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Universidad del País Vasco
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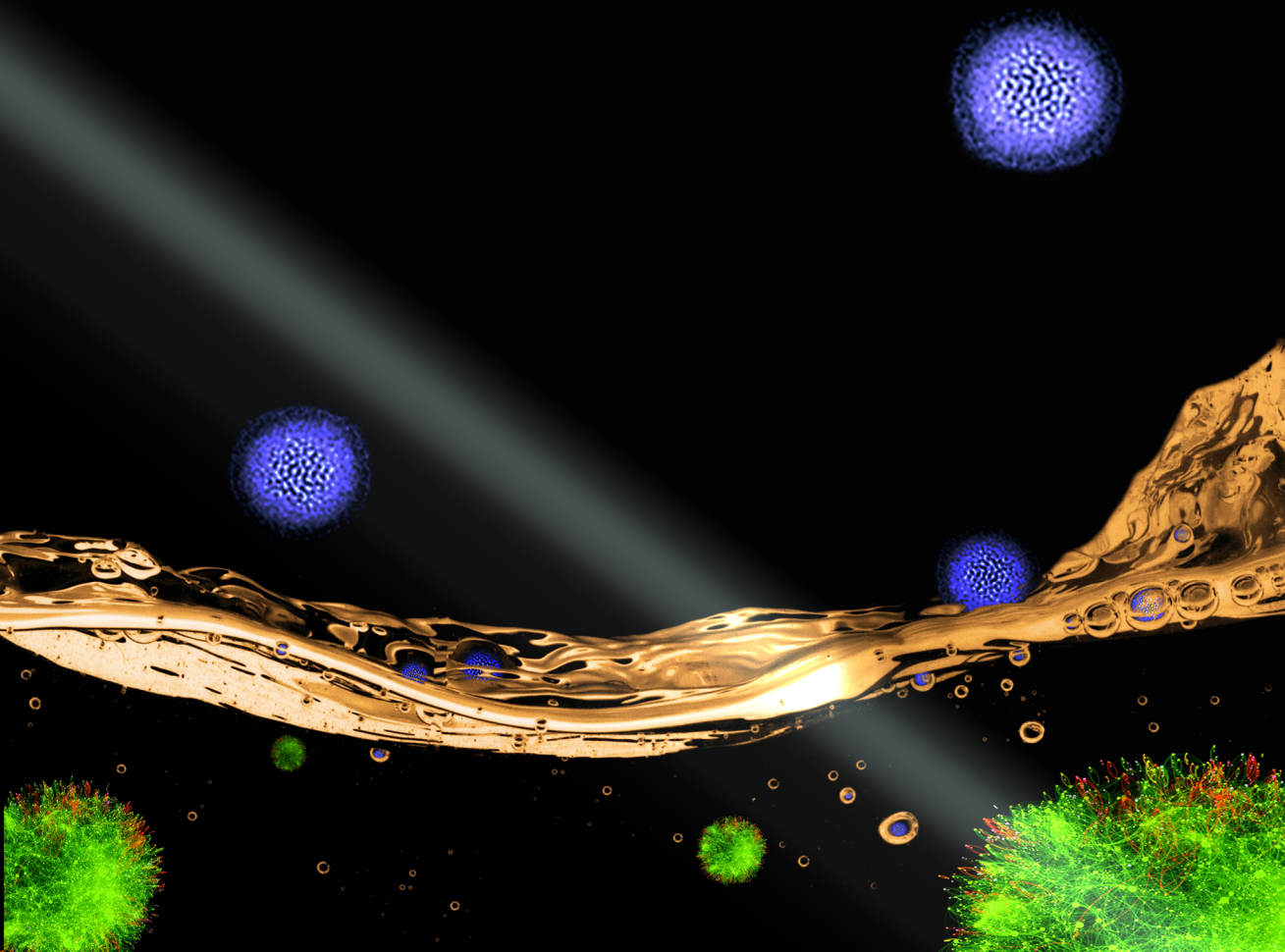
Josune Torrecilla Alzola

Vitoria-Gasteiz 2016



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ESKERRIK ASKO DENOI !!

Josune

Nunca es tarde para intentar lo imposible

El camino a todas las cosas grandes pasa por el silencio
-Friedrich Nietzsche-

Cuando creíamos que teníamos todas las respuestas,
de pronto, cambiaron todas las preguntas
-Mario Benedetti-

GLOSARIO

ADN: ácido desoxirribonucleico

ARN: ácido ribonucleico

ATCC: colección de células americana

BHK-21: células de riñón de hámster

BOS: síndrome de bronquiolitis obliterante

cccDNA: ADN circular covalentemente cerrado

CCK-8: kit de viabilidad celular

CCR5: receptor de quimiocinas beta

CD4: proteína de superficie activadora de los linfocitos T

CD4+ T: linfocitos T que expresan activamente CD4

CD44: receptor de membrana de ácido hialurónico

CD8+ T: linfocitos T que expresan activamente CD8

CLSM: microscopía láser confocal

CN2-29: transgen para las proteínas estructurales de HCV

DDAB: bromuro de dimetil-dioctadecil amonio

DMEM: medio de cultivo Dulbecco's Modified Tagle

DNA: ácido desoxirribonucleico

DNasa: enzima desoxirribonucleasa

DNase: enzima desoxirribonucleasa

DODAG: N',N´-dioctadecil-N-4,8-diaza-10-aminodecanoilglicina

DOPC: 1,2-oleoil-sn-Glicero-3-fosfocolina a

DOPE: dioleoilfosfatidiletanolamina

DOTAP: N-[1-(2,3-Dioleoiloxi)propil]-N,N,N-trimetilamonio

dsRNA: ARN largo de doble hebra

DX: dextrano

EBOV: virus del Ébola

EMA: agencia europea de medicamentos

EMA: monoazida de etidio

EMEM: medio de cultivo Eagle's Minimal Essential

FBS: suero bovino fetal

FDA: agencia de alimentos y medicamentos del gobierno de los Estados Unidos

FLR3-1: células derivadas de la línea célular Huh-7 que soportan un replicón subgenómico de HCV

GAPDH: gliceraldehído-3-fosfato deshidrogenasa

GFP o EGFP: proteína verde fluorescente

HA: ácido hialurónico

HBV: virus de la hepatitis B

HCMV: citomegalovirus humano

HCV: virus de la hepatitis C

HDL: lipoproteína de alta densidad

HEK293: células humanas de riñón embrionario

HeLa: línea celular de adenocarcinoma de cérvix

HIV: virus de la inmunodeficiencia humana

HPAI: Subtipo H5N1 del virus de la influenza aviar tipo A

HPV: virus del papiloma humano

HSV-2: virus herpes simple tipo 2

Huh-7: células de hepatocarcinoma humano

Huh-7 NS3-3': línea celular humana de hepatoma que contiene un transcripto subgenómico de un replicón de HCV

IFN: interferón

IgG: inmunoglobulina G

IgM: inmunoglobulina M

IRES: sitio interno de entrada al ribosoma

LDC: conjugados lípido-fármaco

LDV: velocimetría láser Doppler

LFA-1: receptorde linfocitos T

LNP: nanopartículas lipídicas

mAbd: anticuerpo monoclonal

MDAMB435: línea cellular de cáncer de pecho humano

MDCK: línea celular de epitelio de riñón canino Madin-Darby

miRNA: micro ARN en horquilla

n: número de replicados

NLC: transportadores lipídicos nanoestructurados

NLS: señal de localización nuclear

NP: nucleoproteína

NPC: complejo de poro nuclear

OPTI-MEM: medio de cultivo Dulbecco's Modified Eagle reducido en suero

ORF: marco abierto de lectura

P: protamina

PBS: tampón fosfato salino

PC3: líne celular de cáncer de próstata

PCR: reacción en cadena de la polimerasa

PCS: espectroscopía de correlación fotónica

pDNA: ADN plasmídico

PEG: polietilenglicol

PEG-INT: interferon pegilado

PEG-INT-α: interferón-α pegilado

PFA: paraformaldehido

PLL: poli(L-lisina)

R4-GFP: línea celular derivada de Huh-7 resistente al INF

R6FLR: células derivadas de la línea célular Huh-7 que soportan un replicón subgenómico de HCV

RABV: virus de la rabia

RAV: variantes asociadas a resistencia

rcDNA: DNA circular relajado

RFU: unidades relativas de fluorescencia

RISC: complejo silenciador inducido por ARN

RLB: tampón de lisado celular

RNA: ácido ribonucleico

RNAi: ARN de interferencia

RSV: virus sincitial respiratorio humano

qRT-PCR: reacción en cadena de la polimerasa con transcriptasa inversa

SD: desviación estándar

SDS: laurilsulfato sódico

Sf9: línea celular que deriva de las células IPLB-Sf21-AE de *Spodoptera frugiperda*

shRNA: ARN pequeño en horquilla

siHBV: siRNA específico del gen X del HBV

siRNA: ARN pequeño de interferencia

SLNs: nanopartículas sólidas lipídicas

SNALPs: partículas lipídicas con ácido nucéico estable

sshRNA: shRNA sintéticos

Tat: trans-activador de la transcripción

TLR-9: receptor tipo Toll-9

TEM: microscopio electrónico de transmisión

UTR: región no traducida de los genes

VIH: virus de la inmunodeficiencia humana

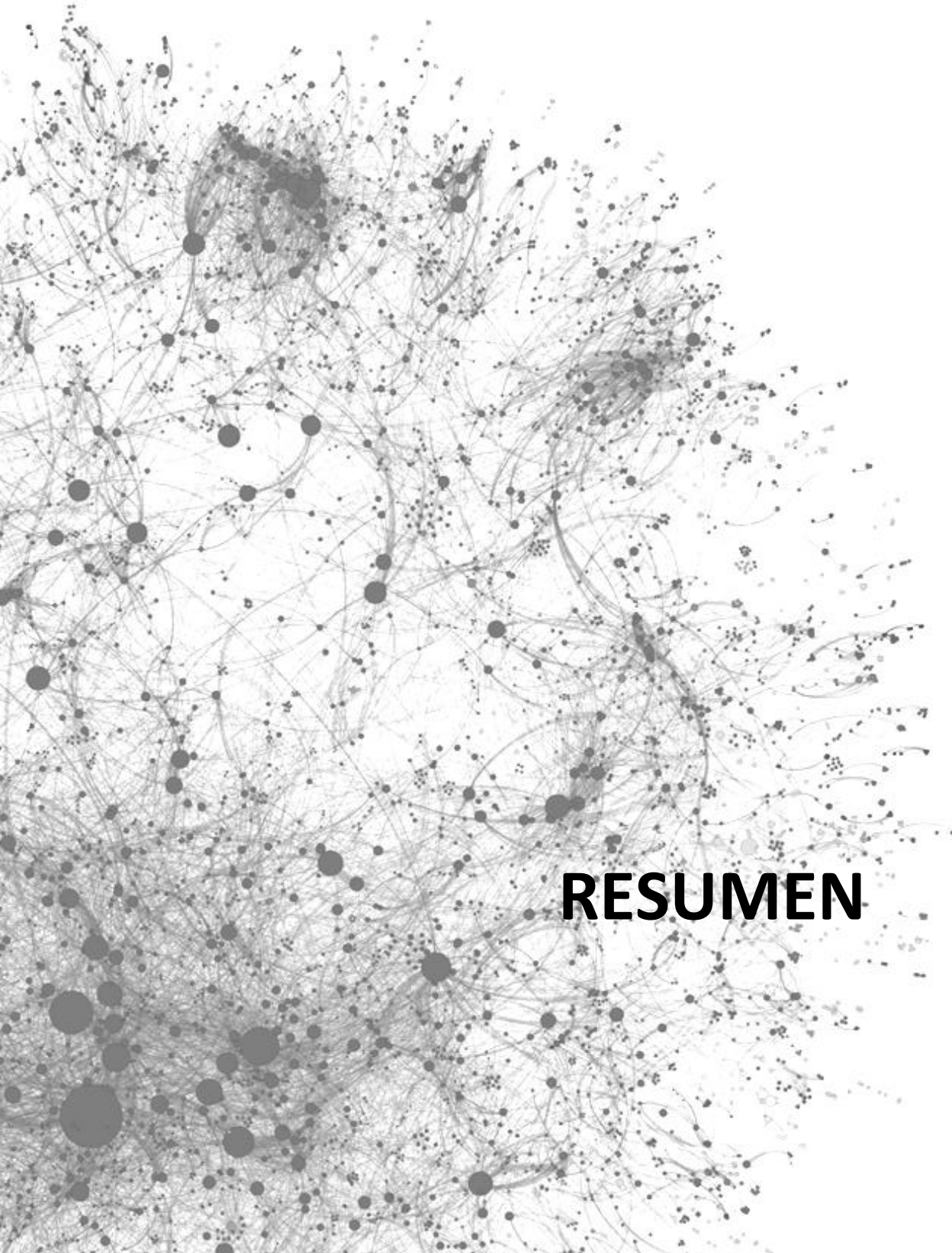
VP: proteina vírica

VP22: proteasa viral del virus herpes simplex

WHO: organización mundial de la salud

ZEBOV: species de Zaire del virus de Ébola

ÍNDICE



RESUMEN

RESUMEN

La hepatitis C sigue siendo hoy en día un problema de salud pública a nivel mundial. Se estima que hay 170 millones de personas infectadas por el virus de la hepatitis C (HCV, del inglés *hepatitis C virus*) en el mundo, con 3-4 millones de nuevas infecciones cada año. Sólo una fracción de las personas infectadas responden al tratamiento estándar consistente en una triple terapia compuesta de interferón-α (INT-α) o interferón-α pegilado (PEG-INT-α), ribavirina e inhibidores de la proteasa (como telaprevir, boceprevir o los recientemente aprobados simeprevir o sofosbuvir). Sin embargo, el éxito del tratamiento depende del genotipo y la carga viral tanto al inicio de la infección como durante el tratamiento. Los efectos adversos, además, son frecuentes y graves. Muchos de los pacientes infectados que no responden a este tratamiento acaban desarrollando una infección crónica caracterizada por una inflamación del hígado que acaba en cirrosis hepática, hepatocarcinoma y finalmente en fallo hepático.

La diversidad de genotipos en los cuales el tratamiento actual no es efectivo, junto con la aparición de resistencias debido a la presión farmacológica selectiva, complican aún más el tratamiento constituyendo esta resistencia al tratamiento antivírico un problema de gran transcendencia sociosanitaria. Existe, por lo tanto, una gran urgencia para desarrollar una estrategia antiviral alternativa a los tratamientos actuales.

Una de las nuevas estrategias propuestas para el tratamiento de la hepatitis C es la terapia génica con ARN de interferencia (RNAi). El RNAi es un mecanismo biológico natural que se basa en la existencia de secuencias de ARN complementarias de manera muy específica con un ARN mensajero (ARNm), de modo que provocan la destrucción del

producto diana o inhiben su síntesis. Desde su descubrimiento a finales del siglo XX, el RNAi ha duplicado su potencial como estrategia terapéutica frente a diferentes enfermedades entre las cuales se encuentran las enfermedades infeccionas.

El HCV es un virus que contiene una sola cadena de ARN (+) con capacidad para replicarse en el citoplasma de las células que infecta, y por tanto el uso del RNAi puede ser una buena estrategia terapéutica capaz de inhibir su replicación. El genoma del virus se compone de secuencias codificantes (tanto de proteínas estructurales del núcleo o de la envoltura del virus, como de proteínas no estructurales), flanqueadas por dos secuencias no codificantes situadas en ambos lados (5´UTR y la 3´UTR). Se trata, además, de un virus con una marcada variabilidad genética y cuyo grado de variabilidad no es homogéneo a lo largo de todo su genoma; no todas las regiones tienen la misma capacidad de mutar. Las regiones más conservadas del genoma son las no codificantes, es decir, la 5´UTR y la 3´UTR y dentro de la región de lectura abierta (ORF, del inglés *open reading frame*) los genes más conservados son los que codifican para proteínas de la nucleocápside, NS3 y NS4, mientras que las zonas más heterogéneas son las que codifican para la envoltura (E1 y E2/NS1) y para las proteínas no estructurales (NS2, NS5). Por lo tanto, debido a las altas tasas de mutación de los virus en respuesta a la presión selectiva, las moléculas de RNAi deberán estar dirigidas a secuencias lo más conservadas posibles dentro del genoma viral.

Otro aspecto a tener en cuenta es el tipo de moléculas de RNAi empleadas. Existe una vía fisiológica de silenciamiento que comienza cuando se expresa un pre-miRNA (micro-RNA precursor), una cadena sencilla que puede plegarse y formar una horquilla de doble cadena denominada miRNA. Otra posibilidad es introducir directamente dsRNAs (del inglés *double-stranded RNA*) sintéticos, o un plásmido que contenga una horquilla que exprese el dsRNA. En este segundo caso, se habla de expresión de shRNAs (siglas en inglés de *short hairpin RNA*). En ambos

casos, estas cadenas dobles de ARN son cortadas por una enzima denominada Dicer en pequeños fragmentos (19 a 24 nt) de doble cadena denominados siRNAs (ARNs cortos de interferencia o *short interfering RNAs*). El bloqueo del ARNm se produce con una alta especificidad con la secuencia diana y es mediado por el complejo RISC (complejo silenciador inducido por ARN o *RNA induced silencing complex*).

La principal ventaja de emplear shRNAs radica en un efecto de silenciamiento más prolongado en el tiempo. Sin embargo, hay que tener en cuenta que para conseguir que la terapia basada en ácidos nucleicos sea efectiva es necesario asegurar que el material genético llegue a la célula diana, así como su protección frente a agentes del medio que podrían degradarlo. Por lo tanto, es preciso diseñar sistemas de administración adecuados para este fin. En este sentido, las nanopartículas sólidas lipídicas (SLNs) son una buena opción debido a su biocompatibilidad, seguridad y facilidad de producción. Otra ventaja importante es la posibilidad de incorporar en su superficie ligandos con el fin de lograr, entre otros, un incremento de la internalización celular o una distribución selectiva a tejidos y órganos concretos, lo que redunda en una mayor eficacia y/o menores efectos adversos.

La protamina, por ejemplo, es un péptido capaz de condensar y proteger el ADN. Además, posee secuencias de localización nuclear (NLS, del inglés *nuclear localization signals*) con un alto contenido en arginina que favorecen la entrada del ADN desde el citoplasma hacia el núcleo celular a través del complejo de poro nuclear (NPC, del inglés *nuclear pore complex*). El dextrano y el ácido hialurónico son polisacáridos biocompatibles que se han usado para incrementar la capacidad de tranfección de las SLNs y que utilizan diferentes mecanismos de entrada celular. Se ha comprobado que el dextrano prolonga el tiempo de circulación de las SLNs *in vivo* dificultando su interacción con los componentes séricos. El ácido hialurónico por su parte, es un polímero

hidrofílico, con carácter mucoadhesivo y que es capaz de prevenir la adsorción de saponinas por repulsión esteárica.

Teniendo esto en cuenta, el objetivo principal de esta tesis es el diseño y evaluación de un vector no viral a base de nanopartículas sólidas lipídicas (SLN) para el tratamiento de la hepatitis C mediante terapia génica basada en RNAi. Las formulaciones se elaboraron utilizando el plásmido shRNA74, complementario a la secuencia IRES (Sitio Interno de entrada al Ribosoma o *internal ribosome entry site*) del genoma vírico situada en el extremo 5´UTR, altamente conservada e imprescindible para la iniciación de la replicación del virus.

En la primera parte del trabajo se evaluaron dos tipos de vectores compuestos por SLNs, protamina (P) y un polisacárido, dextrano (DX) o ácido hialurónico (HA), con diferente carga del plásmido. Para ello los vectores se formaron a dos proporciones shRNA74:SLN : 1:2 (HA-SLN2 o DX-SLN2) y 1:5 (HA-SLN5 o DX-SLN2).

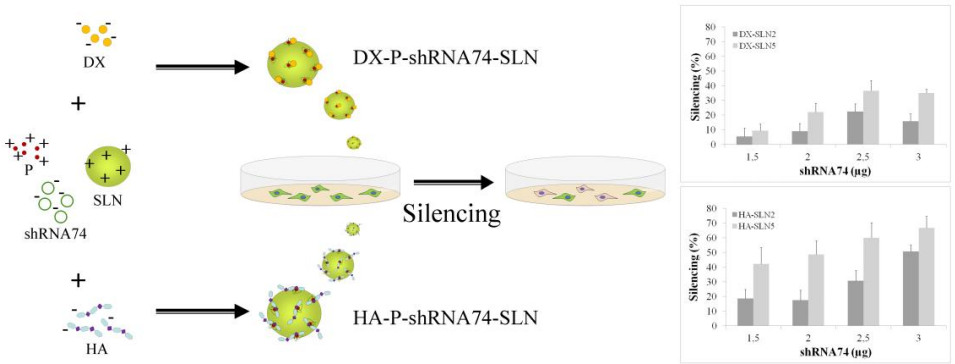


Figura 1. Esquema gráfico de la primera parte del diseño experimental.

Los vectores se caracterizaron en términos de tamaño, carga superficial y capacidad de unión, liberación y protección del plásmido frente a DNasas. El tamaño de todos los complejos fue inferior a 240 nm

de diámetro y la carga superficial en torno a +35 mV. Todos los vectores mostraron una buena capacidad de unión del material genético siendo capaces de protegerlo frente a DNasas y también de liberarlo.

El hígado es el principal órgano diana de la infección crónica por HCV y aunque existe un reservorio extrahepático (células dendríticas y células B, entre otras), el virus se replica preferentemente en el citoplasma del hepatocito. Por lo tanto, el siguiente paso fue la evaluación de la eficacia de silenciamiento de los diferentes vectores en la línea celular de hepatocarcinoma humano HepG2. Para ello, se transfectaron las células con un transfectante comercial para que expresaran el HCV-IRES, que contiene la secuencia IRES del genoma del virus junto con la secuencia que codifica la proteína verde fluorescente (GFP).

Se comparó la eficacia de silenciamiento de los vectores a diferentes dosis de plásmido, así como también la viabilidad celular. En cuanto a la eficacia de silenciamiento, las formulaciones elaboradas con HA resultaron ser más efectivas que las preparadas con DX. Con ambas formulaciones se observó que la eficacia de silenciamiento depende de la dosis de plásmido administrada. El mayor silenciamiento, 66%, se obtuvo con el vector HA-SLN5 a la mayor dosis de shRNA74 (3 µg), frente al 36% que se obtuvo con el vector DX-SLN5 a la misma dosis. La viabilidad celular, indicativa de una potencial toxicidad, también dependió de la dosis de shRNA74; con los vectores preparados con HA descendió desde un 90% con la menor dosis hasta un 65% con la dosis mayor (3 µg de shRNA74). El mismo comportamiento se observó con los vectores preparados con DX, aunque con menor descenso de los valores de viabilidad. Por lo tanto, es tremendamente importante llegar a un equilibrio entre eficacia de silenciamiento y viabilidad, ya que una de las premisas de la utilización de vectores no virales es no comprometer la viabilidad celular.

