based vectors as gene delivery systems for the cornea was initially assessed *in vitro* in Human Corneal Epithelial (HCE-2) cells. Transfection studies were carried out first with the vectors containing the reporter plasmid pcDNA3-EGFP, encoding the intracellular protein GFP, in order to quantify the percentage of transfected cells, and then, with the vectors bearing the therapeutic plasmid pUNO1-IL10. The hybrid promoter EF-1α/HTLV, incorporated in

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20 Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Rodríguez-Gascón A. Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: the importance of the entry pathway. Eur. J. Pharm. Biopharm. 2011; 79:495-502. https://doi.org/10.1016/j.ejpb.2011.06.005.

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28 Tatke A, Dudhipala N, Janga KY, Balguri SP, Avula B, Jablonski MM, Majumdar S. In situ gel of triamcinolone acetonide-loaded solid lipid nanoparticles for improved topical ocular delivery: Tear kinetics and ocular disposition studies. Nanomaterials. 2019; 9, 1–17. https://doi.org/10.3390/nano9010033.

the plasmid that contains the IL-10 gene, will be likely useful to yield persistent expression of the anti-inflammatory cytokine[29]. The levels of IL-10 secreted by the HCE-2 cells treated with the most effective formulations were over 1 ng/ml, which are expected to exert anti-inflammatory effect. In this sense, in a recent work published by Wang et al.[30], IL-10 levels at 0.8 ng/mL in a three-dimensional inflammation model resulted in reduction of proinflammatory cytokines, such as TNF-α, and successful inhibition of inflammation.

The next step was the *ex vivo* evaluation of the formulations in rabbit explanted corneas, an static model that mimics various physiological conditions, since the different layers of the cornea keep intact. As can be seen in Figure I, vector formulated with $SLN_{C\text{-}CH}$ and protamine and BE-SLN_{EE} vectors were only able to transfect the epithelium, while HA-SLN_{EE} also transfected stromal cells. DX-SLN_{EE} was the most effective, since the green protein was detected widely distributed along the epithelium, the stroma and the endothelium. The *ex vivo* transfection obtained using DX-SLN_{EE} vector is in line with previous results obtained in an *in vivo* study in rats[31], in which after topical administration of a SLN-based formulation, GFP expression was detected in three cell corneal layers.

29 Sakaguchi M, Watanabe M, Kinoshita R, Kaku H, Ueki H, Futami J et al. Dramatic increase in expression of a transgene by insertion of promoters downstream of the cargo gene. Mol. Biotechnol. 2014; 56:621-630. https://doi.org/10.1007/s12033-014-9738-0.

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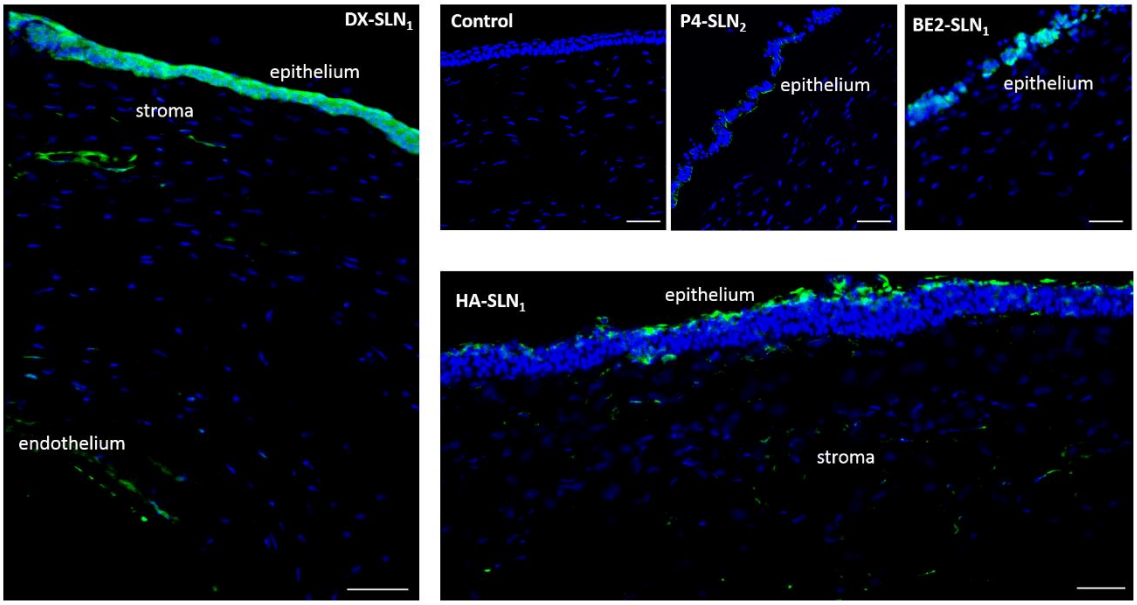

Figure I. GFP transfection in explanted rabbit corneas 48 h after treatment with DX-SLN$_1$ (left), BE2-SLN$_1$ (top right), P4-SLN$_2$ (top middle) and HA-SLN$_1$ (below). As a control (top left), a non-treated cornea immunolabelled with primary and secondary antibodies has been included. Scale bar: 50 µm. BE: Bemiparin; DX: Dextran; GFP: Green fluorescent protein; HA: Hyaluronic acid; SLN: Solid lipid nanoparticle.

Biopharmaceutics plays a significant role in the design and evaluation of gene therapy medicinal products. In our study, the vectors evaluated were able to transfect the epithelium, the stroma, and even the endothelium to varying degrees according to SLN composition and polysaccharide surface coating. On the one hand, the transfection of the epithelium, stratified and renewable, presents the advantage of the high number of cells potentially transfected, and therefore, high production of the protein. On the other hand, transfection of the endothelial cells, with poor division, would contribute to prolong gene expression for longer periods of time (in spite of the fact that this layer contains a low number of cells and more difficult accessibility). Therefore, SLN-based non-viral vectors can be designed to modulate biodistribution and therefore transfection within the different cell layers of the cornea, according to desired therapeutic effect and duration of action. Based on the results from the *in vitro* and *ex vivo* studies, HA-SLN$_{EE}$ and DX-SLN$_{EE}$ vectors were selected for the next stage. In addition, SLN$_C$ was re-formulated and glycol

chitosan was replaced by DEAE-dextran ($SLN_{C\text{-}DX}$), which is a polymer extensively used in nucleic acid delivery[32,33,34,35].

Previously to the *in vivo* studies, technological aspects of the formulations related to the ocular administration route were considered. In this sense, both types of SLNs were biocompatible thanks to the use of well-tolerated physiological lipids in their composition; in addition, their physicochemical stability made possible their use as eye drops. Polyvinyl alcohol (PVA), widely used as viscosity modifier in ophthalmic preparations, was included in the final formulation in order to increase the residence time of the formulation in contact with the cornea and reduce drainage from lachrymal fluid[36,37].

The evaluation of the corneal adhesion of the formulations, with a previously reported *in vitro* model[38], demonstrated that the addition of PVA enhances the adhesive properties of SLN-based vectors. The rheological study showed that all the SLN-based vectors without PVA, as well as those with PVA, presented a pseudoplastic behaviour, except for HA-$SLN_{C\text{-}DX}$ with PVA. Since tear fluid possesses pseudoplastic properties, topical solutions with the same rheological behaviour will be more advantageous[39]. It is well known that non-Newtonian formulations with pseudoplastic behaviour offer less resistance to blinking, making them better accepted than

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Newtonian formulations[39,40,41]. During blinking, which involves high shear rate, low viscosity allows the formulation to spread over the corneal surface. On the contrary, when the shear rate is low, the viscosity increases, improving retention time and avoiding drainage[41,42,43].

pH is another requirement for ophthalmic preparations, which should be as close as possible to that of tears (7.0 – 7.5) to avoid discomfort[44,45], although pH values between 4 and 8 are also well tolerated by the eye[39,41,46]. SLN_{EE}-based formulations showed pH values from 7.3 to 7.5, while the pH of $SLN_{C\text{-}DX}$-based vectors was 4.0, due to the pH-dependency of the coacervation method[47]. The formulation of the vectors with PVA hardly modified the pH values.

The ability of the SLN-based vectors formulated as eye drops to overcome the corneal barriers after topical instillation was assessed in wild type mice. The HA-SLN_{EE} vector was the most effective to penetrate the cornea. The CD44 receptor seems to be involved in the internalization process of the formulations containing HA. Moreover, when both HA and PVA were included in the final formulation, the ability of the vectors to penetrate the cornea improved, which could be related to the higher adhesiveness and the decrease in viscosity observed for that combination in the rheological studies.