Unos de los procesos que condicionan enormemente la capacidad de transfección de los vectores es la capacidad de penetrar en el interior de las células diana. Nuestros vectores fueron captados de manera rápida y efectiva, siendo la captación mayor cuanto mayor era la proporción shRNA74:SLN. Sin embargo, no se observaron diferencias en la captación dependiendo del polisacárido empleado en la preparación de los vectores. Por lo tanto, las diferencias en la eficacia de silenciamiento entre los vectores preparados con HA y los preparados con DX no parecen deberse a diferencias en la capacidad de entrar en las células, sino que podrían estar condicionadas, en parte, por la mayor capacidad de los vectores HA-SLN para proteger el ADN, y en parte por el mecanismo de internalización celular de las nanopartículas, que depende tanto de la línea celular como de la composición del vector. En general, las SLNs son internalizadas por mecanismos de endocitosis, y dependiendo de la línea celular y de la composición de los vectores, pueden utilizar la endocitosis mediada por clatrinas o mediada por caveolas. En las células HepG2 estos mecanismos están presentes y los vectores podrían utilizar estas dos vías. Sin embargo, el vector preparado con HA podría ser captado por las células tras la interacción con el receptor CD44 (presente en las células HepG2), ya que el HA es un sustrato de este receptor. El hecho de que el vector HA-SLN utilizara, al menos en parte, esta vía de entrada y el vector DX-SLN no, podría justificar también las diferencias en la eficacia de silenciamiento. Además, el HA es capaz de modular el alto grado de condensación del plásmido debido al efecto de la protamina, facilitando así la liberación del mismo.

Por lo tanto, este estudio demuestra la capacidad de los vectores no virales compuestos por SLN, protamina y HA o DX, junto con el plásmido shRNA74 para silenciar la expresión de HCV-IRES en la línea celular HepG2, siendo más eficaces aquellos formulados con HA. En base a estos resultados, se seleccionó el vector SLN-HA para los estudios posteriores.

En la segunda parte del diseño experimental de la tesis, con el fin de realizar una aproximación más real, se evaluó la eficacia de los vectores en la línea celular humana de hepatoma (Huh-7 NS3-3') que contiene un transcripto subgenómico de un replicón de HCV.

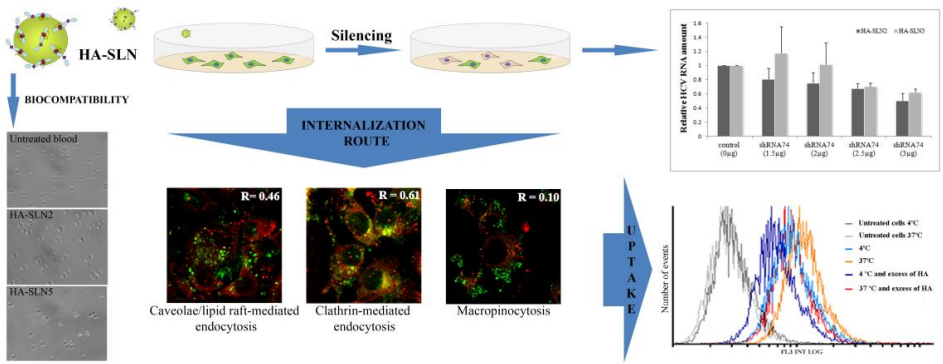


Figura 2. Esquema gráfico de la segunda parte del diseño experimental.

Previamente se comprobó que la eficacia de silenciamiento en la línea celular Huh-7 transfectada con el IRES-GFP era similar a la obtenida en las células HepG2. Posteriormente se evaluó la eficacia de los vectores para inhibir de la replicación del replicón en las células Huh-7 NS3-3'. Mediante qRT-PCR se cuantificó la reducción en la cantidad de ARN correspondiente al replicón subgenómico del virus. Se observó que la mayor reducción se obtiene con el vector HA-SLN5 y con la mayor dosis de shRNA74, dando lugar a una inhibición del 50% de la replicación viral. Estos resultados confirman la potencial utilidad de los vectores en el tratamiento de la infección crónica por el HCV.

Dada la importancia que tiene la capacidad de entrada en las células, ya que condiciona la eficacia de los vectores, el siguiente paso consistió en evaluar en mayor profundidad los mecanismos de entrada y la disposición intracelular de los vectores en las células Huh-7.

En primer lugar se comprobó la presencia en las células Huh-7 de los principales mecanismos de internalización celular utilizados por las nanopartículas lipídicas: macropinocitosis, endocitosis mediada por caveolas y clatrinas, y entrada mediante el receptor CD44. El bloqueo de la endocitosis, proceso dependiente de energía que tiene lugar a 37^{o}C, y uno de los principales mecanismos de entrada de los vectores no virales, produjo una disminución en la captación de los vectores y en el nivel de silenciamiento. El hecho de que el nivel de silenciamiento se redujera en mucho mayor grado que la reducción en la internalización celular indicaría que la endocitosis es un mecanismo muy efectivo para el proceso de transfección y silenciamiento. Entre los diferentes mecanismos de endocitosis, la entrada mediada por clatrinas resultó ser el mecanismo más utilizado por los vectores, en menor medida la endocitosis mediada por caveolas y prácticamente nula la entrada por macropinocitosis.

Otros procesos no dependientes de energía pueden participar en la captación celular de los vectores. El bloqueo de la captación celular mediada por el receptor CD44, presente en las células Huh-7 y al que se une el HA, redujo la internalización de los vectores, pero no dio lugar a un descenso del silenciamiento. Esta vía de entrada por receptor, por lo tanto, parece ser menos efectiva que la entrada por endocitosis. El hecho de que al bloquear el receptor CD44 se inhiba la captación y no el silenciamiento puede ser debido a que al inhibir una vía de entrada se pueden estar activando otros mecanismos. Hay que tener en cuenta que dada la complejidad de los sistemas biológicos, pueden participar simultáneamente varios mecanismos de internalización y que el bloqueo de uno de ellos puede inducir los otros.

También se estudió la disposición intracelular del material genético y cómo está condicionada por la composición del vector. Se comprobó a nivel intracelular el alto grado de condensación del shRNA74, debido en parte al lípido catiónico de la nanopartícula y a la protamina. También se

observó la disminución de la condensación a lo largo del tiempo y su aproximación a la membrana nuclear.

Finalmente, y teniendo en cuenta que el órgano diana en el tratamiento de la hepatitis C es el hígado y considerando una posible administración parenteral, se evaluó la capacidad de los vectores para producir cambios a nivel hematológico que pudieran ser indicativos de una potencial toxicidad. En concreto, evaluamos el efecto hemolítico y el efecto sobre la aglutinación de eritrocitos y no observamos ni hemaglutinación ni actividad hemolítica significativa, probablemente debido a la estabilización estérica del HA, que impide la unión con componentes sanguíneos, lo que hace factible la posibilidad de llevar a cabo estudios *in vivo* en animales modelo de la enfermedad.

En conclusión, esta tesis ha demostrado la capacidad de los vectores no virales basados en SLNs, P y HA, como sistemas de administración del plásmido shRNA74, para inhibir *in vitro* la replicación del HCV. Los resultados aquí presentados sirven como prueba de concepto de la utilidad de los vectores basados en SLNs como una nueva estrategia terapéutica para el tratamiento de la infección crónica por el HCV. Es evidente que son necesarios estudios en modelos animales de infección por HCV para confirmar *in vivo* el efecto antiviral.

INTRODUCCIÓN

Capítulo 1

Lipid nanoparticles as carriers for RNAi against viral infections: current status and future ferspectives

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The efforts made to develop RNAi-based therapies have led to productive research in the field of infections in humans, such as hepatitis C virus (HCV), hepatitis B virus (HBV), human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), herpetic keratitis, human papillomavirus, or influenza virus.

Naked RNAi molecules are rapidly digested by nucleases in the serum, and due to their negative surface charge, entry into the cell cytoplasm is also hampered, which makes necessary the use of delivery systems to exploit the full potential of RNAi therapeutics.

Lipid nanoparticles (LNP) represent one of the most widely used delivery systems for in vivo application of RNAi due to their relative safety and simplicity of production, joint with the enhanced payload and protection of encapsulated RNAs. Moreover, LNP may be functionalized to reach target cells, and they may be used to combine RNAi molecules with conventional drug substances to reduce resistance or improve efficiency. This introduction features the current application of LNP in RNAi mediated therapy against viral infections and aims to explore possible future lines of action in this field.

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1. Introduction

Gene therapy is a relatively recent approach in the management of human diseases and has resulted in an increasingly interest as therapeutic strategy. While traditional drug therapies involve the administration of therapeutic chemicals that have been synthesized outside the body, gene therapy tries to direct patient's cells to produce and deliver a therapeutic agent or to knock down the production of undesirable molecules.

Gene therapy was first defined as the administration of genetic material into a human patient with the intent of correcting a specific genetic defect[1]. This definition appeared in the first gene therapy protocols in the early 90s, related to trials that aimed to correct the effects of some monogenic recessive diseases. Nowadays, the European Agency of medicines (EMA) defines gene therapy as biological medicinal products which fulfils the following two characteristics: (a) the active substance contains or consists of a recombinant nucleic acid applied to human beings in order to regulate, repair, replace, add, or delete a genetic sequence; (b) its beneficial effect relates directly to the recombinant nucleic acid sequence it contains or to the result of genetic expression of this sequence[2].

Since the first FDA-approved gene therapy experiment in 1990[3,4], more than 2200 clinical trials have been performed using a number of

[1] United States Congress, Office of Technology Assessment [OTA], Human Gene Therapy-Background Paper, Washington, DC, USA.

[2] Committee for Advanced Therapies (CAT), Reflection paper on classification of advanced therapy medicinal products, EMA/CAT/600280/2010, 2012.

[3]Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC, Topalian SL, et al. Gene transfer into humans-immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med*. 1990 Aug 30;323(9):570-578.

techniques for gene therapy up to July 2015[5]. Table 1 collects the indications addressed by gene therapy clinical trials and the number of events related to each indication. As observed in this table, infectious diseases are in the fourth place in the ranking, with a percentage with respect to total approved clinical trials similar to that of cardiovascular diseases.

Table 1: Indications addressed by gene therapy clinical trials up to July 2015.

Ranking	Indication	Number of trials (n)	Percentage with respect to total gene therapy clinical trials
1	Cancer diseases	1415	64
2	Monogenic diseases	209	9.5
3	Cardiovascular diseases	175	7.9
4	Infectious diseases	174	7.9
5	Healthy volunteers	53	2.4
6	Gene marking	50	2.3
7	Other diseases	46	2.1
8	Neurological diseases	43	1.9
9	Ocular diseases	31	1.4
10	Inflammatory diseases	14	0.6

Nucleic acid-based therapy has been traditionally focused on the use of DNA as the active substance, but since the discovery of the RNA

[4] Sheridan C. Gene therapy finds its niche. *Nat Biotechnol*. 2011 Feb;29(2):121-128.

[5] The Journal of Gene Medicine Clinical Trial site, http://www.abedia.com/wiley/indications.php.

interference (RNAi) pathway[6], RNA has also gained great interest. Among the multiple possible applications of the RNAi-based therapy, numerous studies have demonstrated its potential in the control and treatment of different viral infections in humans, such as hepatitis C virus (HCV), hepatitis B virus (HBV), human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), herpetic keratitis, human papillomavirus, or influenza virus[7,8,9,10,11,12,13].

A key challenge to exploit the full potential of RNAi therapeutics is their efficient delivery to the target cells. On the one hand, naked RNAi molecules are rapidly digested by nucleases in the serum after systemic administration; on the other hand, due to the negative surface charge of RNA, the entry into the cell cytoplasm is hampered. A number of techniques have been attempted to overcome these problems: physical methods such as electroporation or hydrodynamic injection[14,15], viral

[6] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by doublestranded RNA in caenorhabditis elegans. *Nature*. 1998 Feb 19;391(6669):806-811.

[7] Kim B, Tang Q, Biswas PS, Xu J, Schiffelers RM, Xie FY, Ansari AM, Scaria PV, Woodle MC, Lu P, Rouse BT. Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. *Am J Pathol.* 2004 Dec;165(6):2177-2185.

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[11] Singhania R, Khairuddin N, Clarke D, McMillan NA. RNA interference for the treatment of papillomavirus disease. *Open Virol J*. 2012;6:204-215.

[12] Vlachakis D, Tsiliki G, Pavlopoulou A, Roubelakis MG, Tsaniras SC, Kossida S. Antiviral stratagems against HIV-1 using RNA interference (RNAi) technology. *Evol Bioinform Online*. 2013 May 16;9:203-213.

[13] Betáková T, Svančarová P. Role and application of RNA interference in replication of influenza viruses. *Acta Virol*. 2013;57(2):97-104.

[14] Ovcharenko D, Jarvis R, Hunicke-Smith S, Kelnar K, Brown D. High-throughput RNAi screening “in vitro”: from cell lines to primary cells. *RNA*. 2005 Jun;11(6):985-993.

[15] Dyer V, Ely A, Bloom K, Weinberg M, Arbuthnot P. TRNALys3 promoter cassettes that efficiently express RNAiactivating antihepatitis B virus short hairpin RNAs. *Biochem Biophys Res Commun*. 2010 Aug 6;398(4):640-646.

vectors[16], or lipid and polymeric nanoparticles[17,18]. Among them, lipid nanoparticles (LNP) represent one of themost widely studied delivery systems for *in vivo* application of RNAi[19,20]. The aim of this review is to collect the state of the art and the future perspectives of the utility LNP as RNAi vectors for human viral infections.

2. RNA interference (RNAi)

RNAi is a naturally occurring process of gene regulation present in plants and mammalian cells. The first evidence of the existence of this mechanism appeared in 1998, when Fire et al.[6] observed in *Caenorhabditis elegans* that doublestranded RNAs (dsRNAs) were the basis of sequence-specific inhibition of protein expression. Subsequent works demonstrated that the molecules that induced RNAi were short dsRNAs, of 21 nucleotides in length, called short interfering RNAs

[16] Machitani M, Sakurai F, Katayama K, Tachibana M, Suzuki T, Matsui H, Yamaguchi T, Mizuguchi H. Improving adenovirus vector-mediated RNAi efficiency by lacking the expression of virus-associated RNAs. *Virus Res*. 2013 Dec 26;178(2):357-363.

[17] Novobrantseva TI, Borodovsky A, Wong J, Klebanov B, Zafari M, Yucius K, Querbes W, Ge P, Ruda VM, Milstein S, Speciner L, Duncan R, Barros S, Basha G, Cullis P, Akinc A, Donahoe JS, Narayanannair Jayaprakash K, Jayaraman M, Bogorad RL, Love K, Whitehead K, Levins C, Manoharan M, Swirski FK, Weissleder R, Langer R, Anderson DG, de Fougerolles A, Nahrendorf M, Koteliansky V. Systemic RNAi-mediated gene silencing in nonhuman primate and rodent myeloid cells. *Mol Ther Nucleic Acids*. 2012 Jan 24;1:e4.

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[20] Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. *Nat Rev Drug Discov*. 2009 Feb;8(2):129-138.

(siRNAs), and that siRNAs were able to start the RNAi process in mammalian cells[21].

The RNAi response is activated when the dsRNA is processed by a ribonuclease III-like enzyme called Dicer, resulting in the formation of a siRNA. The siRNA is incorporated into the RNA induce silencing complex (RISC), where a helicase unwinds the duplex siRNA. The resulting antisense strand guides the RISC to its complementary mRNA, which will be cleaved[22]. Typically, there are three different types of commonly used RNAi molecules: siRNA, short-hairpin RNA (shRNA, also named expressed RNAi activators), or microRNA (miRNA).

siRNAs, as mentioned before, are dsRNA molecules of about 19–23 base pair nucleotides in length, able to mediate site specific cleavage and destruction of the targeted mRNA[23].

shRNAs consist of two complementary 19–22 bp RNA sequences linked by a short loop of 4–11. These RNAs are synthesized within the cell by DNA vector-mediated production[24]. shRNAs can be transcribed through either RNA polymerase II or III. The first transcript generates a hairpin like stem-loop structure and then is 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. shRNAs represent an important tool in the assessment of gene function in mammals and are largely used as a research tool.

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[22] Hammond SM. Dicing and slicing: the core machinery of the RNA interference pathway. *FEBS Lett*. 2005 Oct 31;579(26):5822-5829.

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[24] Moore CB, Guthrie EH, Huang MT, Taxman DJ. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. *Methods Mol Biol*. 2010;629:141-158.

miRNAs are single stranded RNAs of about 20–24 nucleotides. This kind of RNAs acts as endogenous posttranscriptional repressors to down regulate gene expression[25]. miRNAs are transcribed from DNAas primary miRNA (pri-miRNA); in this case, primiRNAs are processed into precursor miRNA (pre-miRNA) by two proteins: Drosha and Pasha. Pre-miRNAs are then transported to the cytoplasm, and after processing by Dicer and unwinding to obtain the miRNA, the following steps are identical to those that occur with siRNA and shRNA as it is illustrated in Figure 1.

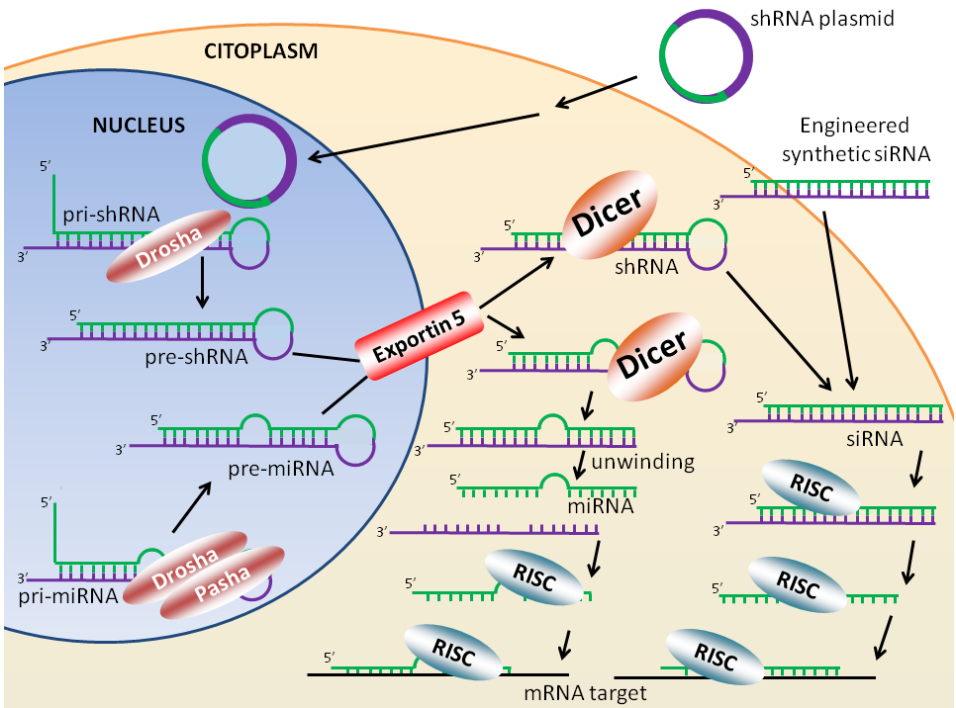

Figure 1: RNAi mechanism. Differences between siRNA, shRNA, and miRNA as therapeutic tools.

[25] Zhang Y, Wang Z, Gemeinhart RA. Progress in microRNA delivery. *J Control Release*. 2013 Dec 28;172(3):962-974.

Recent observations have shown that both miRNAs and siRNAs can suppress translation of mRNAs (in the case of an imperfect match) and can cleave target RNAs (in the case of a perfect match) and play a decisive role in gene and genome regulation[26]. It has been also observed that shRNA can act via miRNA- or siRNA-like mechanism. It is believed that the miRNA-like mechanism is faster than siRNA-like mechanism, because the latter acts via perfect complementarity for a target message[27].

The main advantage of synthesized siRNAs is that these molecules do not need to reach the nucleus to exert effect. However, modifications are necessary to increase their stability, resulting in some cases in loss of siRNA function[28]; there must be a balance between stability and efficacy when modifications are introduced in siRNA. When miRNAs or shRNA expression plasmids are used, they must initially reach the nucleus of cells to be processed. The resulting molecules are then transported to the cytoplasm and finally incorporated into the RISC for activity. Therefore, a limitation of miRNA and shRNA is the need to be delivered into the cell nucleus, although they have a greater durability and higher silencing capacity than siRNAs[27].

RNAi seems to play an important role in the antiviral defense mechanism in human cells, suggesting its potential use as therapeutic in human infectious diseases. For instance, HIV-1 shows higher replication capacity in cells that have suffered knockout of Dicer and Drosha

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[27] Rao DD, Vorhies JS, Senzer N, Nemunaitis J. siRNA vs. shRNA: similarities and differences. *Adv Drug Deliv Rev*. 2009 Jul 25;61(9):746-759.

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expression[29]. In this sense, the mammalian stomatitis virus achieved increased accumulation in *Caenorhabditis elegans* with defective RNAi machinery[30]. It has been also observed that the interferon (IFN) pathway works in coordination with miRNA to control viral infections. IFN-β can induce the expression of several cellular miRNAs that form almost perfect nucleotide base pair matches with the HCV genome. When these miRNAs are artificially introduced, the antiviral effects of IFN-β in HCV are reproduced, and the IFN response is lost when they are experimentally removed[31].

As mentioned above, to take advantage of the potential of RNAi as antiviral therapy, effective delivery is essential; in this regard, lipid-based systems are being widely used as RNAi delivery vectors.

3. Lipid nanoparticles (LNP)

Lipid-based systems have been increasingly recognized as one of the most promising delivery systems for RNAi. Lipid carriers may be available in solid, semisolid, or liquid state in the form of solid lipid nanoparticles, nanostructured lipid carriers, lipid drug conjugate nanoparticles, liposomes, or nanoemulsions. These lipid-based systems were initially designed to address some of the challenges of conventional drug delivery systems, such as the increase of bioavailability of poorly soluble drugs, among others. Nowadays, the application of LNP in other

[29] Triboulet R, Mari B, Lin YL, Chable-Bessia C, Bennasser Y, Lebrigand K, Cardinaud B, Maurin T, Barbry P, Baillat V, Reynes J, Corbeau P, Jeang KT, Benkirane M. Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science*. 2007 Mar 16;315(5818):1579-1582.

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fields, such as gene therapy, has gained attraction. RNAi-lipid-based nanocarriers are able to provide protection from serum nucleases and extended circulation, which results in a higher access to the target tissue[32]. Sometimes, targeting is achieved by surface modification of nanocarriers with specific ligands to target cell populations, such as mannan-modified nanoparticles to direct vectors to alveolar macrophages[33]. Once in the target tissue, the RNA-delivery system will be internalized by the target cell and, upon receptor-mediated endocytosis, will be able to escape from the endosomal compartment into the cell cytoplasm where RNA machinery is located, while avoiding lysosomal enzymes[34]. When these delivery systems are applied to the treatment of viral infections, multiadministration treatment modalities are possible for improved clinical outcomes[35]. Moreover, due to their biocompatibility and their ease of large-scale production, large batches with reproducible specifications are possible.

3.1. Solid lipid nanoparticles (SLNs)

SLNs are considered to be one of the most effective lipid-based colloidal carriers. SLNs are in the submicron size range of 50–1000 nm and are composed of physiologically compatible lipids recognized as safe, which are in solid state at room temperature. They consist of a solid lipid core surrounded by a layer of surfactants in an aqueous dispersion, with

[32] Gomes-da-Silva LC, Fonseca NA, Moura V, Pedroso de Lima MC, Simões S, Moreira JN. Lipid-based nanoparticles for siRNA delivery in cancer therapy: paradigms and challenges. *Acc Chem Res*. 2012 Jul 17;45(7):1163-1171.

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multiple potential combinations of lipids and surfactants[36,37]. The interest on SLNs has led to the development of different types of production methods (i.e., high-pressure homogenization) successfully implemented in pharmaceutical industry. The SLNs obtained with these techniques showlong-term stability and the possibility to be subjected to commercial sterilization and lyophilized procedures[38,39,40].

SLNs are used not only as conventional drug delivery systems but also as carriers for therapeutic peptides, proteins or antigens, and bioactive molecules. Mannosylated SLNs loaded with hepatitis B surface antigen (HBsAg) were subcutaneously administered *in vivo* in mice and sustained antibody titer was obtained; these results demonstrated the potential of SLNs as carriers for vaccine delivery against HBV[41].

As gene delivery systems, SLNs have been studied over the last years for a large number of diseases[40,42,43] and different routes of administration[44]. Cationic lipids are used to prepare SLNs due to their positive surface charge that interacts electrostatically with the negative

[36] Vighi E, Ruozi B, Montanari M, Battini R, Leo E. pDNA condensation capacity and *in vitro* gene delivery properties of cationic solid lipid nanoparticles. *Int J Pharm*. 2010 Apr 15;389(1-2):254-261.

[37] Cortesi R, Campioni M, Ravani L, Drechsler M, Pinotti M, Esposito E. Cationic lipid nanosystems as carriers for nucleic acids. *N Biotechnol*. 2014 Jan 25;31(1):44-54.

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[44] Priano L, Esposti D, Esposti R, Castagna G, De Medici C, Fraschini F, Gasco MR, Mauro A. Solid lipid nanoparticles incorporating melatonin as new model for sustained oral and transdermal delivery systems. *J Nanosci Nanotechnol*. 2007 Oct;7(10):3596-3601.

charge of the nucleic acids. Our research group has developed nonviral vectors based on cationic SLNs[45,46] decorated with peptides[47,48], dextran[42], oligochitosans[49], or hyaluronic acid[50] able to transfect several cell lines and tissues both *in vitro* and *in vivo*[51,52,53]. To date, vectors that were developed for DNA delivery are being applied for siRNA delivery. In spite of their different physicochemical properties and the need to be transported to different parts of the cell (plasmid DNA needs to be transported into nucleus for gene expression whereas siRNA reaches its target in the cytoplasm), a number of publications describe the use of SLNs for delivery of both DNA[33,54,55,56] and RNAi mediated

[45] Rodríguez-Gascón A, Solinís MÁ, del Pozo-Rodríguez A, Delgado D, Pedraz JL. 2012. Inventors. University of the Basque Country UPV/EHU. Lipid nanoparticles for gene therapy. US 20120183589 A1.

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molecules[57,58,59,60] with successful results. Focusing on RNAi delivery, Montana et al.[60] demonstrated the utility of cationic SLNs, produced by microemulsion using Compritol ATO 888 as matrix lipid, Pluronic F68 as tensioactive, and dimethyldioctadecylammonium bromide (DDAB) as cationic lipid, as RNA carriers. In another study[58], SLNs composed of cholesteryl ester, triglyceride, cholesterol, dioleoylphosphatidylethanolamine (DOPE), and 3beta-[N-(N',N'-dimethylaminoethane) carbamoyl]-cholesterol (DC-chol) were able to bind electrostatically siRNA conjugated with polyethylene glycol (PEG) (siRNA-PEG). When compared to polyethylenimine, a commonly used transfectant polymer, the SLN-based system showed similar gene silencing efficiency and very low cytotoxicity in PC3 (human prostate cancer cell line) and MDAMB435 (human breast cancer cell line) cells. In a later work, these SLNs containing a siRNA anti-c-Met (an oncogene overexpressed in a variety of carcinomas) were evaluated *in vivo* in a glioblastoma multiform mouse model. After intravenous administration to mice, the siRNA-PEG/SLN system specifically crossed the blood-brain barrier to the tumor site with no apparent systemic toxicity. Treatment significantly inhibited tumor growth in a dose dependent manner, and a downregulation of c-Met was observed[59].

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3.2. Nanostructured lipid carriers (NLCs)

NLCs are solid lipid core carriers in the nanometer range composed of a mixture of liquid and solid lipids that are spatially incompatible leading to special structures with improved drug encapsulation and release properties. NLCs were developed as a tool to overcome the drawbacks of SLNs and to increase the oral bioavailability of poorly soluble compounds[61,62,63,64,65]. The choice of the lipid is critical to ensure the stability of the drug[61], and the structure of the lipid core matrix determines the classification of NLCs. Imperfect NLCs are made by mixing small amounts of liquid lipid and fatty acids with different chain lengths as solid lipid. The imperfections that are generated in the lipid core due to the crystallization increase the drug load and reduce, although not completely, the drug expulsion[66,67]. Multiple NLCs are made by mixing solid lipids and an excess of liquid lipid, generating oily nanocompartments into the lipid matrix where the drug is well accommodated[68]. Finally, in structureless or also called amorphous NLCs, special liquids are used, and the expulsion of the drug is avoided due to the lack of crystallization[67].

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The application of NLCs to the treatment and prevention of infectious diseases has been attempted in various studies focused on the development of new antimalarial treatments[69,70,71] or in the improvement of encapsulation o hydrophilic antiretroviral drugs[72,73,74]. Although there are few reports about the application of NLCs as RNAi delivery systems, they could be an interesting alternative in codelivery strategies with conventional drugs. For instance, Taratula et al.[75] have recently described a multifunctional NLC system to improve the efficacy of anticancer drugs in lungs. The system was composed of an anticancer drug (doxorubicin or paclitaxel), joint with siRNA to avoid cellular resistance (siRNA targeted to MRP1 mRNA as a suppressor of pump drug resistance and siRNA targeted to BCL2 mRNA as a suppressor of non pump cellular resistance) and an analog of luteinizing hormone-releasing hormone as a targeting moiety to lungs. The inhalation of the NLCs by an orthotopic mice model of human lung cancer resulted in efficient suppression of tumor growth and prevention of adverse side effects on healthy organs.

[69] Joshi M, Pathak S, Sharma S, Patravale V. Design and *in vivo* pharmacodynamic evaluation of nanostructured lipid carriers for parenteral delivery of artemether: Nanoject. *Int J Pharm*. 2008 Nov 19;364(1):119-126.

[70] Nayak AP, Tiyaboonchai W, Patankar S, Madhusudhan B, Souto EB. Curcuminoids-loaded lipid nanoparticles: novel approach towards malaria treatment. *Colloids Surf B Biointerfaces*. 2010 Nov 1;81(1):263-273.

[71] Aditya NP, Patankar S, Madhusudhan B, Murthy RS, Souto EB. Arthemeter-loaded lipid nanoparticles produced by modified thin-film hydration: Pharmacokinetics, toxicological and *in vivo* anti-malarial activity. *Eur J Pharm Sci*. 2010 Aug 11;40(5):448-455.

[72] Kasongo KW, Pardeike J, Müller RH, Walker RB. Selection and characterization of suitable lipid excipients for use in the manufacture of didanosine-loaded solid lipid nanoparticles and nanostructured lipid carriers. *J Pharm Sci*. 2011 Dec;100(12):5185-5196.

[73] Beloqui A, Solinís MÁ, Gascón AR, del Pozo-Rodríguez A, des Rieux A, Préat V. Mechanism of transport of saquinavir-loaded nanostructured lipid carriers across the intestinal barrier. *J Control Release*. 2013 Mar 10;166(2):115-123.

[74] Beloqui A, Solinís MÁ, des Rieux A, Préat V, Rodríguez-Gascón A. Dextran-protamine coated nanostructured lipid carriers as mucus-penetrating nanoparticles for lipophilic drugs. *Int J Pharm*. 2014 Jul 1;468(1-2):105-111.

[75] Taratula O, Kuzmov A, Shah M, Garbuzenko OB, Minko T. Nanostructured lipid carriers as multifunctional nanomedicine platform for pulmonary co-delivery of anticancer drugs and siRNA. *J Control Release*. 2013 Nov 10;171(3):349-357.

3.3. Lipid drug conjugates (LDCs)

LDCs are the most accepted lipid-based nanoparticle systems for the delivery of hydrophilic drugs, as they improve the drug loading capacity of SLNs and NLCs. In LDC nanoparticles, hydrophilic drugs are first conjugated with lipid components by covalent linking between an amino group or a hydroxyl group of the drug and carboxyl groups of the lipid core to obtain a lipophilic complex[76]. The water insoluble LDC is converted to nanoparticles by means of traditional methods used to prepare SLNs or NLCs[77,78]. LDCs have shown hopeful results as delivery system for hydrophilic antitrypanosomal drugs[79,80], for oral application of methotrexate[77], and, more recently, for chemotherapy agents[81,78], such as decitabine, a drug that shows low oral bioavailability[82]. Neupane et al.[83] developed LDC nanoparticles of decitabine to increase its permeability and protect against chemical degradation. LDCs were obtained by salt formation of DCB with stearic acid to be formulated as LDC nanoparticles by cold high-pressure homogenization after addition of surfactants Tween 80, Poloxamer 188, and Labrasol. *Ex vivo* gut permeation studies proved that the drug in LDC nanoparticles showed

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[78] Sharma P, Dube B, Sawant K. Synthesis of cytarabine lipid drug conjugate for treatment of meningeal leukemia: development, characterization and *in vitro* cell line studies. *J Biomed Nanotechnol*. 2012 Dec;8(6):928-937.

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[80] Olbrich C, Gessner A, Schröder W, Kayser O, Müller RH. Lipid-drug conjugate nanoparticles of the hydrophilic drug diminazene-cytotoxicity testing and mouse serum adsorption. *J Control Release*. 2004 May 18;96(3):425-435.

[81] United States Congress, Office of Technology Assessment [OTA], Human Gene Therapy-Background Paper, Washington, DC, USA

[82] Liu D. Recent advances in myelodysplasia: update from 2011 ASH annual meeting. *J Hematol Oncol*. 2012;5(1):A4.

[83] Neupane YR, Sabir MD, Ahmad N, Ali M, Kohli K. Lipid drug conjugate nanoparticle as a novel lipid nanocarrier for the oral delivery of decitabine: ex vivo gut permeation studies. *Nanotechnology*. 2013 Oct 18;24(41):415102.

nearly fourfold increase in the apparent permeability coefficients with respect to the plain decitabine.

3.4. Cationic emulsions

Emulsions are dispersions of one immiscible liquid in another stabilized by a third component, the emulsifying agent[84]; therefore, they present in their composition three components: oil, water, and surfactants. When cationic surfactants are used, these dispersed systems make them suitable for gene delivery. The presence of cationic surfactants causes the formation of positively charged droplets that promote strong electrostatic interactions between emulsion and the anionic nucleic acid phosphate groups. Cationic emulsions composed of cationic lipids and core oil have been shown to be useful for gene delivery[85,86]. The colipid DOPE is largely used to improve the ability of cationic emulsions and liposomes to transfect cells due to its fusogenic properties. This can be partially explained by the fact that the amine group of DOPE interacts with DNA phosphate groups, thus weakening the binding affinity between cationic lipids and DNA[84].

In spite of the advantages of nanoemulsions for delivery of nucleic acids, only few attempts have been made to use this new delivery system for RNAi. For instance, Kaneda et al.[87] showed the potential application of cationic nanoemulsion prepared with DOTAP, DOPE, and cholesterol for

[84] Verissimo LM, Lima LF, Egito LC, de Oliveira AG, do Egito ES. Pharmaceutical emulsions: a new approach for gene therapy. *J Drug Target*. 2010 Jun;18(5):333-342.

[85] Yi SW, Yune TY, Kim TW, Chung H, Choi YW, Kwon IC, Lee EB, Jeong SY. A cationic lipid emulsion/DNA complex as a physically stable and serum-resistant gene delivery system. *Pharm Res*. 2000 Mar;17(3):314-320.

[86] Yoo HS, Mazda O, Lee HY, Kim JC, Kwon SM, Lee JE, Kwon IC, Jeong H, Jeong YS, Jeong SY. *In vivo* gene therapy of type I diabetic mellitus using a cationic emulsion containing an Epstein Barr Virus (EBV) based plasmid vector. *J Control Release*. 2006 May 1;112(1):139-144.

[87] Kaneda MM, Sasaki Y, Lanza GM, Milbrandt J, Wickline SA. Mechanisms of nucleotide trafficking during siRNA delivery to endothelial cells using perfluorocarbon nanoemulsions. *Biomaterials*. 2010 Apr;31(11):3079-3086.

siRNA delivery. Transfection complexes, with a mean particle diameter of approximately 300 nm, were able to suppress endothelial cell expression of upregulated vascular adhesion molecules. To the knowledge of the authors, no studies about cationic emulsions with RNAi molecules to treat viral infections have been published.

3.5. Liposomes

Liposomes are colloidal lipid- and surfactant-based delivery systems, composed of a phospholipid bilayer surrounding an aqueous compartment. They may present spherical vesicles and can range in size from 20nm to a few microns. Cationic lipid-based liposomes are able to complex with negatively charged nucleic acid via electrostatic interactions, resulting in complexes that offer biocompatibility, low toxicity, and the possibility of large-scale production required for *in vivo* clinical applications[88]. The lipid to RNA ratio and overall lipid concentration used in forming these complexes are very important for efficient gene delivery and vary with applications. Liposomes can fuse with the plasma membrane for uptake; once inside the cell, the liposomes are processed via the endocytic pathway and the genetic material is then released from the endosome/carrier into the cytoplasm.

Compared to polymeric nanoparticles, liposomes have long been perceived as better drug delivery vehicles because of their superior biocompatibility, as liposomes are basically analogues of biological membranes, which can be prepared from both natural and synthetic phospholipids[88]. Neutral lipids are highly nontoxic and do not activate an immune response[89]. 1,2-Oleoyl-sn-glycero-3-phosphocholine (DOPC) and

[88] Rodríguez-Gascón A, del Pozo-Rodríguez A, Solinís MÁ. Development of nucleic acid vaccines: use of self-amplifying RNA in lipid nanoparticles. *Int J Nanomedicine*. 2014 Apr 10;9:1833-1843.

[89] Gavrilov K, Saltzman WM. Therapeutic siRNA: principles, challenges, and strategies. *Yale J Biol Med*. 2012 Jun;85(2):187-200.

DOPE are among the most widely used neutral lipids. Simply mixing siRNA with DOPC results in high encapsulation efficiency[90]. However, neutral liposomes yield relatively low transfection efficiency. Cationic lipids, such as 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), can complex electrostatically with siRNAs and be used to create a more effective liposome as the positively charged lipids provide enhanced cell entry and increased protection against serum enzymes[91].

Although liposomes are one of the most commonly used transfection reagents *in vitro*, safe and efficacious delivery *in vivo* is more difficult to achieve due to toxicity, nonspecific uptake, and unwanted immune response. Much of the nonspecific response and toxicity is directly linked to the positive charge on the surface of the particles necessary for the binding of oligonucleotides. In order to improve their behavior, in recent years, significant effort has been dedicated to modifying the composition and chemical structure of liposomes. For instance, different additives, such as hydrophobic cholesterol, nonionic surfactants, or PEG, can be used to enhance *in vivo* stability after exposure to blood components[92]. It has been reported that the stability-enhanced liposomes have much better transfection efficiencies, especially under *in vivo* conditions[93]. To increase specificity, PEGylated immunoliposomes conjugated with targeting ligands have been

[90] Landen CN Jr, Chavez-Reyes A, Bucana C, Schmandt R, Deavers MT, Lopez-Berestein G, Sood AK. Therapeutic EphA2 gene targeting *in vivo* using neutral liposomal small interfering RNA delivery. *Cancer Res*. 2005 Aug 1;65(15):6910-6918.

[91] Zhang C, Tang N, Liu X, Liang W, Xu W, Torchilin VP. siRNA-containing liposomes modified with polyarginine effectively silence the targeted gene. *J Control Release*. 2006 May 15;112(2):229-239.

[92] Kwon SM, Nam HY, Nam T, Park K, Lee S, Kim K, Kwon IC, Kim J, Kang D, Park JH, Jeong SY. *In vivo* time-dependent gene expression of cationic lipid-based emulsion as a stable and biocompatible non-viral gene carrier. *J Control Release*. 2008 May 22;128(1):89-97.

[93] Hyvönen Z, Rönkkö S, Toppinen MR, Jääskeläinen I, Plotniece A, Urtti A. Dioleoyl phosphatidylethanolamine and PEG-lipid conjugates modify DNA delivery mediated by 1,4-dihydropyridine amphiphiles. *J Control Release*. 2004 Sep 14;99(1):177-190.

developed[94].Liposomes have been widely studied as RNAi carriers as potential treatment of viral infections such as HCV, HBV, and VIH, among others[95,96,97,98,99].

4. Lipid nanoparticles as RNAi carriers against viral infections

As mentioned above, an effective delivery system will have to be developed for exploiting the gene silencing by RNA interference strategy for antiviral therapy. The applicability of LNP for the treatment of viral infections is emerging in the last years. On the one hand, the use of LNP as nonviral nanocarriers of gene material is the most studied approach. In this sense, some researchers suggest the simultaneous administration of different siRNAs or shRNAs to avoid the important payload of viral mutation escape[100]. On the other hand, the prophylaxis for viral infections using RNAi has been considered a promising strategy.

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[94] Deng L, Zhang Y, Ma L, Jing X, Ke X, Lian J, Zhao Q, Yan B, Zhang J, Yao J, Chen J. Comparison of anti-EGFR-Fab' conjugated immunoliposomes modified with two different conjugation linkers for siRNa delivery in SMMC-7721 cells. *Int J Nanomedicine*. 2013;8:3271-3283.

[95] Watanabe T, Umehara T, Yasui F, Nakagawa S, Yano J, Ohgi T, Sonoke S, Satoh K, Inoue K, Yoshiba M, Kohara M. Liver target delivery of small interfering RNA to the HCV gene by lactosylated cationic liposome. *J Hepatol*. 2007 Dec;47(6):744-750.

[96] Kim SI, Shin D, Lee H, Ahn BY, Yoon Y, Kim M. Targeted delivery of siRNA against hepatitis C virus by apolipoprotein A-I-bound cationic liposomes. *J Hepatol*. 2009 Mar;50(3):479-488.

[97] Kim SI, Shin D, Choi TH, Lee JC, Cheon GJ, Kim KY, Park M, Kim M. Systemic and specific delivery of small interfering RNAs to the liver mediated by apolipoprotein A-I. *Mol Ther*. 2007 Jun;15(6):1145-1152.

[98] Carmona S, Jorgensen MR, Kolli S, Crowther C, Salazar FH, Marion PL, Fujino M, Natori Y, Thanou M, Arbuthnot P, Miller AD. Controlling HBV replication *in vivo* by intravenous administration of triggered PEGylated siRNA-nanoparticles. *Mol Pharm*. 2009 May-Jun;6(3):706-717.

[99] Kim SS, Peer D, Kumar P, Subramanya S, Wu H, Asthana D, Habiro K, Yang YG, Manjunath N, Shimaoka M, Shankar P. RNAi-mediated CCR5 silencing by LFA-1-targeted nanoparticles prevents HIV infection in BLT mice. *Mol Ther*. 2010 Feb;18(2):370-376.

[100] Pan Q, Tilanus HW, Janssen HL, van der Laan LJ. Prospects of RNAi and microRNA-based therapies for hepatitis C. *Expert Opin Biol Ther*. 2009 Jun;9(6):713-724.

The RNAi process requires a high specificity of gene sequences and the choice of the target is the most crucial step for a successful viral inhibition. It is very important to design siRNA against very highly conserved sequences o the viral genome in order to optimize efficacy in inhibiting a majority of virus straints. In addition to its utility as a stand-alone strategy, RNAi may have expanded applications as an adjuvant in multipronged treatment settings. Another RNAi adjuvant strategy is the use of dsRNA oligonucleotides as immunostimulatory agonists alongside vaccines, as in the case of a RIG1 agonist to enhance the activity of a DNA vaccine against influenza[101].

4.1. Hepatitis C virus (HCV)

According to the World Health Organization (WHO), every year, 3-4 million people are infected with the HCV, and about 150 million people are chronically infected, which can lead to liver cirrhosis and/or hepatocellular carcinoma (liver cancer). In consequence, more than 350000 people die from hepatitis C-related liver diseases every year[102].

Combination of generic antiviral agents, IFN-α and ribavirin, is the mainstay of current hepatitis C treatment. Unfortunately, IFN-α is not available in some countries, people do not always tolerate well this drug, and many people do not finish their treatment. In addition, some virus genotypes do not respond well to IFN-α[103]. Recently new antiviral drugs, telaprevir or boceprevir, have been added to set up the so-called triple therapy; the experience in patients with chronic hepatitis C genotype 1 shows that this new combination is superior to dual therapy in terms of

[101] Luke JM, Simon GG, Söderholm J, Errett JS, August JT, Gale M Jr, Hodgson CP, Williams JA. Coexpressed RIG-I agonist enhances humoral immune response to influenza virus DNA vaccine. *J Virol*. 2011 Feb;85(3):1370-1383.

[102] Hepatitis C Fact sheet No. 164, http://www.who.int/mediacentre/factsheets/fs164/en/.

[103] Hnatyszyn HJ. Chronic hepatitis C and genotyping: the clinical significance of determining HCV genotypes. *Antivir Ther*. 2005;10(1):1-11.

sustained virologic response[104]. However, genotype independent alternatives should be more effective in the treatment and prevention of this liver infection. In this regard, the RNAi technology is an attractive strategy.

HCV is a RNA virus belonging to Flaviviridae family. The single-stranded RNA genome of this virus acts also as mRNA, which makes it an attractive target for RNAi based therapy. Several works show potent HCV replication inhibition by using chemically synthesized siRNAs targeted against sequences in the protein-coding regions of core, E2, NS3, NS5B, or NS4[105,106,107]. However, these viral coding sequences suffer variations among different HCV genotypes. Therefore, highly conserved regions, such as 5'untranslated regions (5'UTR), seem to be better targets for developing a rational antiviral strategy[108]. The replication of different HCV genotypes has been inhibited by targeting 5'UTR with synthetic siRNA or shRNA expression[109,110,111]. The main limitation of all these new strategies lies in the emergence of resistant virus variants due to the high specificity of RNAi and the prolonged treatment.

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[104] Park C, Jiang S, Lawson KA. Efficacy and safety of telaprevir and boceprevir in patients with hepatitis C genotype 1: a meta-analysis. *J Clin Pharm Ther*. 2014 Feb;39(1):14-24.

[105] Takigawa Y, Nagano-Fujii M, Deng L, Hidajat R, Tanaka M, Mizuta H, Hotta H. Suppression of hepatitis C virus replicon by RNA interference directed against the NS3 and NS5B regions of the viral genome. *Microbiol Immunol*. 2004;48(8):591-598.

[106] Prabhu R, Vittal P, Yin Q, Flemington E, Garry R, Robichaux WH, Dash S. Small interfering RNA effectively inhibits protein expression and negative strand RNAsynthesis from a full-length hepatitis C virus clone. *J Med Virol*. 2005 Aug;76(4):511-519.

[107] Kim M, Shin D, Kim SI, Park M. Inhibition of hepatitis C virus gene expression by small interfering RNAs using a tri-cistronic full-length viral replicon and a transient mouse model. *Virus Res*. 2006 Dec;122(1-2):1-10.