Preliminary *in vivo* transfection studies were conducted in wild type mice with the plasmid encoding the intracellular reporter GFP to identify the transfected corneal cells. The distribution of GFP matched with the distribution of the vectors previously observed in the localization study, as can be observed in Figure II, confirming that only the epithelial cells were transfected. HA-SLN_{EE} and HA-$SLN_{C\text{-}DX}$ combined with PVA resulted in higher protein expression than DX-SLN_{EE}

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vectors. These results are not completely consistent with those observed in explanted corneas from rabbits, in which some vectors transfected even the endothelial layer. This fact could be due to the lack of tear washing and to a higher contact time between the formulations and the corneal tissue in the static chamber used in the *ex vivo* studies.

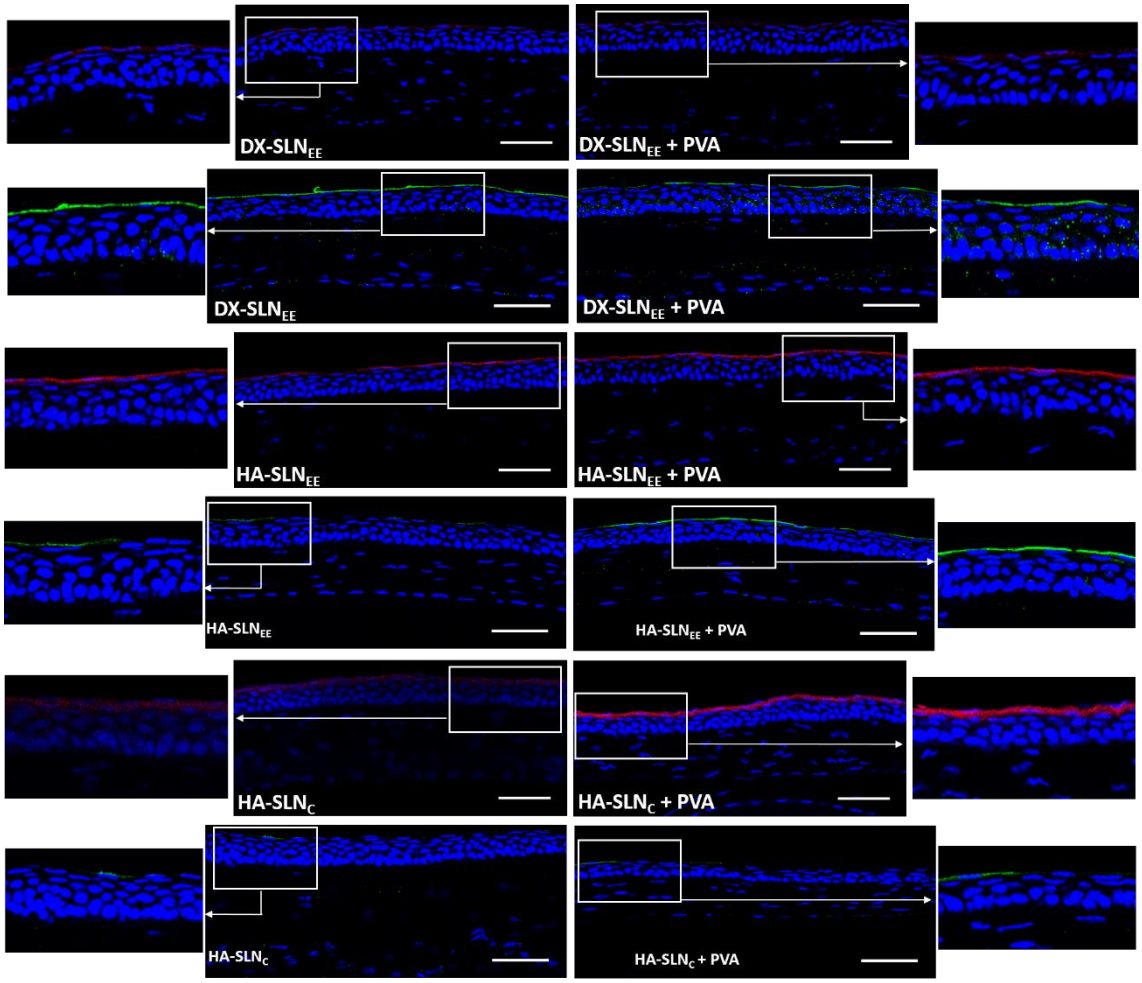

Figure II. Localization of the formulations 2 h after instillation of the last dose and corneal transfection with vectors bearing the plasmid pcDNA3-EGFP with and without PVA in vivo in wild type mice. (20x). Blue: nuclei stained with DAPI. Red: vectors with IR780 iodide labelled SLNs. Green: green fluorescent protein. Scale bar: 50 µm. DX: Dextran; SLN: Solid lipid nanoparticle; PVA: polyvinyl alcohol; HA: Hyaluronic acid.