[108] Prabhu R, Garry RF, Dash S. Small interfering RNA targeted to stem-loop II of the 5' untranslated region effectively inhibits expression of six HCV genotypes. *Virol J*. 2006 Nov 27;3:100.

[109] Yokota T, Sakamoto N, Enomoto N, Tanabe Y, Miyagishi M, Maekawa S, Yi L, Kurosaki M, Taira K, Watanabe M, Mizusawa H. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep*. 2003 Jun;4(6):602-608.

[110] Kanda T, Steele R, Ray R, Ray RB. Small interfering RNA targeted to hepatitis C virus 5' nontranslated region exerts potent antiviral effect. *J Virol*. 2007 Jan;81(2):669-676.

[111] Zekri AR, Bahnassy AA, El-Din HM, Salama HM. Consensus siRNA for inhibition of HCV genotype-4 replication. *Virol J*. 2009 Jan 27;6:13.

In order to prevent escape mutants, some approaches have been proposed. The internal ribosome entry site (IRES) in the 5'UTR is required for the synthesis of viral proteins and, therefore, for viral replication. Mutations in these structures might lead to loss of function[112], making IRES an excellent target for anti-HCV drugs, which might prevent viral escape; it has also been targeted by the RNAi technology[113]. Another possibility to reduce resistant variants is the combination of two or more RNAi molecules with different specificities[114] targeted to separated regions of the HCV genome. Targeting multiple sites of the HCV genome and host genes necessary for virus replication has also been documented as a valid approach to prevent the development of resistance[115,116].

Among the RNAi delivery systems against HCV, cationic lipid-based nanoparticles play an interesting role; these lipid based vectors are well characterized as nanocarriers for systemic delivery of RNAi molecules to the liver, due to their safety profile and simplicity of production. Table 2 summarizes the strategies employed by different authors to improve the efficacy of lipid nanosystems in RNAi-mediated therapies against HCV.

Watanabe et al.[117] designed lactosylated cationic liposomes to deliver siRNA targeting the 5'UTR and 3'UTR of the HCV genome into mouse liver hepatocytes; the galactose terminus of lactose is a ligand of

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[112] Motavaf M, Safari S, Alavian SM. Therapeutic potential of RNA interference: a new molecular approach to antiviral treatment for hepatitis C. *J Viral Hepat*. 2012 Nov;19(11):757-765.

[113] Ray RB, Kanda T. Inhibition of HCV replication by small interfering RNA. *Methods Mol Biol*. 2009;510:251-262.

[114] De Francesco R, Migliaccio G. Challenges and successes in developing new therapies for hepatitis C. *Nature*. 2005 Aug 18;436(7053):953-960.

[115] Zender L, Hutker S, Liedtke C, Tillmann HL, Zender S, Mundt B, Waltemathe M, Gosling T, Flemming P, Malek NP, Trautwein C, Manns MP, Kuhnel F, Kubicka S. Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc Natl Acad Sci U S A*. 2003 Jun 24;100(13):7797-7802.

[116] Henry SD, van der Wegen P, Metselaar HJ, Tilanus HW, Scholte BJ, van der Laan LJ. Simultaneous targeting of HCV replication and viral binding with a single lentiviral vector containing multiple RNA interference expression cassettes. *Mol Ther*. 2006 Oct;14(4):485-493.

[117] Watanabe T, Umehara T, Yasui F, Nakagawa S, Yano J, Ohgi T, Sonoke S, Satoh K, Inoue K, Yoshiba M, Kohara M. Liver target delivery of small interfering RNA to the HCV gene by lactosylated cationic liposome. *J Hepatol*. 2007 Dec;47(6):744-750.

the asialoglycoprotein receptor, which is specifically expressed in the surface of hepatocytes. Intravenous administration of the mentioned vectors into HCV-transgenic mice resulted in a decrease of HCV core protein.

Another possibility is the functionalization of LNP with apolipoproteins. Kim et al.[118] developed liver-specific siRNA delivery vectors composed of cationic liposomes and apolipoprotein A-1 (apo A-1) derived from human plasma. This protein, component of high density lipoprotein (HDL), has been proposed as a targeting ligand to hepatocytes[119]. After intravenous administration of the lipid-based systems containing HCV-core specific siRNA into an HCV mouse model, viral expression was inhibited by 65–75% in the liver on day 2. In the same work, the chemical modification of the HCV-core specific siRNA to increase its serum stability resulted in gene silencing efficacy up to 95% for at least 6 days. In order to avoid safety problems related to the risk of pathogen contamination associated with the use of plasma-derived protein, in a posterior work[120] apo A-1 was substituted by a recombinant human apo A-1 (rhapo A-1). This new apolipoprotein of low endotoxin grade was expressed and purified from an *Escherichia coli* expression system. The use of rhapo A-1 to deliver siRNA to the liver resulted as effective and selective as plasma-derived apo A-1, without affecting the normal liver function.

The choice of an adequate RNAi molecule is also crucial. HCV replication has also been inhibited both *in vitro* and *in vivo* by delivery of different siRNAs with lipid nanosomes composed of the cationic lipid DOTAP, the helper lipid cholesterol, and the peptide protamine.

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[118] Kim SI, Shin D, Lee H, Ahn BY, Yoon Y, Kim M. Targeted delivery of siRNA against hepatitis C virus by apolipoprotein A-I-bound cationic liposomes. *J Hepatol*. 2009 Mar;50(3):479-488.

[119] Kim SI, Shin D, Choi TH, Lee JC, Cheon GJ, Kim KY, Park M, Kim M. Systemic and specific delivery of small interfering RNAs to the liver mediated by apolipoprotein A-I. *Mol Ther*. 2007 Jun;15(6):1145-1152.

[120] Lee H, Kim SI, Shin D, Yoon Y, Choi TH, Cheon GJ, Kim M. Hepatic siRNA delivery using recombinant human apolipoprotein A-I in mice. *Biochem Biophys Res Commun*. 2009 Jan 9;378(2):192-196.

Nanosomes are nanosize unilamellar vesicles obtained by high-pressure homogenization of lipid dispersions[121,122]. In a first work, nanovectors were optimized in terms of lipid-to-siRNA ratio and favorable particle size depending on sonication time. Cell viability was maintained about 90% and HCV inhibition reached approximately 85%[122]. Later on, the cationic lipid nanosomes were used to complex different siRNA targeted to the 5'UTR of the HCV genome. Chandra et al. compared repeated treatments with two-siRNA versus a single siRNA treatment. A reduction in the development of resistant mutants to the siRNA therapy was observed when the combinatorial strategy was used, and after systemic injection in a liver tumor-xenotransplant mouse model of HCV, significant inhibition of virus replication was obtained[121].

More recently, short synthetic shRNAs (sshRNAs) that target a sequence within IRES have been formulated into LNP by the process of step-wise ethanol dilution and spontaneous vesicle formation. sshRNA was dissolved in an aqueous solution containing 30% ethanol and added to preequilibrated LNP at 35°C. After reaching the final sshRNA to lipid ratio, the mixture was incubated for further 30min at 35°C to allow vesicle reorganization and encapsulation of the RNA. Finally, LNP were dialyzed against PBS and filter sterilized through a 0.2 μm filter. The intravenous injection of this vector resulted in enough uptake by the hepatocytes to substantially suppress gene expression in a rapid and durable manner[123].

[121] Chandra PK, Kundu AK, Hazari S, Chandra S, Bao L, Ooms T, Morris GF, Wu T, Mandal TK, Dash S. Inhibition of hepatitis C virus replication by intracellular delivery of multiple siRNAs by nanosomes. *Mol Ther*. 2012 Sep;20(9):1724-1736

[122] Kundu AK, Chandra PK, Hazari S, Pramar YV, Dash S, Mandal TK. Development and optimization of nanosomal formulations for siRNA delivery to the liver. *Eur J Pharm Biopharm*. 2012 Feb;80(2):257-267.

[123] Dallas A, Ilves H, Shorenstein J, Judge A, Spitler R, Contag C, Wong SP, Harbottle RP, Maclachlan I, Johnston BH. Minimal-length Synthetic shRNAs Formulated with Lipid Nanoparticles are Potent Inhibitors of Hepatitis C Virus IRES-linked Gene Expression in Mice. *Mol Ther Nucleic Acids*. 2013 Sep 17;2:e123.

Table 2: Studies carried out with lipid-based nanosystems as RNAi delivery vectors against hepatitis C (HCV).

Lipid nanosystem	RNAi	Targeting molecule	Culture cells	*In vivo* model	Ref.
Cationic liposomes	siRNAs against the 5´-UTR and 3´-UTR of the HCV genome	Lactosylated -PE	FLR3-1 and R6FLR-N cells	CN2-29 transgenic mice	117
Cationic liposomes	HCV-core specific siRNA (siHCc)	Apo A-I	Huh-7	HCV mouse model constructed by hydrodynamic injection of DNA plasmid expressing viral proteins	118
Cationic liposomes	HCV-core specific siRNA (siHCc)	Recombinat human apo A-I	-	HCV mouse model constructed by hydrodynamic injection of DNA plasmid expressing viral proteins	120
Cationic nanosomes	siRNAs against the stem-loop domains II–IV of HCV 5´UTR	-	Huh-7.5 and R4-GFP cells	HCC tumor-xenograft mice model for HCV	121
Cationic LNP	sshRNA targeting the HCV IRES volunteers	-	-	Reporter mice that express in the liver firefly luciferase under the control of the HCV IRES	123

4.2. Hepatitis B virus (HBV)

WHO estimates that about 600000 people die every year due to the consequences of HBV infection (mainly cirrhosis of the liver and liver cancer). A vaccine against hepatitis B has been available since 1982. Hepatitis B vaccine is 95% effective in preventing infection but offers

scarce therapeutic benefit to chronic carriers[124]. Some people with chronic hepatitis B are treated with drugs, including IFN, nucleotide/nucleoside analogues, and immunomodulators, but there is no specific treatment for acute hepatitis B[125]. Moreover, current therapies have limited efficacy, are expensive, produce side effects, and are associated with viral resistance[126]. The emergence of resistance has been reduced by new antivirals (entecavir or tenofovir), but patients usually need to take these drugs for life, because interruption of treatment quickly reactivates viral replication[127]. Therefore, there is need for finding effective therapies against HBV, with the use of RNAi being an attractive possibility. RNAi has also been studied as a possible vaccine against HBV[128], but its main application is the treatment of chronic HBV infection.

HBV viron contains a partly double-stranded relaxed circular DNA (rcDNA) that is encapsidated by core proteins and enveloped with S proteins and membrane lipids from the host to form viral particles[129]. When a hepatocyte is infected endogenous repair enzymes convert rcDNAin a fully double-stranded, circular, and supercoiled DNA (cccDNA), which serves as template for transcription of HBV RNA. Expression of viral proteins and viral replication may be potentially knocked down by RNAi-based therapeutics.

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[124] Ivacik D, Ely A, Arbuthnot P. Countering hepatitis B virus infection using RNAi: how far are we from the clinic? *Rev Med Virol*. 2011 Nov;21(6):383-396.

[125] Hepatitis B, Fact sheet No. 204, 2013, http://www.who.int/mediacentre/factsheets/fs204/en/.

[126] Keeffe EB, Dieterich DT, Pawlotsky JM, Benhamou Y. Chronic hepatitis B: preventing, detecting, and managing viral resistance. *Clin Gastroenterol Hepatol*. 2008 Mar;6(3):268-274.

[127] Zoulim F. Hepatitis B virus resistance to antiviral drugs: where are we going? *Liver Int*. 2011 Jan;31 Suppl 1:111-116.

[128] Jiang W. Blockade of B7-H1 enhances dendritic cell-mediated T cell response and antiviral immunity in HBV transgenic mice. *Vaccine*. 2012 Jan 17;30(4):758-766.

[129] Wooddell CI, Rozema DB, Hossbach M, John M, Hamilton HL, Chu Q, Hegge JO, Klein JJ, Wakefield DH, Oropeza CE, Deckert J, Roehl I, Jahn-Hofmann K, Hadwiger P, Vornlocher HP, McLachlan A, Lewis DL. Hepatocyte-targeted RNAi therapeutics for the treatment of chronic hepatitis B virus infection. *Mol Ther*. 2013 May;21(5):973-985.

RNAi activators used against HBV include expressed or engineered synthetic RNAi intermediates; each class of silencing molecules has advantages and disadvantages. Expressed anti-HBV sequences, as plasmids that produce specific siRNAs[130] or as shRNA[131,132,133,134,135], have demonstrated efficacy *in vitro* and *in vivo* and they achieve more sustained silencing effect, whereas synthetic siRNAs require repeated administration to provide a long-term suppression of HBV replication[136,137,138]. However, expressed RNAi activators are complex in terms of delivery and dose control, which have led to the development of chemically modified synthetic siRNAs with the aim of improving silencing efficacy, specificity for a particular target mRNA, avoidance of innate immunostimulation, stability, and delivery to target tissue[139,140].

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[130] Shlomai A, Shaul Y. Inhibition of hepatitis B virus expression and replication by RNA interference. *Hepatology*. 2003 Apr;37(4):764-770.

[131] Uprichard SL, Boyd B, Althage A, Chisari FV. Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNAs. *Proc Natl Acad Sci U S A*. 2005 Jan 18;102(3):773-778.

[132] Carmona S, Ely A, Crowther C, Moolla N, Salazar FH, Marion PL, Ferry N, Weinberg MS, Arbuthnot P. Effective inhibition of HBV replication *in vivo* by anti-HBx short hairpin RNAs. *Mol Ther*. 2006 Feb;13(2):411-421.

[133] Sun D, Rösler C, Kidd-Ljunggren K, Nassal M. Quantitative assessment of the antiviral potencies of 21 shRNA vectors targeting conserved, including structured, hepatitis B virus sites. *J Hepatol*. 2010 Jun;52(6):817-826.

[134] He F, Chen EQ, Liu L, Zhou TY, Liu C, Cheng X, Liu FJ, Tang H. Inhibition of hepatitis B Virus replication by hepatocyte nuclear factor 4-alpha specific short hairpin RNA. *Liver Int*. 2012 May;32(5):742-751.

[135] Wang XJ, Li Y, Huang H, Zhang XJ, Xie PW, Hu W, Li DD, Wang SQ. A simple and robust vector-based shRNA expression system used for RNA interference. *PLoS One*. 2013;8(2):e56110.

[136] Giladi H, Ketzinel-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E. Small interfering RNA inhibits hepatitis B virus replication in mice. *Mol Ther*. 2003 Nov;8(5):769-776.

[137] Moore MD, McGarvey MJ, Russell RA, Cullen BR, McClure MO. Stable inhibition of hepatitis B virus proteins by small interfering RNA expressed from viral vectors. *J Gene Med*. 2005 Jul;7(7):918-925.

[138] Wu KL, Zhang X, Zhang J, Yang Y, Mu YX, Liu M, Lu L, Li Y, Zhu Y, Wu J. Inhibition of Hepatitis B virus gene expression by single and dual small interfering RNA treatment. *Virus Res*. 2005 Sep;112(1-2):100-107.

[139] Hean J, Crowther C, Ely A, Ul Islam R, Barichievy S, Bloom K, Weinberg MS, van Otterlo WA, de Koning CB, Salazar F, Marion P, Roesch EB, Lemaitre M, Herdewijn P, Arbuthnot P. Inhibition of hepatitis B virus replication *in vivo* using lipoplexes containing altritol-modified antiviral siRNAs. *Artif DNA PNA XNA*. 2010 Jul;1(1):17-26.

[140] Marimani MD, Ely A, Buff MC, Bernhardt S, Engels JW, Arbuthnot P. Inhibition of hepatitis B virus replication in cultured cells and *in vivo* using 2'-O-guanidinopropyl modified siRNAs. *Bioorg Med Chem*. 2013 Oct 15;21(20):6145-6155.

The delivery of these RNAi molecules against HBV has been addressed by several research groups by means of cationic lipid-based systems, recapitulated in Table 3. In an early study, Morrissey et al.[141] entrapped a couple of chemically modified synthetic anti-HBV siRNAs in stable nucleicacid- lipid particles (SNALPs). SNALPs consist of a lipid bilayer composed of cationic and fusogenic lipids, which are coated with a PEG-lipid. Inner lipids enable cellular uptake and endosomal release of the molecules payload, whereas the coating stabilizes the particles during formulation and shields the particles *in vivo* to avoid rapid systemic clearance. Following administration, the PEG-lipid dissociates from the nanoparticles and the SNALPs become a transfection competent entity[142]. After intravenous administration of SNALPs-siRNA vectors to mice carrying the replicating virus, encapsulated siRNA presented a longer half-life in plasma and liver than nonencapsulated. Sustained specific reduction in HBV titers, for up to 7 days, and reduced toxic and immunostimulatory side effects were achieved[141].

Like in the case of HCV, in order to improve liver-targeting, apo A-1 has been combined with the cationic lipid DOTAP and the helper lipid cholesterol to form lipoplexes with siRNAs against HBV[143]; apo A-1 was incorporated into the formulation by reassembling the liposomes with a solution of the protein at 4^{o}C overnight. Intravenous injection of these lipoplexes into a HBV mouse model significantly reduced viral protein expression during at least 8 days in only a single treatment.

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[141] Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, Breen W, Hartsough K, Machemer L, Radka S, Jadhav V, Vaish N, Zinnen S, Vargeese C, Bowman K, Shaffer CS, Jeffs LB, Judge A, MacLachlan I, Polisky B. Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol*. 2005 Aug;23(8):1002-1007.

[142] Ambegia E, Ansell S, Cullis P, Heyes J, Palmer L, MacLachlan I. Stabilized plasmid-lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression. *Biochim Biophys Acta*. 2005 May 20;1669(2):155-163.

[143] Kim SI, Shin D, Choi TH, Lee JC, Cheon GJ, Kim KY, Park M, Kim M. Systemic and specific delivery of small interfering RNAs to the liver mediated by apolipoprotein A-I. *Mol Ther*. 2007 Jun;15(6):1145-1152.

In two subsequent studies, lipid-based systems demonstrated more effective or comparable inhibition of viral proliferation than lamivudine, a licensed HBV drug[144,145]. In one case[144], lipoplexes were prepared by adding an aqueous solution of siRNA targeting conserved regions of the HBV genome to a dispersion of lipid vesicles with constant vortex mixing. Thereafter, a PEG-lipid was added to the lipoplexes and the mixture was incubated for 16 h. These vectors were also lyophilized in presence of trehalose for long-term storage. In the second case[145], the siRNA targeting conserved regions of the HBV genome was combined with the cationic lipid N',N'-dioctadecyl-N-4,8-diaza-10-aminodecanoylglycine amide (DODAG) under conditions of rapid vortex mixing to produce siRNA-DODAG nanoparticles.

More recently, a liver-targeting cholesterol galactoside was incorporated into lipoplexes to obtain an altriol-modified siRNA delivery system[139]. The galactoseconjugated cholesterol was synthesized by a cooper-mediated "click" reaction between the 2-propynylcarbamate derivative of cholesterol and *O*-tetraacetate galactose azide. This lipid was mixed with a nucleic acid binding cholesterol derivative and the helper lipid to obtain liposomes capable of binding the siRNA. The improved hepatotropism and attenuated immunostimulatory properties of the vectors demonstrated that galactose functionalization has also potential for delivery of RNAs to hepatocytes in the treatment of hepatic viral infections and other liver diseases.

[144] Carmona S, Jorgensen MR, Kolli S, Crowther C, Salazar FH, Marion PL, Fujino M, Natori Y, Thanou M, Arbuthnot P, Miller AD. Controlling HBV replication *in vivo* by intravenous administration of triggered PEGylated siRNA-nanoparticles. *Mol Pharm*. 2009 May-Jun;6(3):706-717.

[145] Mével M, Kamaly N, Carmona S, Oliver MH, Jorgensen MR, Crowther C, Salazar FH, Marion PL, Fujino M, Natori Y, Thanou M, Arbuthnot P, Yaouanc JJ, Jaffrès PA, Miller AD. DODAG; a versatile new cationic lipid that mediates efficient delivery of pDNA and siRNA. *J Control Release*. 2010 Apr 19;143(2):222-232.

Table 3: Studies carried out with lipid-based nanosystems as RNAi delivery vectors against hepatitis B (HBV).

Lipid nanosystem	RNAi	Targeting molecule	Culture cells	*In vivo* model	Ref.
SNALP	HBV siRNAs chemically stabilized for nuclease resistance	-	HBV-replicating HepG2	HBV mouse model constructed by hydrodynamic injection of HBV vector DNA	141
Cationic liposomes	HBV-X specific siRNA (siHBV)	Apo A-I	HepG2 and Huh-7	Acute HBV-infected mouse model by hydrodynamic injection of a plasmid	143
PEGylated cationic liposomes	HBV specific siRNA	-	Huh-7 cells previously transfected with HBV replication target plasmid	HBV transgenic mice	144
DODAG 8 lipid	HBV specific siRNA	-	-	HBV transgenic mice	145
Cationic liposomes	Altriol modified HBV-X siRNA	Galactose	Huh-7 cells previously transfected with HBV target DNA plasmid	HBV transgenic mice	139

4.3. Human immunodeficiency virus (HIV)

HIV continues to be a major global public health issue, having claimed more than 36 million lives so far. In 2012, approximately 35.3 million people lived infected by HIV[146]. Although effective treatment with

[146] HIV/AIDS Fact sheet No.360, 2013, http://www.who.int/mediacentre/factsheets/fs360/en.

antiretroviral drugs can control the virus, there is no cure for HIV infection; therefore, novel and more promising strategies must be developed.

HIV belongs to retroviruses, which are viruses that contain RNA as genetic material. Throughout its life cycle, the RNA of HIV is not protected during viral uncoating and reverse transcription. At this point, therapeutic RNAi molecules may interact with viral RNA and blockage or reduce HIV infection. Synthetic and expressed RNAi have been evaluated preclinically to target HIV-encoded RNA such as rev, nef, or integrase[147,148,149] or host factors, mainly the chemokine receptor 5 (CCR5)[150,151,152], a promising target because it is apparently not important for human physiology[153]. Some of these targets have also been evaluated in the clinical practice, by using lentiviral or *ex vivo* strategies, but the drawbacks related to immunogenicity of the vectors and/or inconveniences for patients call for new delivery systems[154,155,156,157]. In

[147] Lee NS, Dohjima T, Bauer G, Li H, Li MJ, Ehsani A, Salvaterra P, Rossi J. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol*. 2002 May;20(5):500-505.

[148] Naito Y, Nohtomi K, Onogi T, Uenishi R, Ui-Tei K, Saigo K, Takebe Y. Optimal design and validation of antiviral siRNA for targeting HIV-1. *Retrovirology*. 2007 Nov 8;4:80.

[149] ter Brake O, Legrand N, von Eije KJ, Centlivre M, Spits H, Weijer K, Blom B, Berkhout B. Evaluation of safety and efficacy of RNAi against HIV-1 in the human immune system (Rag-2(-/-)gammac(-/-)) mouse model. *Gene Ther*. 2009 Jan;16(1):148-153.

[150] Kim SS, Peer D, Kumar P, Subramanya S, Wu H, Asthana D, Habiro K, Yang YG, Manjunath N, Shimaoka M, Shankar P. RNAi-mediated CCR5 silencing by LFA-1-targeted nanoparticles prevents HIV infection in BLT mice. *Mol Ther*. 2010 Feb;18(2):370-376.

[151] An DS, Donahue RE, Kamata M, Poon B, Metzger M, Mao SH, Bonifacino A, Krouse AE, Darlix JL, Baltimore D, Qin FX, Chen IS. Stable reduction of CCR5 by RNAi through hematopoietic stem cell transplant in non-human primates. *Proc Natl Acad Sci USA*. 2007 Aug 7;104(32):13110-13115.

[152] Anderson JS, Walker J, Nolta JA, Bauer G. Specific transduction of HIV-susceptible cells for CCR5 knockdown and resistance to HIV infection: a novel method for targeted gene therapy and intracellular immunization. *J Acquir Immune Defic Syndr*. 2009 Oct 1;52(2):152-161.

[153] Boutimah F, Eekels JJ, Liu YP, Berkhout B. Antiviral strategies combining antiretroviral drugs with RNAi-mediated attack on HIV-1 and cellular co-factors. *Antiviral Res*. 2013 Apr;98(1):121-129.

[154] Amado RG, Mitsuyasu RT, Rosenblatt JD, Ngok FK, Bakker A, Cole S, Chorn N, Lin LS, Bristol G, Boyd MP, MacPherson JL, Fanning GC, Todd AV, Ely JA, Zack JA, Symonds GP. Anti-human immunodeficiency virus hematopoietic progenitor cell-delivered ribozyme in a phase I study: myeloid and lymphoid reconstitution in human immunodeficiency virus type-1-infected patients. *Hum Gene Ther*. 2004 Mar;15(3):251-262.

addition, as with other viruses, when only one RNAi is targeted escape mutants can be generated[158]; targeting multiple viral targets, combinations of host and viral proteins, even combining host, viral proteins, and clinically approved antiretroviral drugs have demonstrated improved efficacy against HIV[153,159,160,161].

In the field of vaccines against HIV, RNAi may also play an important role. On the one hand, RNAi technology has been proposed as a strategy to block genes related to the suppression of immune response mediated by DNA vaccines, which are limited in nonhuman primates and humans, probably due to the relative brief duration of vaccine antigen expression *in vivo*. After intramuscular (i.m.) administration of plasmid DNA in mice, an adaptive immune response that mediates the apoptotic destruction of vaccine antigen expressing myocytes was detected[162]. The use of a shRNA targeted to caspase-12, a cell death mediator activated after plasmid DNA vaccination, resulted in increased HIV-gp120 Env antigen expression

[155] Levine BL, Humeau LM, Boyer J, MacGregor RR, Rebello T, Lu X, Binder GK, Slepushkin V, Lemiale F, Mascola JR, Bushman FD, Dropulic B, June CH. Gene transfer in humans using a conditionally replicating lentiviral vector. *Proc Natl Acad Sci USA*. 2006 Nov 14;103(46):17372-17377.

[156] DiGiusto DL, Krishnan A, Li L, Li H, Li S, Rao A, Mi S, Yam P, Stinson S, Kalos M, Alvarnas J, Lacey SF, Yee JK, Li M, Couture L, Hsu D, Forman SJ, Rossi JJ, Zaia JA. RNA-based gene therapy for HIV with lentiviral vector-modified CD34(+) cells in patients undergoing transplantation for AIDS-related lymphoma. *Sci Transl Med*. 2010 Jun 16;2(36):36-43.

[157] Zeller SJ, Kumar P. RNA-based gene therapy for the treatment and prevention of HIV: from bench to bedside. *Yale J Biol Med*. 2011 Sep;84(3):301-309.

[158] Lu X, Yu Q, Binder GK, Chen Z, Slepushkina T, Rossi J, Dropulic B. Antisense-mediated inhibition of human immunodeficiency virus (HIV) replication by use of an HIV type 1-based vector results in severely attenuated mutants incapable of developing resistance. *J Virol*. 2004 Jul;78(13):7079-7088.

[159] ter Brake O, Konstantinova P, Ceylan M, Berkhout B. Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol Ther*. 2006 Dec;14(6):883-892.

[160] Kumar P, Ban HS, Kim SS, Wu H, Pearson T, Greiner DL, Laouar A, Yao J, Haridas V, Habiro K, Yang YG, Jeong JH, Lee KY, Kim YH, Kim SW, Peipp M, Fey GH, Manjunath N, Shultz LD, Lee SK, Shankar P. T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell*. 2008 Aug 22;134(4):577-586.

[161] Liu YP, Haasnoot J, ter Brake O, Berkhout B, Konstantinova P. Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron. *Nucleic Acids Res*. 2008 May;36(9):2811-2824.

[162] Greenland JR, Geiben R, Ghosh S, Pastor WA, Letvin NL. Plasmid DNA vaccine-elicited cellular immune responses limit *in vivo* vaccine antigen expression through Fas-mediated apoptosis. *J Immunol*. 2007 May 1;178(9):5652-5658.

and higher CD8 T cell and antibody responses[163]. On the other hand, siRNA can be used in a prophylactic manner. For instance, siRNAs designed to knock down CCR5 and/or viral genes in CD4+ T cells, macrophages, and dendritic cells have shown protection capacity against HIV vaginal transmission when applied intravaginally to humanized mice[164]. In this regard, LNP may be the delivery system of choice, as they have demonstrated adjuvant properties for HIV vaccine.

Anionic SLNs prepared with an emulsifying wax and coated with the HIV Tat (transactivator of transcription) protein and administered subcutaneously twice to mice at an interval of 2 weeks elicited IgG and IgM responses similar to the commonly used adjuvant Alum and a higher release of IFN-γ in splenocytes[165]. In addition, anti-Tat IgG titers obtained with Alum carrying Tat were lower than those obtained with a reduced dose of the peptide adjuvanted with the SLN[166]. Other administration routes have also been explored. Intradermal and intranasal administration in mice of carnauba wax based SLN coated with HIV gp140 antigen and toll-like receptor-9 (TLR-9) yielded higher systemic andmucosal immunity than the antigen alone[167]. In another work, liposomes bearing anti-CCR5 siRNA were functionalized with the monoclonal antibody (mAbd) against lymphocyte function-associated antigen-1 (LFA-1) integrin. After intravenous administration into

[163] Geiben-Lynn R, Frimpong-Boateng K, Letvin NL. Modulation of plasmid DNA vaccine antigen clearance by caspase 12 RNA interference potentiates vaccination. *Clin Vaccine Immunol*. 2011 Apr;18(4):533-538.

[164] Wheeler LA, Trifonova R, Vrbanac V, Basar E, McKernan S, Xu Z, Seung E, Deruaz M, Dudek T, Einarsson JI, Yang L, Allen TM, Luster AD, Tager AM, Dykxhoorn DM, Lieberman J. Inhibition of HIV transmission in human cervicovaginal explants and humanized mice using CD4 aptamer-siRNA chimeras. *J Clin Invest*. 2011 Jun;121(6):2401-2412.

[165] Cui Z, Patel J, Tuzova M, Ray P, Phillips R, Woodward JG, Nath A, Mumper RJ. Strong T cell type-1 immune responses to HIV-1 Tat (1-72) protein-coated nanoparticles. *Vaccine*. 2004 Jun 30;22(20):2631-2640.

[166] Patel J, Galey D, Jones J, Ray P, Woodward JG, Nath A, Mumper RJ. HIV-1 Tat-coated nanoparticles result in enhanced humoral immune responses and neutralizing antibodies compared to alum adjuvant. *Vaccine*. 2006 Apr 24;24(17):3564-3573.

[167] Arias MA, Loxley A, Eatmon C, Van Roey G, Fairhurst D, Mitchnick M, Dash P, Cole T, Wegmann F, Sattentau Q, Shattock R. Carnauba wax nanoparticles enhance strong systemic and mucosal cellular and humoral immune responses to HIV-gp140 antigen. *Vaccine*. 2011 Feb 1;29(6):1258-1269.

humanized mice, leukocyte-specific gene silencing was obtained during 10 days, and when challenged with HIV plasma viral load and loss of CD4 T cells were reduced[150].

4.4. Other viral infections.

Although HCV, HBV, and HIV are the most studied, RNAi with lipid formulations has also been applied to the potential treatment of other viral infections such as herpes simplex virus, Ebola virus, human papillomavirus (HPV), or rabies virus, among others.

Herpes simplex virus-2 (HSV-2) infection causes significant morbidity and is an important cofactor for the transmission of HIV infection. In a study carried out by Palliser et al.[168], seven siRNAs targeting three essential HSV-2 genes (UL5 (a component of the helicase-primase complex), UL27 (envelope glycoprotein B), and UL29 (a DNA-binding protein)) were prepared and assayed for viral protection. siRNAs lipoplexes were efficiently taken up by epithelial and lamina propria cells and silenced gene expression in the mouse vagina and ectocervix for at least nine days. Intravaginal application of siRNAs targeting the HSV-2 UL27 and UL29 genes was well tolerated, did not induce IFN-responsive genes or cause inflammation, and protected mice when administered before and/or after lethal HSV-2 challenge.

In a later study[169], one of the viral siRNAs was combined with a siRNA targeting the HSV-2 receptor nectin-1. Cholesterol-conjugated-(chol-) siRNAs silenced gene expression in the vagina without causing inflammation or inducing IFNs. The viral siRNA prevented transmission

[168] Palliser D, Chowdhury D, Wang QY, Lee SJ, Bronson RT, Knipe DM, Lieberman J. An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature*. 2006 Jan 5;439(7072):89-94.

[169] Wu Y, Navarro F, Lal A, Basar E, Pandey RK, Manoharan M, Feng Y, Lee SJ, Lieberman J, Palliser D. Durable protection from Herpes Simplex Virus-2 transmission following intravaginal application of siRNAs targeting both a viral and host gene. *Cell Host Microbe*. 2009 Jan 22;5(1):84-94.

within a day of challenge, whereas the siRNA targeting the HSV-2 receptor nectin-1 protected for a week, but protection is delayed for a few days until the receptor is downmodulated. Combining siRNAs targeting a viral and host gene protected mice from HSV-2 for a week, irrespective of the time of challenge.

Ebola virus (EBOV) infection causes a frequently fatal hemorrhagic fever that is refractory to treatment with currently available antiviral therapeutics. Geisbert et al.[170] prepared four siRNAs targeting the polymerase gene of the Zaire species of EBOV (ZEBOV) and complexed them with polyethylenimine or formulated them in SNALPs. Guinea pigs were treated with these siRNAs either before or after lethal ZEBOV challenge. Treatment of guinea pigs with a pool of the L gene-specific siRNAs delivered by polyethylenimine polyplexes reduced plasma viremia levels and partially protected the animals from death when administered shortly before the ZEBOV challenge. Evaluation of the same pool of siRNAs delivered using SNALPs proved that this system was more efficacious, as it completely protected guinea pigs against viremia and death when administered shortly after the ZEBOV challenge. Additional experiments showed that 1 of the 4 siRNAs alone could completely protect guinea pigs from a lethal ZEBOV challenge. In a later study[171], the same research group assessed the efficacy of modified nonimmunostimulatory siRNAs in a uniformly lethal nonhuman primate model of ZEBOV haemorrhagic fever. Two (66%) of three rhesus monkeys given four postexposure treatments of the pooled anti-ZEBOV siRNAs were protected from lethal ZEBOV infection, whereas all macaques given seven postexposure treatments were protected. The treatment was well

[170] Geisbert TW, Hensley LE, Kagan E, Yu EZ, Geisbert JB, Daddario-DiCaprio K, Fritz EA, Jahrling PB, McClintock K, Phelps JR, Lee AC, Judge A, Jeffs LB, MacLachlan I. Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. *J Infect Dis*. 2006 Jun 15;193(12):1650-1657.

[171] Geisbert TW, Lee AC, Robbins M, Geisbert JB, Honko AN, Sood V, Johnson JC, de Jong S, Tavakoli I, Judge A, Hensley LE, Maclachlan I. Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet*. 2010 May 29;375(9729):1896-1905.

tolerated with minor changes in liver enzymes that might have been related to viral infection. From results obtained in these studies, in January 2014, a Phase I clinical trial commenced, as it will be mentioned in Section 5 of this review.

The human papillomavirus (HPV) is the etiologic agent of cervical cancer. The E7 gene is a promising target for RNAi application in the management of HPV infection[172]. It plays an important role in cell-cycle regulation. Moreover, E7 inhibits the therapeutic effect of antiviral agents (such as IFN-α) and suppresses local immunity through functional inhibition of antigen-presenting cells and cytotoxic T lymphocytes. E7 gene silencing in high risk HPV types such as HPV-16 and HPV-18 by siRNAs suppressed cell growth *in vitro* and *in vivo*. siRNA duplexes or shRNA-expressing plasmids targeting the E7 genes of HPV-6b or HPV-11 were inoculated into cultured E7-expressing cells via cationic liposomes or into E7 gene-expressing mouse tumor models intratumorally or intravenously[171]. Both siRNAs and shRNA-expressing plasmids reduced *in vitro* the mRNA levels of HPV-6b or HPV-11 E7 to 20–40%. E7 mRNA expression in tumor models was reduced to 45–50% after three intratumoral injections. Intratumoral injections of RNAi effectors induced greater inhibition than did intravenous injections.

Rabies virus (RABV) infection continues to be a global threat to human and animal health, yet no curative therapy has been developed. siRNAs that target the conserved region of the RABV challenge virus standard (CVS)-11 strain nucleoprotein gene represent a promising approach for treating RABV infections[173]. Using a plasmid-based transient expression model, these siRNAs were capable of significantly inhibiting viral replication *in vitro* and *in vivo*. They effectively suppressed RABV

[172] Chen XZ, Zhu KJ, Xu Y, Tang XY, Cai XZ, Zhang X, Cheng H. RNA interference silences the human papillomavirus 6b/11 early gene E7 *in vitro* and *in vivo*. *Clin Exp Dermatol*. 2010 Jul;35(5):509-515.

[173] Yang YJ, Zhao PS, Zhang T, Wang HL, Liang HR, Zhao LL, Wu HX, Wang TC, Yang ST, Xia XZ. Small interfering RNAs targeting the rabies virus nucleoprotein gene. *Virus Res*. 2012 Oct;169(1):169-174.

expression in infected baby hamster kidney-21 (BHK-21) cells, as evidenced by direct immunofluorescence assay, viral titer measurements, real-time PCR, and Western blotting. In addition, liposome mediated siRNA expression plasmid delivery to RABV infected mice significantly increased survival, compared to a nonliposome-mediated delivery method.

siRNA immunoliposomes have also been shown as a therapeutic agent against H5N1 influenza virus infection. In a recent study[174], siRNA specific for influenza virus nucleoprotein (NP) mRNA was employed as the key antiviral agent to inhibit viral replication. A humanized single-chain Fv antibody (huscFv) against the hemagglutinin (HA) of H5N1 highly pathogenic avian influenza virus (HPAI) was used as the targeting molecule to HA of H5N1 virus, which is abundantly expressed on the surface of infected cells (the HA target cells). The immunoliposomes were shown to specifically bind HA-expressing Sf9 cells and demonstrated enhanced siRNA transfection efficiency. Furthermore, the siRNA silencing effect was more pronounced when the immunoliposomes were administered 6 to 12 h after H5N1 infection in MDCK cells compared with the nontargeted liposomes.

In a recent study[175], liposomes were used to deliver a self-amplifying RNA vaccine for respiratory syncytial virus (RSV). The vaccine potently induced neutralizing antibodies in cotton rats, as well as antigen-specific IFN-γ-producing CD4+ and CD8+ T cells in mice. These responses were comparable to or exceeding those elicited by RNA delivered by viral particles or electroporation of pDNA and provided protection against subsequent RSV infection.

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[174] Khantasup K, Kopermsub P, Chaichoun K, Dharakul T. Targeted small interfering RNA-immunoliposomes as a promising therapeutic agent against highly pathogenic Avian Influenza A (H5N1) virus infection. *Antimicrob Agents Chemother*. 2014 May;58(5):2816-2824.

[175] Geall AJ, Verma A, Otten GR, Shaw CA, Hekele A, Banerjee K, Cu Y, Beard CW, Brito LA, Krucker T, O'Hagan DT, Singh M, Mason PW, Valiante NM, Dormitzer PR, Barnett SW, Rappuoli R, Ulmer JB, Mandl CW. Nonviral delivery of self-amplifying RNA vaccines. *Proc Natl Acad Sci USA*. 2012 Sep 4;109(36):14604-14609.

5. Clinical development of LNP-RNAi for viral infections

The exhaustive work undertaken in preclinical studies (both *in vitro* in culture cells and *in vivo* in animals) has shown that RNAi therapeutic against viral infections is highly effective at reducing virus replication and also useful as a tool to rapidly identify novel antiviral drug targets via large-scale screens for a number of viral infections[176]. Several novel viral targets have been identified and are the subject of intense research and development, but definitive evidence is lacking from well-controlled studies that demonstrate the effectiveness in these infection diseases[177].Therefore, the next logical step must consist of human clinical trials that depict the realistic possibilities of this kind of therapy in viral infections.

The number of RNAi-based clinical trials for viral infections has grown over the past several years and has included studies against respiratory syncytial virus (RSV), HCV, HBV, HIV, and Ebola virus[178], some of them using lipid-based systems as vectors. Table 4 collects the clinical trials evolving the application of RNAi molecules to the treatment of viral infections.

Alnylam Pharmaceuticals Inc. has carried out clinical trials with a naked siRNA against RSV nucleocapsid (ALNRSV01). A Phase IIb study in adult lung transplant patients showed that this candidate is a promising alternative for RSV induced bronchiolitis obliterans syndrome (BOS) that causes significant morbidity and mortality in this group of patients;

[176] Blake SJ, Bokhari FF, McMillan NA. RNA interference for viral infections. *Curr Drug Targets*. 2012 Oct;13(11):1411-1420.

[177] DeVincenzo J, Lambkin-Williams R, Wilkinson T, Cehelsky J, Nochur S, Walsh E, Meyers R, Gollob J, Vaishnaw A. A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus. *Proc Natl Acad Sci USA*. 2010 May 11;107(19):8800-8805.

[178] Kanasty R, Dorkin JR, Vegas A, Anderson D. Delivery materials for siRNA therapeutics. *Nat Mater*. 2013 Nov;12(11):967-977.

moreover, the treatment with the siRNA was safe and well tolerated, and it was associated with more than 50%reduction in the incidence of new or progressive BOS at days 90 and 180[179].

The international clinical-stage biopharmaceutical company Santaris Pharma A/S has developed an anti-miRNA drug candidate currently in clinical testing (Phase II) for treatment of HCV infections (Miravirsen, SPC3649). This drug acts against MiR-122, a liver-specific miRNA that the HCV requires for replication[180]. Data from the Phase IIa trial showed that Miravirsen was well tolerated by patients with chronic HCV genotype 1, given as weekly subcutaneous injections, over 4 weeks. Antiviral activity was continued and prolonged well beyond the end of active therapy. These data provide clinical evidence about the potential of Miravirsen as once weekly monotherapy for chronic HCV[181].

Although in a less advanced state, Benitec Biopharma Ltd., with its subsidiary Tacere Therapeutics, has designed a shRNAibased multicassette vector called TT-034, which targets 3 well-conserved regions of HCV simultaneously, thus preventing generation of drug-resistant mutants. In addition, the targeted regions are conserved across all genotypes. Recently Benitec Biopharma Ltd. has announced that the first patient enrolled in the "first in man" Phase I/II clinical trial for TT-034 has received the first dose[182].

The biopharmaceutical company Arrowhead Research Corporation has recently presented data on the Phase I clinical study of ARC-520, the

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[179] Simon A, Karsten V, Cehelsky J, Shah S, Gollob J, Meyers R, Vaishnaw A, Glanville A, Zamora M, DeVincenzo J, Arcasoy S, Musk M, Sommerwerk U, Gottlieb J. Results of a phase 2b multi-center trial of ALN-RSV01 in respiratory syncytial virus (RSV)-infected lung transplant patients, *Eur Respir J*. 2012 Sep;40 (Suppl 56):267s.

[180] Gebert LF, Rebhan MA, Crivelli SE, Denzler R, Stoffel M, Hall J. Miravirsen (SPC3649) can inhibit the biogenesis of miR-122. *Nucleic Acids Res*. 2014 Jan;42(1):609-621

[181] Janssen HL, Reesink HW, Zeuzem S, Lawitz E, Rodriguez-Torres M, Chen A, Davis C, King B, Levin AA, Hodges MR. Randomized, double-blind, placebo-controlled safety, anti-viral, proof of concept study of miravirsen, an oligonucleotide targeting miR-122, in treatment-naive patients with genotype 1 chronic HCV infection. *Hepatology*. 2011;54(Suppl. 4) Abstr. LB-6.

[182] http://www.benitec.com/documents/140529HEPATITISCPATGCOMMENCEDFINAL.pdf.

company's clinical candidate for the treatment of chronic HBV infection. ARC-520 is a siRNA-based therapeutic composed of a hepatocyte-targeted peptide (NAG-MLP) that promotes the endosomal escape of the liver tropic HBV cholesterol-siRNA. Although additional blinding results are still missing, initial results in 36 healthy volunteers receiving different doses indicate that ARC-520 is well tolerated at doses expected to be efficacious in patients with chronic HBV[183], and a Phase IIa clinical trial has recently begun[184]. The study is planned to enroll up to 16 chronic HBV patients in two-dose cohorts with patients receiving either ARC-520 or placebo in combination with entecavir. The study is designed to evaluate the depth and duration of hepatitis B surface antigen (HBsAg) decline, among other measures, in response to a single dose of ARC-520.

In the case of HIV an *ex vivo* silencing approach has been developed by the company Calimmune, by using shRNA licensed by Benitec Biopharma. T cells are extracted from HIV patients, the gene that codes for the CCR5 receptor protein is silenced *ex vivo* and re-injecting the modified cells, resistance to HIV is conferred to the patients. In its Phase I/II clinical trial of the treatment, the enrolment of the first cohort of patients, which corresponds to the group that will receive modified CD4+ T cells and CD34+ stem cells, is about to be completed[185].

Finally, siRNA delivery against Ebola virus is also being under subject of a Phase I clinical trial recently initiated. Tekmira Pharmaceuticals has developed a therapeutic product composed of a combination of modified siRNAs targeting the Zaire Ebola polymerase, viral protein (VP) 24, and VP35 formulated in LNP (TKM-Ebola). The Ebola Phase I clinical trial is a randomized, single-blind, placebo-controlled

[183] Schluep T, Kalinoski L, Wooddell C, Lewis D, Gish R, Lickliter J. A Phase I, first in human clinical trial of ARC-520, an siRNA-based therapeutic for the treatment of chronic hepatitis B virus infection, in normal healthy volunteers. *Global Antiviral Journal HEP DART*. 2013;9(2):57.

[184] http://www.arrowheadresearch.com/press-releases/arrowhead-begins-dosing-phase-2a-trial-rnai-therapeutic-arc-520-chronic-hepatitis-b.

[185] http://www.benitec.com/documents/1311_CalimmunePresn_to_SIS.pdf.

study involving single ascending doses and multiple ascending doses of the lipid nanoparticle based formulation (TKMEbola). The study will assess the safety, tolerability, and pharmacokinetics of administering TKM-Ebola to healthy adult subjects[186].