The incorporation of PVA to the eye drops increased the transfection efficacy of all the vectors. Accordingly, PVA-containing formulations were selected for the following studies with the therapeutic plasmid pUNO1-hIL10 in wild type and in IL-10 KO mice. After three days of topical treatment, IL-10 expression was detected in all the sections analysed from both mice. No signs of irritation in the external ocular tissues, nor histological changes, were detected in the treated corneas. Hence, formulations seem to be well tolerated after repeated topical administration.

In wild type mice, HA-SLN$_{EE}$ and HA-SLN$_{C\text{-}DX}$ provided the highest intensity of fluorescence, indicative of a raised IL-10 synthesis; these results are consistent with the localization and transfection pattern of vectors bearing the plasmid pcDNA3-EGFP. The presence of the secreted

IL-10 in a certain layer does not necessarily mean that it was expressed there. In fact, IL-10 was detected not only in the epithelium but also in the endothelial layer with all the vectors, being the intensity of fluorescence significantly higher in the endothelium of corneas transfected with the HA-SLN_{EE} vector.

The location profile of the secreted IL-10 was similar in IL-10 KO and wild type mice, although a lower intensity of fluorescence was observed in the former, maybe related to a lower capacity of the IL-10-deficient cells to produce the transgene protein. HA-SLN_{EE} was also the most effective formulation, although the differences among vectors were less marked than in wild type mice, probably due to the high disorganization of the corneal layers in IL-10 KO mice.

Additional studies are necessary to follow up the impact of the expression of IL-10 on corneal inflammation, but, overall, from the obtained results, it can be concluded that gene augmentation strategy is a feasible therapy to address corneal inflammation by *de novo* IL-10 production in corneal cells, through the topical administration of SLN-based gene delivery systems formulated as eye drops.

CONCLUSIONS

1. Gene therapy medicinal products for cornea, based on different types of SLNs, were designed with a particle size in the nanometre range, from 150 to 500 nm, and positive superficial charge, from +15 to +40 mV, making them suitable for promoting corneal retention and permeation after topical administration. These nanosystems properly protected and released the genetic material transported, were efficiently uptaken by human corneal epithelial cells (HCE-2) *in vitro*, and they were able to transfect them and to produce the therapeutic protein interleukin-10 (IL-10). The intracellular behaviour of the formulations and their efficacy in the *in vitro* studies were highly dependent on the composition of the SLNs and on the ligands included in the final formulation.

2. SLN-based vectors were able to transfect *ex vivo* explanted corneas from rabbits without affecting the corneal structure. The transfection efficacy and the type of corneal cell transfected depended on formulation factors, and in particular, on the polysaccharide used, being the vectors formulated with dextran or hyaluronic acid the most effective. All the vectors evaluated were able to induce the production of the intracellular green fluorescent protein in epithelial cells, whereas those containing hyaluronic acid also transfected the stroma. However, with vectors prepared with dextran, the protein was widely produced in the epithelium, in the stroma and in the endothelium as well.

3. SLN-based vectors formulated as eye drops were localized mainly in the corneal epithelium after topical administration to wild type mice. The vector based on SLNs prepared with Precirol® ATO5, DOTAP and Tween 80 by emulsification/solvent evaporation, combined with protamine and hyaluronic acid was the most effective to overcome corneal barriers. The localization into the corneal tissue depended not only on the type of vector but also on the presence of the viscosity modifier polyvinyl alcohol (PVA). The higher uptake of these vectors *in vivo* by corneal epithelial cells seemed to be related to the rheological behaviour of the SLN-based vectors formulated with PVA.

4. The formulations containing PVA were able to transfect the corneal epithelium of wild type and IL-10 KO mice, when administered by topical instillation for three days. The formulations containing hyaluronic acid showed the highest production efficiency of IL-10 in both types of mice. The IL-10 cytokine, secreted by the epithelial cells, was able to reach even the endothelial layer. Furthermore, no signs of irritation nor histological changes were detected in the external ocular tissues of the treated corneas.

5. Topical administration of eye drops containing SLN-based gene delivery systems has shown to be a feasible strategy to address corneal inflammation by *de novo* IL-10 production. The results obtained highlight the possible contribution of non-viral gene augmentation therapy

to the future clinical approach of corneal inflammation, although additional studies are necessary to improve and follow up the expression of IL-10 and its impact on corneal histology.

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