Table 4: Clinical trials evolving the application of RNAi in the treatment of viral infections.

Clinical candidate	Targeting virus	RNAi molecule	Clinical phase	Company	Ref.
ALN-RSV01	RSV	siRNA	IIb	Alnylam Pharmaceuticals Inc.	179
Miravirsen, SPC3649	HCV	antimiRNA	IIa	Santaris Pharma A/S	180,181
TT-034	HCV	shRNA	I/IIa	Benitec Biopharma Ltd.	182
ARC-520	HBV	siRNA	II	Arrowhead Research Corporation	184
CCR5 negative cells (generated *ex vivo*)	HIV	shRNA	I/II	Calimmune and Benitec Biopharma Ltd.	185
TKM-Ebola	Ebola virus	siRNAs	I	Tekmira Pharmaceuticals	186

CCR5: chemokine receptor 5; RSV: respiratory syncytial virus; HCV: hepatitis C virus; HBV: hepatitis B virus; HIV: human immunodeficiency virus; siRNA: short interfering RNA; miRNA: microRNA; shRNA: short hairpin RNA.

[186] Tam YY, Chen S, Cullis PR. Advances in Lipid Nanoparticles for siRNA Delivery. *Pharmaceutics*. 2013 Sep 18;5(3):498-507.

6. Conclusions and future perspectives

As summarized in this review, RNAi is a promising therapeutic strategy due to its ability to silence any gene with a known sequence. However, the use of RNAi in the clinic is limited by the complexity of effective and well-controlled delivery *in vivo*. Naked RNAs have potential toxicities such as saturation of the innate RNAi machinery, stimulation of the immune response, and off-target effects. Moreover, systemic administration of RNAs encounters several obstacles that reduce their therapeutic efficacy: they are highly unstable intravascularly, with a short half-life due to their susceptibility to serum nucleases and rapid renal clearance. Consequently, RNAs do not accumulate in target tissues and cannot readily cross target cell membranes to access their cytoplasmic site of action. In addition, in the field of viral infections, it is necessary to find out new specific and effective targets and alternative combined targets to avoid mutant escapes.

The efforts to achieve a realistic clinical application of RNAi as therapeutic for viral infections should be focused mainly on three areas: reducing toxicity of RNAs while increasing stability, avoiding mutant escapes, and delivery to target tissues. RNA design and chemical modifications increase stability, reduce immunogenicity, and may help to reduce viral escapes; however, even modified, naked RNAs have poor cellular uptake due to their small size, net negative charge, renal clearance, and hydrophilicity. A range of delivery vectors such as liposomes, polymers, and nanoparticles have been developed to facilitate efficient cellular uptake and target site accumulation as well as to provide a degree of protection.

Among all delivery platforms, safe and easily manufactured LNP are good candidates, which can additionally be functionalized to achieve appropriate tropism, especially when the RNAi molecules are not tissue-

specific. In addition, the availability of different types of LNP favors the chance of attaining synergistic effects between RNAi molecules and conventional drugs, which may be greatly useful in the case of drugs that develop resistances or do not result effective. All these considerations, joint with data from ongoing clinical trials, suggest that in an early future the delivery of RNAi therapeutics by LNP could be a first-line treatment in several human diseases, such as viral infections.

OBJETIVOS

OBJETIVOS

La infección por el virus de la hepatitis C sigue siendo hoy en día un problema de salud pública a nivel mundial para el que los tratamientos existentes no siempre resultan del todo efectivos, debido a la diversidad de genotipos virales y a la presión farmacológica, que generan la aparición de resistencia. Por lo tanto, es necesario desarrollar estrategias terapéuticas alternativas a los tratamientos actuales.

Puesto que el virus de la hepatitis C es un virus que contiene una sola cadena de ARN (+) con capacidad para replicarse en el citoplasma de las células que infecta, el uso del ARN de interferencia puede ser una buena estrategia terapéutica capaz de inhibir su replicación. Un aspecto importante a tener en cuenta para explotar todo el potencial que tiene el uso del ARN de interferencia como estrategia terapéutica, es conseguir que el material genético entre de manera eficaz en las células diana. Para ello es indispensable diseñar un sistema de administración adecuado. Entre estos sistemas de administración, las nanopartículas sólidas lipídicas (SLNs) han demostrado ser una buena alternativa debido a su biocompatibilidad, seguridad y facilidad de producción. Otra ventaja importante es la posibilidad de incorporar en su superficie ligandos con el fin de lograr, entre otros, un incremento de la internalización celular o una distribución selectiva a tejidos y órganos concretos, lo que redunda en una mayor eficacia y/o menores efectos adversos.

Teniendo todo esto en cuenta, el objetivo principal de esta tesis es el diseño y evaluación de un vector no viral a base de SLNs para el tratamiento de la hepatitis C mediante terapia génica basada en ARN de interferencia. Para ello, el trabajo experimental se ha diseñado considerando los siguientes objetivos específicos:

1. Diseño y caracterización de vectores no virales basados en SLNs, protamina y un oligosacárido, dextrano o ácido hialurónico. Los vectores se prepararán con un plásmido que codifica un ARN de interferencia frente a la secuencia IRES (del inglés *internal ribosome entry site*), necesaria para la replicación del virus (shRNA74).

Se estudiará la influencia de la carga del material genético en las SLNs. Los vectores se caracterizarán en términos de tamaño, carga superficial, capacidad de unión y posterior liberación y protección del material genético frente a DNasas.

2. Evaluación de la capacidad de entrada, de la eficacia de silenciamiento *in vitro* de los vectores y de la viabilidad celular. Se compararán los diferentes vectores y se estudiará también el efecto de la dosis. Los estudios se realizarán en células hepáticas HepG2 que expresan el IRES y, tras el tratamiento con los vectores, se cuantificará el porcentaje de silenciamiento.

3. Evaluación de la capacidad de inhibir la replicación del virus de la hepatitis C *in vitro*. Se utilizará la línea celular Huh-7 NS3-3´, que expresa un replicón subgenómico del virus de la hepatitis C. Se evaluará el efecto de la carga del material genético y de la dosis en la eficacia de silenciamiento.

4. Estudio de los mecanismos de internalización y distribución intracelular en células hepática Huh-7, utilizando para ello técnicas de microscopía y citometría de flujo.

5. Estudio de la toxicidad de los vectores. Se evaluará *in vitro* la viabilidad celular de las células Huh-7 tratadas con los vectores, y la capacidad de hemaglutinación y hemólisis en muestras de eritrocitos.

DISEÑO EXPERIMENTAL

Capítulo 2

Solid lipid nanoparticles as non-viral vector for the treatment of chronic hepatitis C by RNA interference

RNA interference (RNAi) is a promising strategy to treat the chronic infection by hepatitis C virus (HCV). The objective of this work was to develop a non-viral vector based on solid lipid nanoparticles (SLN) and RNAi to inhibit the internal ribosome entry site (IRES) mechanism of the HCV.

The vectors were prepared with SLN, protamine, hylauronic acid (HA) or dextran (DX), and a short-hairpin RNA expression plasmid targeted to the stem loop II of the 50 UTR (shRNA74). The particle size, surface charge, and capacity to bind, release and protect the shRNA74 against nucleases were evaluated. Cell uptake, silencing capacity and cell viability were evaluated in HepG2 cells.

All the vectors presented particle size in the range of nanometers and positive surface charge, and they were able to protect the shRNA74 against DNase. An effective and rapid uptake into the cells was observed. Silencing capacity ranged from 3% to 67% depending on the presence of DX or HA in the vector, the shRNA74 to SLN ratio, and the shRNA74 dose.

Vectors prepared with HA showed to be twice more effective than those prepared with DX. Differences in the intracellular trafficking may justify the higher efficacy of the HA-prepared vectors.

1. Introduction

It is estimated that about 200 million people worldwide, 3% of the world's population, have been infected with the Hepatitis C virus (HCV), and as many as 170 million are chronically infected[1]. Only a fraction of infected people can clear the virus by developing immunity and responding to the triple therapy of interferon-α (INT-α) or pegylated interferon-α (PEG-INT-α), ribavirin and protease inhibitors (such as telaprevir or boceprevir), which is the standard of care for the treatment of HCV infection[2]. Many of infected patients do not respond to this combination therapy[3,4], resulting in a lifelong infection characterized by chronic liver inflammation that ultimately progresses to liver cirrhosis, hepatocellular carcinoma and liver failure[5].

The use of different protease inhibitors such as simeprevir and sofosbuvir, which have been recently approved in the USA and Europe, in combination with PEG-INT-α and ribavirin has been one of the more recent approaches[6]. However, the success of the therapy is dependent on the genotype and the viral load at its onset and during treatment. Furthermore, the treatment often causes severe side effects, limiting their broader use. Therefore, the development of an alternative antiviral strategy becomes necessary.

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[1] Chung RT, Baumert TF. Curing chronic hepatitis C – the arc of a medical triumph. *N Engl J Med*. 2014 Apr 24;370(17):1576-1578.

[2] Ding Y, Zhang H, Li Y, Wu D, He S, Wang Y, Li Y, Wang F, Niu J. Inhibition of HCV 5'-NTR and core expression by a small hairpin RNA delivered by a histone gene carrier. HPhA. *Int J Med Sci*. 2013 Jun 9;10(8):957-964.

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[6] Liu CH, Kao JH. Nanomedicines in the treatment of hepatitis C virus infection in Asian patients: optimizing use of peginterferon alfa. *Int J Nanomedicine*. 2014 Apr 25;9:2051-2067.

RNA interference (RNAi) is a natural mechanism based on the complementarity between RNA and target messenger RNA (mRNA) with perfect sequence specificity to cause destruction of the target without activating an interferon response[7,3,2]. Since the discovery of this process in animals[8], many applications have been studied and represent an exciting new technology for the molecular-targeted of various diseases including cancer and viral infections[9,10]. Since HCV is a positive-stranded RNA virus of about 9.6 kb, and its replication cycle occurs in the cytoplasm of infected cells, sequence-specific gene silencing based on RNAi offers a promising approach to inhibit HCV replication.

Within the viral genome, there are different sequences that have been used as target for RNAi both in the single open reading frame encoding a polyprotein precursor composed by structural and non-structural proteins, and in non-coding sequences, also called untranslated regions (UTR). The first studies showing efficacy of RNAi used HCV non-structural proteins sequences of the polyprotein region (including NS3, NS4B, NS5A and NS5B). After that, more efficient results were obtained using non-coding regions[11]. The highly conserved 50 and 30 UTR of the HCV genome are required for both protein translation by an internal ribosome entry site (IRES) mechanism and for virus replication[12,13], and

[7] Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. *Nat Rev Drug Discov*. 2009 Feb;8(2):129-138.

[8] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*. 1998 Feb 19;391(6669):806-811.

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[10] Huang PI, Lo WL, Cherng JY, Chien Y, Chiou GY, Chiou SH. Non-viral delivery of RNA interference targeting cancer cells in cancer gene therapy. *Curr Gene Ther*. 2012 Aug;12(4):275-284.

[11] Pan Q, Tilanus HW, Janssen HL, van der Laan LJ. Prospects of RNAi and microRNA-based therapies for hepatitis C. *Expert Opin Biol Ther*. 2009 Jun;9(6):713-724.

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efficient inhibition of HCV replication has been demonstrated targeting IRES region[14,15,16].

A key challenge to exploit the full potential of RNAi therapeutics is the efficient delivery of the genetic material to the target cells. Actually, due to the negative surface charge, entry of the naked RNAi molecules into the cytoplasm is hampered and must resist the digestion of nucleases into the serum. For this purpose, the use of an appropriate delivery system becomes essential.

Lipid-based systems, which include solid lipid nanoparticles (SLNs), have been increasingly recognized as one of the most promising non-viral delivery systems for RNAi due to their biocompatibility, their relative safety and simplicity of production[17,7], joint with the non-limit in the size of the genetic material they can transport. In addition, SLNs may be decorated with components such as protamine, dextran, oligochitosans or hyaluronic acid to improve their efficacy both *in vitro* and *in vivo*[18,19,20,21,22,23].

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[14] Hamazaki H, Ujino S, Abe E, Miyano-Kurosaki N, Shimotohno K, Takaku H. RNAi expression mediated inhibition of HCV replication. *Nucleic Acids Symp Ser (Oxf)*. 2004;(48):307-308.

[15] Ilves H, Kaspar RL, Wang Q, Seyhan AA, Vlassov AV, Contag CH, Leake D, Johnston BH. Inhibition of hepatitis C IRES-mediated gene expression by small hairpin RNAs in human hepatocytes and mice. *Ann N Y Acad Sci*. 2006 Oct;1082:52-55.

[16] Kanda T, Steele R, Ray R, Ray RB. Small interfering RNA targeted to hepatitis C virus 5' nontranslated region exerts potent antiviral effect. *J Virol*. 2007 Jan;81(2):669-676.

[17] de Fougerolles AR. Delivery vehicles for small interfering RNA 'in vivo'. *Hum Gene Ther*. 2008 Feb;19(2):125-132.

[18] 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 Nov;79(3):495-502.

[19] Delgado D, del Pozo-Rodríguez A, Solinís MÁ, Avilés-Triqueros M, Weber BH, Fernández E, Gascón AR. Dextran and protamine-based solid lipid nanoparticles as potential vectors for the treatment of X-linked juvenile retinoschisis. *Hum Gene Ther*. 2012 Apr;23(4):345-355.

[20] Delgado D, Gascón AR, Del Pozo-Rodríguez A, Echevarría E, Ruiz de Garibay AP, Rodríguez JM, Solinís MÁ. Dextran-protamine-solid lipid nanoparticles as a non-viral vector for gene therapy: *in vitro* characterization and *in vivo* transfection after intravenous administration to mice. *Int J Pharm*. 2012 Apr 4;425(1-2):35-43.

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The objective of this work was to design a non-viral vector based on SLN containing a short-hairpin RNA (shRNA) expression plasmid targeted to the HCV IRES region, as a new strategy to treat the chronic infection by HCV.

2. Materials and methods

2.1. Materials

For the elaboration of the nanocarriers, DOTAP (1,2-Dioleoyl-3-trimethylammonium-propane chloride salt) was purchased from Avanti Polar-lipids Inc. (AL, USA), Precirol® ATO 5 was generously provided from Gattefossé (Madrid, Spain), Tween 80 and dichloro-methane were obtained from Panreac (Madrid, Spain) and protamine sulfate salt Grade X (P) and the polysaccharides hyaluronic acid (HA) and dextran (DX) were purchased from Sigma–Aldrich (Madrid, Spain). The materials for the agarose electrophoresis gel were purchased from Bio-Rad (Madrid, Spain).

The human hepatocellular carcinoma HepG2 cell line and Eagle's Minimum Essential Medium EMEM (30-2003) were supplied by American Type Culture Collection (ATCC). The fetal bovine serum (FBS) and Reduced-Serum Minimal Essential Medium (OPTI-MEM) were purchased from Invitrogen. The antibiotic solution Normocín™ was obtained from Invivogen and the Cell Counting viability Kit 8 (CCK-8) was purchased from Sigma–Aldrich (Spain) as all other reagents of analytical grade. The rat monoclonal CD44 antibody and Alexa Fluor 488-conjugated goat anti-

[22] Ruiz de Garibay AP, Solinís MA, 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 Mar;11(3):500-511.

[23] Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Gascón AR, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. *Int J Pharm*. 2014 Apr 25;465(1-2):413-426.

rat IgG were obtained from Abcam. The transfection reagent Lipofectamine® 2000 was obtained by Invitrogen.

Finally, functional HCV-IRES 1b U6-Promoter-GFP-Neo plasmid, silencer shRNA74 (sequence of the siRNA74 that encodes; 3'-TCGCAGATCGGTACCGCAA-5'), negative control shRNAscramble and positive control shRNA74-GFP plasmids were purchased from GeneCust Europe (Luxembourg).

2.2. Preparation of shRNA74-containing vectors

The SLNs were prepared by the solvent emulsification-evaporation technique previously described by del Pozo-Rodríguez et al.[24]. Briefly, an aqueous phase containing DOTAP (0.4%, w/v) and Tween 80 (0.1%, w/v) was added to the lipid Precirol® ATO 5 dissolved in the organic solvent dichloromethane (5%, w/v). The mixture of the two phases was immediately emulsificated by sonication (Branson Sonifier 250, Danbury) and, after the evaporation of the dichloromethane using a magnetic agitator during 1 h and keeping later in vacuum conditions for 15 min, nanoparticles were obtained.

HA-P-shRNA74-SLN and DX-P-shRNA74-SLN vectors were prepared as described by Apaolaza et al.[23]. Firstly, a P-shRNA74 complex at a fixed ratio of 2:1 (w/w) was prepared. Then, an aqueous solution of HA or DX was added to form the HA-P- shRNA74 and DX-P-shRNA74 complexes at 0.5:2:1 and 1:2:1 ratios (w/w/w), respectively. Finally, the solution of the obtained complexes was put in contact with the previously prepared SLNs suspension, and vectors were formed by electrostatic interactions at two shRNA74 to SLN ratios, expressed as the ratio of DOTAP to shRNA74 (w:w): 1:2 (HA-SLN2 or DX-SLN2) and 1:5 (HA-SLN5 or DX-SLN2). This

[24] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles: formulation factors affecting cell transfection capacity. *Int J Pharm*. 2007 Jul 18;339(1-2):261-268.

difference in the SLN ratio brings about a difference in the shRNA74 load of each vector.

2.3. Characterization of the nanocarriers

Size of the vectors was determined by Photon Correlation Spectroscopy (PCS) and Z potential was measured by laser Doppler velocimetry (LDV). All samples were diluted in Milli-QTMwater and both measurements were performed on a Zetasizer Nano series- Nano ZS (Malvern Instruments, Worcestershire, UK).

2.4. Agarose gel electrophoresis

The capacity of the vector to bind the shRNA74, to protect it against DNase I (Sigma-Aldrich, Spain) digestion and to release it was evaluated by electrophoresis during 30 min at 120 V on a 1% agarose gel containing Gel Red™ (Biotium) for visualization of the DNA.

Complexes were diluted in MilliQ™ water up to a final concentration of 0.03 µg DNA/ml. A concentration of 1 U DNase I/ 2.5 µg DNA was added to HA-P-shRNA74-SLN or DX-P-shRNA74- SLN vectors, and the mixtures were then incubated at 37°C during 30 min. Afterwards, 4% SDS solution was added to the samples to a final concentration of 1% to release the shRNA74 from the SLNs. Finally, the bands were observed with an Uvitec Uvidoc D-55-LCD- 20 M auto transilluminator and the integrity of the DNA in each sample of binding, protection and release was compared to a control of untreated shRNA74 plasmid.

2.5. Cell culture conditions

The human hepatocellular carcinoma cell line HepG2 was maintained in EMEM, supplemented with 10% (v/v) heat-inactivated FBS

and 1% of Normocín™ antibiotic solution at 37ºC in a 5% CO_2 atmosphere and subcultured every 2–3 days using trypsin-EDTA (Lonza).

2.6. Cellular uptake of non-viral vectors

Entry of vectors into HepG2 cells was studied qualitatively and semi-quantitatively by an Axio Observer Inverted Microscope Z.1 with ApoTome (Zeiss) using 63x magnification and Gallios™ flow cytometer (Beckman Coulter, FLorida, Miami, US), respectively. For this purpose, SLNs were labelled with the fluorescent dye Nile Red (λ = 590 nm) as described in a previous study[25].

To analyze the uptake by fluorescence microscopy, cells were seeded in Millicell® EZ slides (Millipore) in a density of 1.2 x 10^5 cells/well. Cells were allowed to adhere overnight and then were treated with the different formulations (shRNA74 dose: 2.5 µg). Two hours after the addition of the labelled vectors, the cells were washed three times with PBS, fixed with paraformaldehyde (PFA) 4% (Panreac) and the nuclei were dyed with the mounting media DAPI-Fluoromount-G® (SouthernBiotech) for microscopy.

For the semi-quantitative analysis, the cells treated with the vectors were washed three times with PBS and detached from plates and analysed by flow cytometry at 650 nm (FL3) collecting 1 x 10^4 events per sample.

2.7. Detection of CD44 expression by immunocytochemistry

The presence of the HA specific receptor CD44 was also studied in HepG2 by inmunocytochemistry. For this purpose 1.2 x 10^5 cells/ well

[25] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles for retinal gene therapy: transfection and intracellular trafficking in RPE cells. *Int J Pharm*. 2008 Aug 6;360(1-2):177-183.

were seeded in a 24 wells plate with cover glasses in the bottom and previously treated with poli-L-lysine (Sigma-Aldrich). After 24 h of incubation (37ºC, 5% CO_2), the cells were washed three times with PBS and fixed with 4% paraformaldehyde (Panreac) during 10 min. Afterwards, the cells were washed three times with PBS and 200 ml of a blocking solution (PB buffer, 0.3% Triton X-100, 10% goat serum) was added in order to block and permeabilize the cells during 30 min. An antibody solution containing the rat monoclonal CD44 antibody in PB buffer (2.5% goat serum, 0.1% triton X-100) was then added keeping it in contact during 1 h. After 3 more washes with PBS, the cells were dyed with the secondary antibody Alexa Fluor 488-conjugated goat anti-rat IgG for 1 h in the dark. Finally, the nuclei were dyed with the mounting media DAPI-Fluoromount-G® (SouthernBiotech). The images were captured with an Axio Observer Inverted Microscope Z.1 with Apotome (Zeiss) using 40x magnification.

2.8. Silencing protocol

For silencing studies the cells were seeded in a 24 wells plate (1.2 x 10^5 cells/well) with EMEM complemented with 10% FBS and were allowed to grow overnight.

The cells were firstly transfected with HCV-IRES 1b U6-Promoter-GFP-Neo vector (IRES-GFP) to obtain the model of infected hepatocytes. Lipofectamine® 2000 transfection reagent was used according to the manufacturer's instructions. Briefly, the IRES-GFP plasmid and Lipofectamine® 2000 were mixed separately but in an equal total volume of 50 ml with OPTI-MEM at room temperature during 5 min. Then, the solution of the transfection reagent was added to the plasmid solution and, after 20 min of incubation at room temperature, 0.8 µg of IRES-Lipofetamine®2000 was added to each well based on 0.5 ml cell culture media.

After 2 h the media was replaced and the cells were transfected with the four silencing vectors HA-SLN2, HA-SLN5, DX-SLN2 and DX-SLN5 at different shRNA74 doses: 1.5 µg, 2 µg, 2.5 µg and 3 µg, corresponding to an IRES to shRNA74 ratio of 1:1.8; 1:2.5; 1:3.1; and 1:3.7 (w/w), respectively. Transfections with a shRNA scramble (shRNAscr) and with the naked shRNA74 plasmid were used as internal controls. After 4 h, 1 ml of fresh media was added.

2.9. *In vitro* HCV inhibition efficacy

The silencing efficiency of HCV by the four formulations at different shRNA74 doses were evaluated *in vitro* at 48 h post-transfection by measuring the inhibition of GFP expression previously induced with the IRES-GFP plasmid. Inhibition of GFP expression was qualitatively monitored under a fluorescence microsope (4x), and a fluorometric analysis was performed to quantify the intracellular GFP expression after the silencing treatment. Briefly, the culture media was replaced with 300 ml of Reported Lysis Buffer (RLB, Promega) and incubated for 30 min at 37^{o}C in a 5% CO_2 atmosphere. The cells were then scraped and centrifugated at 12000 g during 2 min. The supernatant was recovered and the GFP fluorescence of each sample was measured in a Glomax™ Multi detection System (Promega). The results were then corrected by the total amount of protein of each sample quantified by Micro BCA™ Protein Assay kit (Thermo Scientific). The silencing percentage was calculated from the fluorescent amounts (RFU/mg total protein).

2.10. Cell viability

The viability of the HepG2 cells treated with the vectors was studied with the CCK-8 assay following the manufactture's protocol. Briefly, the cells (5 x 10^3 cells/well) were cultured on a 96-well culture

plate for 24 h followed by transfection with an IRES-GFP and Lipofectamine® 2000 transfection reagent. After 2 h the media was replaced and the cells were transfected with the different vectors. At 48 h post-transfection, the CCK-8 reactive was added. After 4 h incubating the plate at 37ºC, cell viability was measured by reading the absorbance at 450 nm. Untreated cells and cells treated with Triton-X 10% were used as positive and negative control, respectively.

2.11. Statistical analysis

The results were expressed as mean _ standard deviation. Statistical significance between groups during analysis of HCV inhibition and CCK-8 assay of cell viability was performed with IBM SPSS Statistics 21. Normal distribution of samples was assessed by the Shapiro–Wilk test, and homogeneity of variance, by the Levene test. The formulations were compared with ANOVA test. A p value of <0.05 was considered as evidence of a statistically significant difference.

3. Results

3.1. Characterization of the nanocarriers

We have prepared four vectors with different shRNA74 to SLN ratio and with HA or DX. As can be seen in Table 1, particle size of the two vectors prepared with HA was around 240 nm without significant differences between them. The particle size of the vectors prepared with DX was lower than those of vectors bearing HA, although significant differences were only observed between the particle size of HA-SLN5 and DX-SLN2 (p < 0.05). No significant differences were found between the two vectors prepared with DX. All the vectors showed cationic surface

charge, and the DX- SLN5 formulation presented surface charge statistically higher to the others ($p < 0.01$). The polydispersity index was lower than 0.25 in all cases.

Table 1. Physical characterization of SLNs-based vectors bearing the shRNA74 plasmid. PA (polyanion); Particle size (nm); Polydispersity index (PdI); Zeta potential (mV). Mean ± SD (n=3).

Vector	PA:P:shRNA74:SLN ratio	Size (nm)	Zeta potential (mV)	PdI
HA-SLN2	0.5:2:1:2	233 ± 13	+29 ± 0.46	0.21 ± 0.012
HA-SLN5	0.5:2:1:5	242 ± 16	+30.97 ± 0.45	0.23 ± 0.004
DX-SLN2	1:2:1:2	208 ± 9.9*	+31.58 ± 0.86	0.23 ± 0.006
DX-SLN5	1:2:1:5	219± 3.5	+38.87 ± 0.64 **	0.22 ± 0.018

* $p < 0.05$ respect to HA-SLN5; ** $p < 0.01$ respect to all other formulations.

3.2. Agarose gel electrophoresis

The vectors were subjected to electrophoresis on agarose gel to assess the capacity of the nanocarriers to effectively bind the plasmid, to protect it from the degradation of DNase I and to release it. Figure 1 shows that in all formulations, shRNA74 was fully bound (lanes 2–5). After treatment with DNase I, the DNA bands were detected in all lanes, which indicates that all formulations protected the shRNA74 from nuclease action (lanes 7–10); however, in the lanes corresponding to the vectors prepared with DX a light tail was also detected, which indicates a slight degradation of the plasmid. After the treatment with SDS, shRNA74 was successfully released (lanes 11–14).

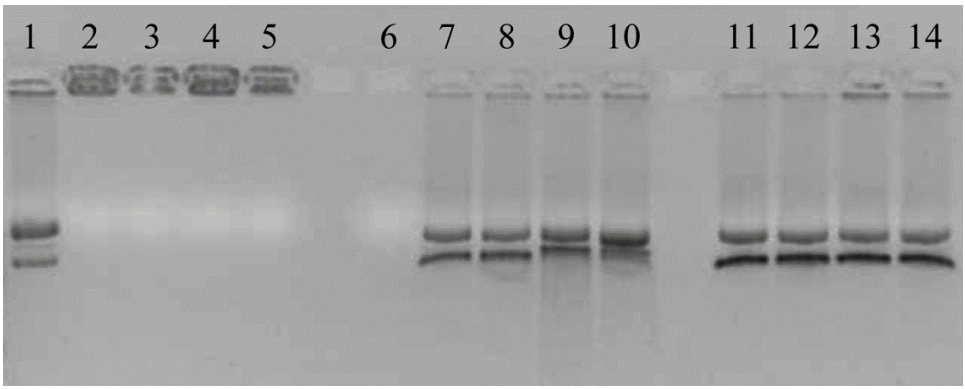

Figure 1. Binding, protection and release of shRNA74 by HA-P-SLNs and DX-P-SLNs vectors as visualized by agarose (1%) gel electrophoresis; lane 1: free shRNA74; lane 2: shRNA74 bound to HA-P-SLNs in 1:2 ratio (HA-SLN2); lane 3: shRNA74 bound to HA-P-SLNs in a 1:5 ratio (HA-SLN5); lane 4: shRNA74 bound to DX-P-SLNs in 1:2 ratio DX-SLN2); lane 5: shRNA74 bound to DX-P-SLNs in 1:5 ratio (DX-SLN5); lane 6: DNase I - treated free shRNA74; lane 7: DNase I - treated HA-SLN2 vector; lane 8: DNase I - treated HA-SLN5 vector; lane 9: DNase I - treated DX-SLN2 vector; lane 10: DNase I - treated DX-SLN5 vector; lane 11: shRNA74 released from HA-SLN2; lane 12: shRNA74 released from HA-SLN5; lane 13: shRNA74 released from DX-SLN2 and lane 14: shRNA74 released from DX-SLN5.

3.3. Cellular uptake of non-viral vectors

The capacity of the vectors to enter the cells was studied by flow cytometry and fluorescence microscopy (Figures 2 and 3). The fluorescence intensity corresponding to Nile Red-labelled nano-carriers was higher with the vectors prepared with a shRNA74 to SLN ratio of 1:5, irrespective of the polysaccharide HA or DX, which is related to a higher entry ($p < 0.01$). However, no differences were observed between HA and DX formulations with the same SLN proportion.

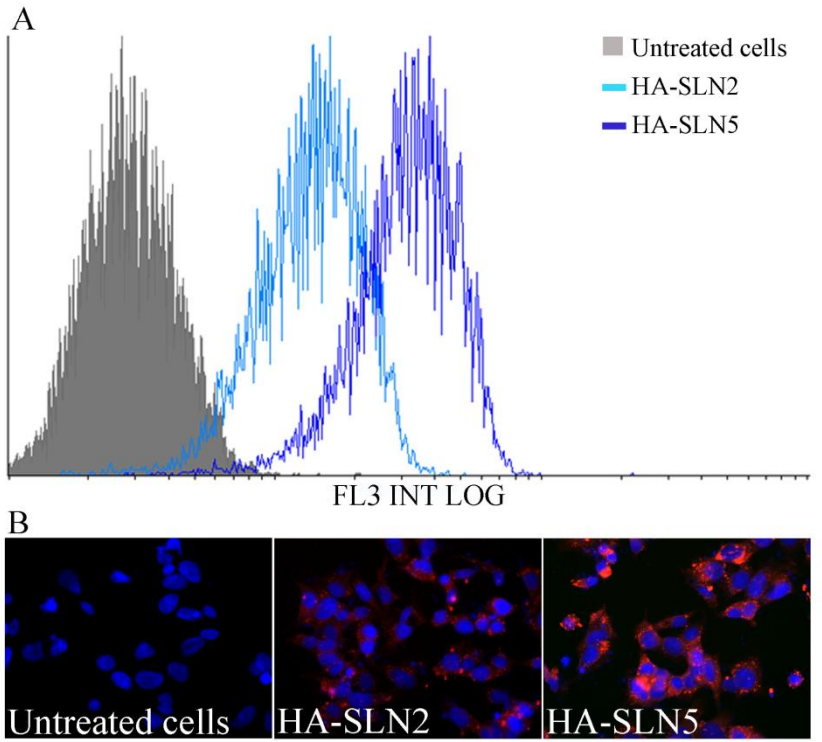

Figure 2. Cellular uptake of non-viral vectors in HepG2 cells 2 h after addition of Nile Red-labelled HA vectors. (A) Flow cytometry histograms. (B) Fluorescence microscopy (40x).

3.4. Detection of CD44 expression by immunocytochemistry

Figure 4 demonstrates the presence of the specific HA receptor CD44 in HepG2 cell line. The fluorescence images show the nuclei of the cells treated with DAPI-Fluoromount-G® in blue. The expression of CD44 in the cells treated with the rat monoclonal CD44 antibody and with the Alexa Fluor 488-conjugated goat anti-rat IgG (B) is shown in green.

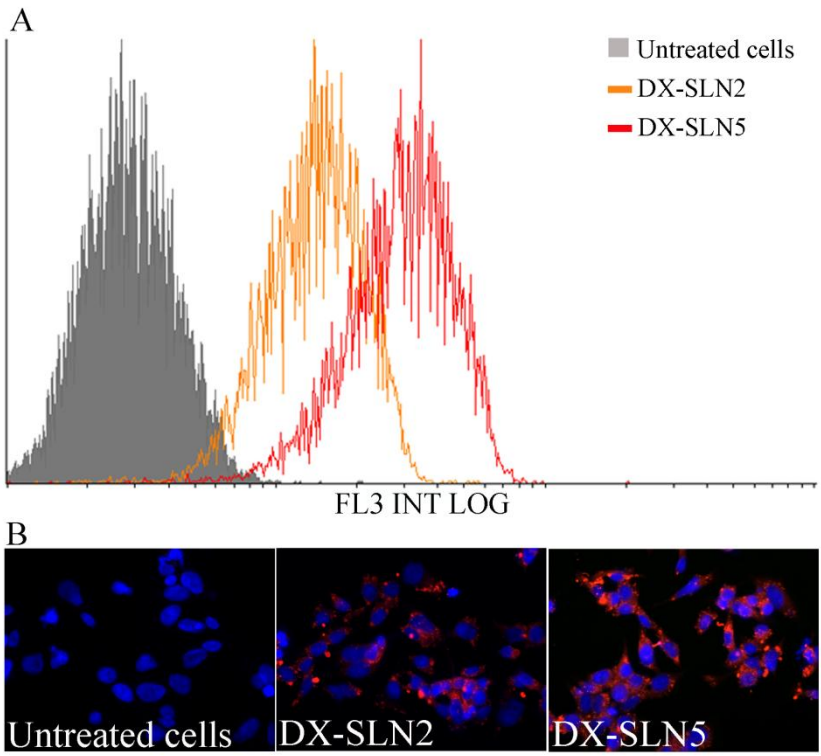

Figure 3. Cellular uptake of non-viral vectors in HepG2 cells 2 h after addition of Nile Red-labelled DX vectors. (A) Flow cytometry histograms. (B) Fluorescence microscopy (40x).

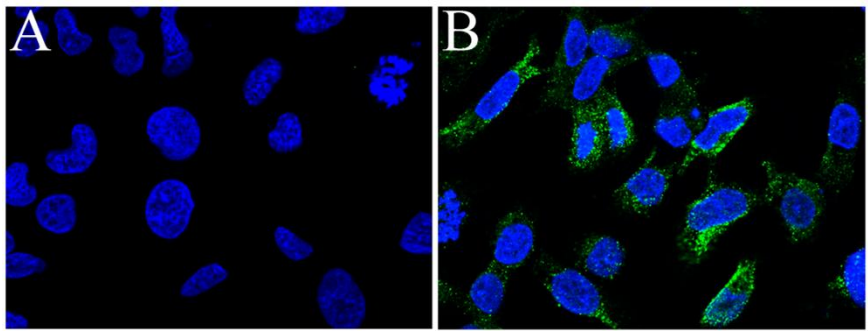

Figure 4. Fluorescence microscopy images (63x) of Alexa Fluor 488-CD44 receptor (green) in HepG2 cell line. Cells were treated with DAPI-Fuoromont-G® (blue). (A) Control sample. (B) Treated cells.

3.5. *In vitro* inhibition of IRES-GFP

Figures 5 and 6 feature the capacity of the vectors to inhibit the expression of GFP, and therefore, of IRES, in HepG2 cells. No silencing was observed when the cells were treated with shRNAscr, and naked shRNA74 induced a negligible silencing rate (< 2%). Silencing capacity of the vectors prepared with HA ranged from 19% to 67%, depending on the HA formulation and the shRNA74 dose (Figure 5).

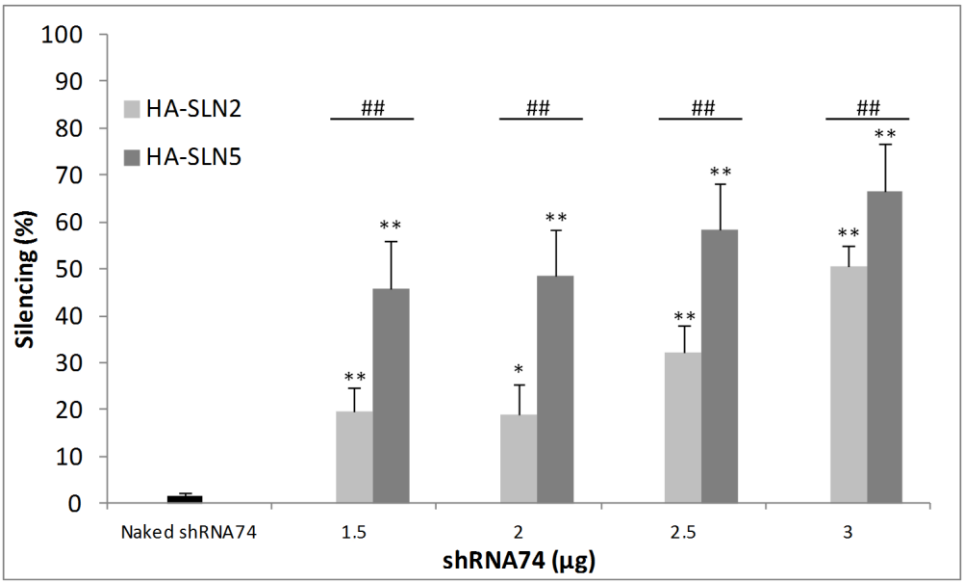

Figure 5. Silencing of IRES expression in HepG2 cells, 48 h after the treatment with HA-SLN2 and HA-SLN5 vectors, at different doses of shRNA74. Error bars represent SD (n = 3). ##p < 0.01 between formulations; **p < 0.01 respect to naked shRNA74; *p < 0.05 respect to naked shRNA74.

The silencing rate increased with the shRNA74 dose with the two formulations, and for every shRNA74 dose, the HA-SLN5 vector provided a higher silencing than the HA-SLN2 vector (p < 0.01). Regarding the HA-

SLN5 vector, there was no significant difference in the silencing rate between the two higher doses, 2.5 and 3 µg.

Figure 6 shows the ability of the vectors prepared with DX to silence the GFP expression. As with HA, the silencing rate increased with the shRNA74 dose, and the DX-SLN5 vector induced a higher silencing rate than the DX- SLN2 vector.

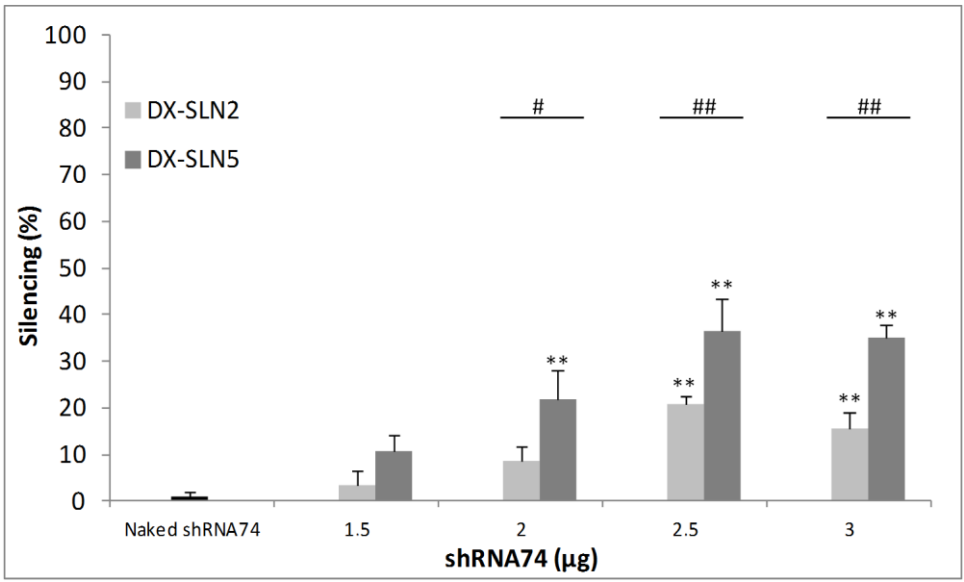

Figure 6. Silencing of IRES expression in HepG2 cells, 48 h after the treatment with DX-SLN2 and DX-SLN5 vectors, at different doses of shRNA74. Error bars represent SD (n = 3). ##$p < 0.01$; #$p < 0.05$; **$p < 0.01$ respect to naked shRNA74.

The higher silencing level (around 36%) was obtained with the DX-SLN5 vector at the higher doses (2.5 µg and 3 µg). When comparing the vectors prepared with DX and HA, we can see that HA vectors were more effective. Contrary to DX vectors, HA vectors were able to silence GFP

even with the lower dose (1.5 µg), and at the highest shRNA74 dose level, HA vectors induced a silencing rate two-fold higher than the DX-vectors.

3.6. Cell viability

Figures 7 and 8 show the cell viability of HepG2 cells 48 h after the treatment with the HA and DX vectors, respectively. The viability of the cells treated with the HA-SLN2 vector was around 90%, without significant differences depending on the shRNA74 dose. When the cells were transfected with the HA-SLN5 vector, the viability significantly decreased with the shRNA74 dose, with the 3 µg dose having a 64%.

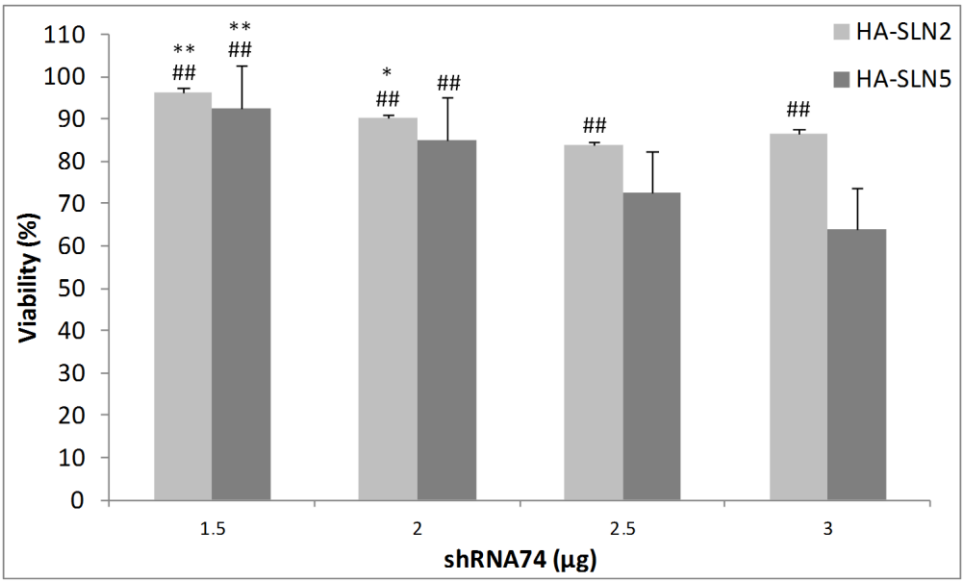

Figure 7. Cell viability 48 h after treatment of HepG2 cells with the HA-SLN2 and HA-SLN5 vectors, at different doses of shRNA74. Error bars represent SD (n = 3). ##$p < 0.01$ respect to HA-SLN5, dose 3 µg; **$p < 0.01$ respect to HA-SLN5, dose 2.5 µg; *$p < 0.05$ respect to HA-SLN5, dose 2.5 µg.

Regarding the DX-SLN vectors, cell viability was around 90% except with the formulation DX-SLN5 at the higher dose (3 µg), which induced a viability of 76%, significantly lower than the viability measured when the cells were treated with the DX-SLN2 vectors at the shRNA74 dose level of 1.5 µg ($p < 0.01$) and 2 µg ($p < 0.05$).

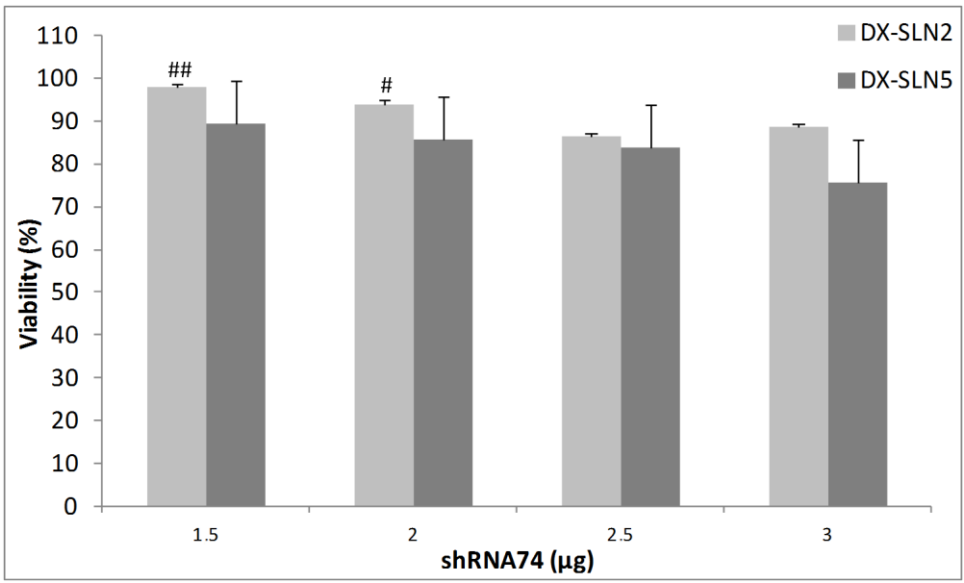

Figure 8. Cell viability 48 h after treatment of HepG2 cells with the DX-SLN2 and DX-SLN5 vectors, at different doses of shRNA74. Error bars represent SD (n = 3). ##$p < 0.01$ respect to DX-SLN5, dose 3 µg; #$p < 0.05$ respect to DX-SLN5, dose 3 µg.

4. Discussion

Many of infected patients do not respond to the standard of care for HCV. In this regard, the use of multiple viral targets, combined

targeting of viral and host factors, or the combination of advanced therapies with conventional treatments could be a good option to achieve the best therapeutic effect[26]. Among the new antiviral strategies, RNAi appears to be a powerful therapeutic approach for a variety of viral diseases, and can be especially interesting to treat chronic HCV[27,26].

One of the possibilities to silence genes is the use of shRNA expression plasmids, which have greater durability and higher efficacy than other RNAi molecules, although they must reach the nucleus of cells to be processed[28,29]. In this work we have formulated the shRNA74 targeted to the stem loop II of the 5' UTR in SLNs, and we have demonstrated the ability to inhibit the expression of IRES in HepG2 cells previously transfected with a plasmid encoding this viral protein.

SLNs are a commonly used non-viral vector for gene therapy. They have shown a high capacity to protect the genetic material from degradation, and are also able to efficiently internalize into the cells and deliver the genetic material[30,31]. In previous studies, we have demonstrated the ability of SLNs to transfect *in vitro* several cell lines and after systemic administration to mice[32,33]. The incorporation of different

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components such as DX or oligochitosans to the SLNs resulted in longer-term transfection in different organs, including the liver, one of the organs where the HCV remains in chronic infection[34].

In this study, we have designed a vector composed by SLNs, protamine and a polysaccharide (DX or HA). The peptide protamine has a great capacity to condense and protect nucleic acids, facilitate their transport to the nucleus and improve intranuclear transcription[35,36]. DX and HA are biocompatible polysaccharides previously used to improve the transfection capacity of SLNs[37,38,39]. DX has been shown to induce a longer circulation time of SLNs *in vivo*, hampering the interactions with serum components[32].

The size of the vectors ranged from 200 nm to 240 nm depending on the polysaccharide incorporated to the SLNs. The vectors were prepared at two shRNA74 to SLN ratios, 1:2 and 1:5. DX-SLN vectors containing the highest proportion of cationic nanoparticles (DX-SLN5) showed higher surface charge than DX- SLN2; however, a higher

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[33] del Pozo-Rodríguez A, Delgado D, Solinís MA, Pedraz JL, Echevarría E, Rodríguez JM, Gascón AR. Solid lipid nanoparticles as potential tools for gene therapy: *in vivo* protein expression after intravenous administration. *Int J Pharm*. 2010 Jan 29;385(1-2):157-162.

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proportion of cationic nanoparticles in the HA-SLN5 vector in comparison with the HA-SLN-2 vector did not lead to a change in the surface area (Table 1). It is important to take into account that the zeta potential reflects the net surface charge of the vectors and, therefore, changes in the amount of charges do not always imply modifications in the charge distribution at surface.

According to the agarose gel electrophoresis (Figure 1), nano-carriers showed suitable features as non-viral vectors: ability to bind, to release and, to protect the shRNA74 against nucleases, although vectors prepared with HA provided a higher protection degree than those containing DX.

The internalization of vectors into the cells, necessary for transfection, is initiated by the binding of the vectors to the cell surface. Cationic surface charges of the vectors facilitate the interaction with the cell membrane, negatively charged, inducing the internalization of the nanocarriers into the cells. Our vectors were very effective and rapidly internalized, and the higher the SLN to plasmid ratio was, the higher the cell uptake was (Figures 2 and 3). No difference in cell uptake was found depending on the polysaccharide used to prepare the vectors. Differences in cell uptake depending on the shRNA74 to SLN ratio could explain, al least in part, the higher silencing rate of the vectors prepared at a shRNA74 to SLN ratio of 1:5 (Figures 5 and 6), although they do not explain the lower silencing ability of the vectors prepared with DX in comparison with the vectors prepared with HA.

The silencing capacity depended on the presence of DX or HA in the vector, the shRNA74 to SLN ratio, and the shRNA74 dose. With both polysaccharides the higher silencing efficacy was obtained with the DX-SLN5 and HA-SLN5 vectors at the higher shRNA74 doses (2.5 μg and 3 μg). These dose levels correspond to an IRES:shRNA74 ratio of 1:3.1–

1:3.7. This ratio is similar to the 1:4 ratio used by Prabhu et al.[40] in a previous study, in which siRNA74 was intracellulary delivered in Huh-7 cells to inhibit GFP expression from HCV-IRES. A control shRNAscr did not inhibit the GFP expression, indicating that the silencing activity of shRNA74 is highly specific. Nacked shRNA74 induced very small inhibition of GFP expression (< 2%), confirming the importance of the delivery system for efficient transfection.

Additionally to the silencing efficacy, the potential toxicity of the vectors is also crucial. Cell viability of the HepG2 cells treated with the vector HA-SLN2 was around 90%; however, viability decreased with the HA-SLN5 vector when increasing the shRNA74 dose (Figure 7). Although less pronounced, the same behavior is observed with the formulations prepared with DX (Figure 8). It is important to consider that when increasing the plasmid dose, the number of nanoparticles added to the cell culture increases, and for a certain dose of plasmid a higher amount of nanoparticles is added to the cultured cells in the case of the vectors prepared with a shRNA74 to SLN ratio of 1:5. As a result, viability of the cells is affected by the amount of nanoparticles. Therefore, although HA-SLN5 and DX-SLN5 are more effective than HA-SLN2 and DX-SLN2, respectively, they induce lower cell viability, mainly at the higher doses.

As previously mentioned, cell uptake does not explain the difference in the silencing rate of the HA-vectors in comparison with the DX-SLN vectors. The study of the capacity of the formulations to protect the plasmid revealed that the formulations prepared with HA provided a higher protection degree, and this higher protection degree may contribute to explain the higher silencing efficacy. On the other hand, differences in the intracellular fate of the vectors inside the cells, which is

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conditioned among other reasons by the entry mechanism, may also explain the difference in the silencing efficacy.

Endocytosis has been claimed as the major mechanism of cell uptake of non-viral vectors. Certain endocytic mechanisms are ubiquitous to all cells, but others are cell-specific or play an enhanced role in certain cells. In a previous work, we demonstrated the presence in HepG2 cells of clathrin and caveolae mediated endocytosis mechanisms in a similar extend[34].

In the present study we have confirmed the presence of the CD44 receptor in the HepG2 cells (Figure 4), which may be also involved in the internalization of the vectors. It is well known that HA is able to interact with the CD44 receptor and it has been used to enhance the cell uptake of nanocarriers by inducing the CD44-mediated endocytosis[41,42]. When HA interacts with CD44[43], the caveolin-mediated endocytosis is activated and this endocytic route has the ability to bypass lysosomes. However, vectors containing DX are internalized mainly by the endolysosomal pathway[35,38]. Therefore, differences in the internalization mechanism may lead to a higher lysosomal degradation of the plasmid when formulated in the DX-SLN vector.

Additionally, HA is able to modulate the high condensation degree of the plasmid due to the protamine, which facilitates the release of the plasmid from the vector inside the cell and therefore the transfection, especially when the vector is uptaken by caveolae/lipid raft-mediated endocytosis[39].

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5. Conclusion

In conclusion, we have demonstrated the capacity of non-viral vectors composed by SLNs, protamine and HA or DX, bearing the shRNA74 plasmid, to silence HCV-IRES expression in HepG2 cells. Vectors prepared with HA showed to be almost twice more effective than those prepared with DX. However, further studies are needed to confirm the potential of this strategy to inhibit the HCV replication and therefore to treat patients with chronic hepatitis C.

Acknowledgments

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Capítulo 3

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)

Gene silencing mediated by (RNAi) has gained increasing interest as an alternative for the treatment of infectious diseases such as refractory hepatitis C virus (HCV) infection. In this work we have designed and evaluated a non-viral vector based on solid lipid nanoparticles (SLN) bearing hyaluronic acid, protamine and a short hairpin RNA (shRNA74) targeted to the Internal Ribosome Entry Site (IRES) of the HCV.

The vector was able to inhibit the expression of the HCV IRES in Huh-7 cells, with the inhibition level dependent on the shRNA74 to SLN ratio and on the shRNA74 dose added to the culture cells. The nanocarrier was also able to inhibit the replication in human hepatoma cells supporting a subgenomic HCV replicon (Huh-7 NS3-3'). The vector was quickly and efficiently internalized by the cells, and endocytosis was the most productive uptake mechanism for silencing. Clathrin-mediated endocytosis and to a lesser extent caveolae/lipid raft-mediated endocytosis were identified as endocytic mechanisms involved in the cell uptake. Internalization via the CD44 receptor was also involved, although this entry route seems to be less productive for silencing than endocytosis.

The vector did not induce either hemolysis or agglutination of red cells *in vitro*, which was indicative of good biocompatibility. In summary, we have shown for the first time the ability of a non-viral SLN-based vector to silence a HCV replicon.

1. Introduction

Hepatitis C Virus (HCV) infection is a major health challenge worldwide because approximately 80% of infections persist to chronicity and can lead to hepatic cirrhosis and hepatocellular carcinoma and, ultimately, to liver failure and death[1]. The new protease inhibitors, such as simeprevir (Olysio®) or sofosvubir (Sovaldi®), have successfully improved sustained antiviral responses against HCV infection[2]. However, resistance to these inhibitors is expected to emerge in response to the pharmacological selection pressure[3,4] due the high mutation rate of the virus[5]. Moreover, the treatment is still very expensive and must be administered in combination with other drugs, such as α-interferon conjugated with poly(ethylene glycol) (PEG-INF-α) and/or ribavirin, which increases the probability of side effects[6,7]. Therefore, resistance-associated variants (RAV) remain an important concern[6].

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Taking this into account, novel, safe and economically supportable therapies for refractory HCV, in combination with currently available therapies, are needed to treat HCV infection.

The use of interference RNA (RNAi) as anti-HCV agent has been extensively reported as a promising strategy in therapeutics[8,9]. HCV, which possesses a genome of 9.6 kb long single-stranded RNA molecule and has a replication cycle that occurs into the cytoplasm, is an ideal candidate for this therapeutic approach. Different sequences within the viral genome have been used as target for RNAi. Specifically, targeting the Internal Ribosome Entry Site (IRES) has demonstrated efficient inhibition of HCV replication[10,11,12]. Because of its important roles in translation and replication as well as its high sequence conservation across all HCV genotypes (seven genotypes and a series of subtypes), HCV IRES has been considered as a promising target for RNAi-mediated antiviral therapy[13,14]. Additionally, IRES is the most sequence-conserved region of the HCV genome, suggesting that clinical resistance against IRES inhibitors might be slow to develop[14].

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[13] Dibrov SM, Parsons J, Carnevali M, Zhou S, Rynearson KD, Ding K, Garcia Sega E, Brunn ND, Boerneke MA, Castaldi MP, Hermann T. Hepatitis C virus translation inhibitors targeting the internal ribosomal entry site. *J Med Chem*. 2014 Mar 13;57(5):1694-1707.

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Among the different types of commonly used RNAi molecules, short-hairpin RNA (shRNA) is considered to be an attractive strategy against HCV[7]. 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. Since shRNA is constantly synthesized in host cells, more durable gene silencing is achieved in comparison to other forms of RNAi[15,16]. shRNA requires entering the cell and must reach the nucleus to be effective, and a major challenge for its therapeutic use is the development of a suitable delivery system. In this sense, solid lipid nanoparticles (SLNs) have been increasingly recognized as one of the most promising non-viral vectors for gene therapy due to their biocompatibility and the ease of large-scale production[17,18,19].

In a previous study[20], we demonstrated the capacity of non-viral vectors composed by SLNs, protamine, and hyaluronic acid (HA) or dextran (DX) to silence HCV IRES in HepG2 cells. The efficacy of the nanocarriers prepared with HA turned out to be higher than those prepared with DX. In order to confirm the capacity of these HA vectors to inhibit HCV replication, the objective of the present work was to evaluate the silencing efficacy in the human hepatoma cell line Huh-7

[15] 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.

[16] Jin X, Sun T, Zhao C, Zheng Y, Zhang Y, Cai W, He Q, Taira K, Zhang L, Zhou D. Strand antagonism in RNAi: an explanation of differences in potency between intracellularly expressed siRNA and shRNA. *Nucleic Acids Res*. 2012 Feb;40(4):1797-1806.

[17] Rodríguez-Gascón A, Solinís MÁ, del Pozo-Rodríguez A, Delgado D, Pedraz JL. 2012. Inventors. University of the Basque Country UPV/EHU. Lipid nanoparticles for gene therapy. US 20120183589 A1..

[18] Rodríguez-Gascón A, Solinís MÁ, del Pozo-Rodríguez A, Delgado D, Fernández E. 2012. Inventors. University of the Basque Country UPV/EHU and Universidad Miguel Hernández de Elche. Lipid nanoparticles for treating ocular diseases. WO 2012085318 A1.

[19] Gascón AR, del Pozo-Rodríguez A, Solinís MÁ. Non-viral delivery systems in gene therapy. *In: Martin, F. (Ed.), Gene Therapy - Tools and Potential Applications*. 2013; pp. 3 - 33.

[20] Torrecilla J, del Pozo-Rodríguez A, Apaolaza PS, Solinís MÁ, Rodríguez-Gascón A. Solid lipid nanoparticles as non-viral vector for the treatment of chronic hepatitis C by RNA interference. *Int J Pharm*. 2015 Feb 1;479(1):181-188.

NS3-3' supporting a subgenomic HCV replicon[21]. Due to the importance of cell uptake and intracellular trafficking for transfection efficacy, we studied the mechanisms of cell internalization and the intracellular disposition of the vectors. Additionally, we also studied *in vitro* the hemaglutination capacity and the haemolytic activity of the vector, as indicative of biocompatibility.

2. Materials and methods

2.1. Materials

Precirol® ATO 5 was generously provided by Gattefossé (Madrid, Spain). 1,2-Dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) was purchased from Avanti Polar Lipids (AL, USA). Tween 80, dichloromethane and paraformaldehyde (PFA) were obtained from Panreac (Madrid, Spain) and the fluorescent dye Nile Red from Sigma–Aldrich (Madrid, Spain). For the preparation of the vectors, protamine sulfate salt Grade X (P) and the Select-HA™ Hyaluronan 150 kDa (HA) were purchased from Sigma–Aldrich (Madrid, Spain).

For cell culture Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Reduced-Serum Minimal Essential Medium (OPTI-MEM) were purchased from Invitrogen. The antibiotic solution Normocin™ was obtained from Invivogen and the Cell Counting viability Kit 8 (CCK-8) was purchased from Sigma-Aldrich (Madrid, Spain) as all other reagents of analytical grade.

[21] Lohmann V, Körner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science*. 1999 Jul 2;285(5424):110-113.

The Reporter Lysis Buffer (RLB) was acquired from Promega (Madrid, Spain) and the Micro BCA™ Protein Assay Kit from Thermo Scientific (Madrid, Spain).

The rat monoclonal CD44 antibody and Alexa Fluor 488-conjugated goat anti-rat IgG were obtained from Abcam. The transfection reagent Transfectin™ was obtained from Bio-Rad (Madrid, Spain). Alexa Fluor®488-Cholera Toxin, Alexa Fluor®488-Transferrin, Lysotraker® and FITC-Dextran 70000 MW were provided by Molecular Probes (Barcelona, Spain), and DAPI-Fluoromount-G® by SouthernBiotech (Birmingham, USA).

Functional HCV IRES 1b U6-Promoter-GFP-Neo plasmid, silencer shRNA74 (sequence of the siRNA74 that encodes; 3'-TCGCAGATCGGTACCGCAA-5')[22], negative control (shRNAscramble) and positive control (shRNA74-GFP) plasmids were purchased from GeneCust Europe (Luxembourg).

The subgenomic replicon carries the HCV IRES of genotype 1b, followed by the neomycin phosphotransferase gene (*neo*), the IRES from encephalomyocarditis virus (EMCV IRES), the coding sequence for non-structural HCV proteins (NS3-NS5) and the HCV 3'UTR[21,23].

2.2. Elaboration of the shRNA74-bearing vectors

The nanocarriers were prepared with SLNs, P, HA and shRNA74 plasmid. The SLNs were elaborated by solvent emulsification-evaporation technique as previously described[24,15] using the cationic lipid

[22] Prabhu R, Garry RF, Dash S. Small interfering RNA targeted to stem-loop II of the 5' untranslated region effectively inhibits expression of six HCV genotypes. *Virol J*. 2006 Nov 27;3:100.

[23] Larrea E, Aldabe R, Molano E, Fernandez-Rodriguez CM, Ametzazurra A, Civeira MP, Prieto J. Altered expression and activation of signal transducers and activators of transcription (STATs) in hepatitis C virus infection: *in vivo* and *in vitro* studies. *Gut*. 2006 Aug;55(8):1188-1196.

[24] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles: formulation factors affecting cell transfection capacity. *Int J Pharm*. 2007 Jul 18;339(1-2):261-268.

DOTAP, the surfactant Tween 80 and Precirol® ATO 5 as solid lipid core. The vectors were formed by electrostatic interactions at two different shRNA74 to SLN ratios, expressed as the ratio of shRNA74 to DOTAP (w:w): 1:2 (HA-SLN2) and 1:5 (HA-SLN5). This difference in the shRNA74 to SLN ratio brings a difference in the shRNA74 load of each vector.

2.3. Characterization of the nanocarriers

In order to characterize the nanocarriers, the measurements of the size and Z potential were performed by Photon Correlation Spectroscopy (PCS) and Laser Doppler Velocimetry (LDV), respectively. The samples were diluted in MilliQ™ water to perform the measurements on a Zetasizer Nano series- Nano ZS (Malvern Instruments, Worcestershire, UK).

Electron microscopy negative staining was performed for the visualization of the vectors. For that purpose, 10 µl of the vector suspension were adhered onto glow discharged carbon coated grids for 60 s. The remaining liquid was removed by blotting on filter paper and stained with 2% uranyl acetate for 60 s. Samples were visualized in a Philips EM208S Transmission electron microscope (TEM) and digital images were acquired with an Olympus SIS purple digital camera.

2.4. Cell culture conditions

The human hepatoma cell line Huh-7 was maintained in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% of Normocin™ antibiotic solution at 37ºC in a 5% CO_2 atmosphere and subcultured every 2 to 3 days using trypsin-EDTA (Lonza).

The human hepatoma cell line Huh-7 NS3-3' supporting a subgenomic HCV replicon was cultured with DMEM-high glucose

supplemented with 20% heat-inactivated fetal bovine serum, 1 mM sodium piruvate and 0.5 mg/ml of G-418 (Sigma-Aldrich Chemie)[25].

2.5 Silencing studies in Huh-7 cell line

2.5.1. Silencing protocol

The cells were seeded in a 24-well plate (1 x 10^5 cells/well) with complete medium and were allowed to grow overnight. Afterwards, the Transfectin™ Lipid Reagent was used to transfect the HCV-IRES 1b U6-Promoter-GFP-Neo plasmid (IRES-GFP), according to the manufacturer's instructions. Briefly, the IRES-GFP plasmid and Transfectin™ were mixed separately in an equal total volume of 50 µl with OPTI-MEM at room temperature during 5 min at the appropriate concentration to achieve a final IRES-GFP to Transfectin™ ratio of 1:3. Then, the solution of the lipid reagent was added to the plasmid solution and, after 20 min of incubation at room temperature, the complex was added to each well into 500 µl cell culture media (0.8 µg of IRES-GFP/well).

Two hours later the media was replaced and the cells were treated with the HA-SLN2 or HA-SLN5 vectors bearing the shRNA74. The doses of silencing plasmid were 1.5, 2, 2.5 and 3 µg, corresponding to an IRES-GFP to shRNA74 ratio of 1:1.8, 1:2.5, 1:3.1 and 1:3.7 (w/w), respectively. After 4 h, 1 ml of fresh media was added to each well. The specificity of the silencing plasmid was assayed by using a shRNA scramble (shRNAscr). Naked shRNA74 plasmid was also tested.

The silencing efficacy of the formulations at different shRNA74 doses was evaluated *in vitro* at 48 h post-transfection by assessing the inhibition of GFP expression under the control of the HCV IRES. Inhibition

[25] Romero-López C, Díaz-González R, Barroso-delJesus A, Berzal-Herranz A. Inhibition of hepatitis C virus replication and internal ribosome entry site-dependent translation by an RNA molecule. *J Gen Virol*. 2009 Jul;90(Pt 7):1659-1669.

of GFP expression was qualitatively monitored under a fluorescence microscope (4x), and a fluorometric analysis was performed to quantify the intracellular GFP expression after the silencing treatment. Briefly, the culture media was replaced with 300 µl of RLB, and incubated for 30 min at 37^{o}C in a 5% CO_2 atmosphere. The cells were then scraped and centrifuged at 12000 g during 2 min. The supernatant was recovered and the GFP fluorescence of each sample was measured in a Glomax™ Multi detection System (Promega). The results were then corrected by the total amount of protein of each sample quantified by Micro BCA™ Protein Assay kit. The silencing efficacy was expressed as the percentage of fluorescent amount (RFU/mg total protein) with regard to cells non-treated with HA-SLN vectors.

2.5.2. Cell viability

The viability of the Huh-7 cells treated with the vectors was studied with the CCK-8 assay as it is described in a previous study[15]. In all cases, cell viability was measured 48 h after the addition of the vectors or after treatment with Triton-X as control.

2.6. Silencing studies in Huh-7 NS3-3', HCV replication system

2.6.1. Silencing protocol

Analysis of the inhibitory effect exerted by the formulations on HCV replication was performed by transfecting the cell line Huh-7 NS3-3'[21,23]. Cells were grown as described above in 1.5 cm diameter dishes for 24 h up to 70-80% confluence. The silencing plasmid was added to the cells at the same doses as previously described with Huh-7 cells: 1.5, 2, 2.5 and 3 µg with the two formulations HA-SLN2 and HA-SLN5. Forty-eight hours after transfection, the cells were harvested and processed for analysis.

2.6.2. Relative quantification of HCV RNA replicon

The intracellular levels of the positive strand of HCV subgenomic replicon RNA were quantified by qRT-PCR and normalized to those obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cellular mRNA, as described[26]. Briefly, cellular RNA was extracted using Trizol, as recommended by the manufacturer. RNA (50 ng) was then reverse-transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). A molar excess of random primers was hybridized with 50 ng of the cellular RNA by denaturation at 95ºC and slow cooling. MultiScribe reverse transcriptase was then added to a final concentration of 0.5 U/µl and extension of the cDNA mix was performed by fast extension at 42ºC for 30 min. The reverse transcriptase was heat-inactivated at 85ºC for 5 min; 1/5 of the cDNA mix was then diluted with the SsoFast™ EvaGreen® Supermix (BioRad).

HCV RNA was quantified using the previously reported specific primers C-149 and C-342[27], which target the IRES region. For GAPDH quantification, qRT-PCR of the mRNA encoding for the human GADPH (hGAPDH) was performed in parallel with the primers hGAPDH_Fw and hGAPDH_Rev[28]. Reactions were performed using a CFX96™ Real-Time PCR Detection System and data were analysed using CFX Manager software v.3.0 (both from Bio-Rad).

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[26] Marton S, Berzal-Herranz B, Garmendia E, Cueto FJ, Berzal-Herranz A. Anti-HCV RNA Aptamers Targeting the Genomic cis-Acting Replication Element. *Pharmaceuticals (Basel)*. 2011 Dec 28;5(1):49-60.

[27] Martell M, Gómez J, Esteban JI, Sauleda S, Quer J, Cabot B, Esteban R, Guardia J. High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. *J Clin Microbiol*. 1999 Feb;37(2):327-332.

[28] Barroso-delJesus A, Romero-López C, Lucena-Aguilar G, Melen GJ, Sanchez L, Ligero G, Berzal-Herranz A, Menendez P. Embryonic stem cell-specific miR302-367 cluster: human gene structure and functional characterization of its core promoter. *Mol Cell Biol*. 2008 Nov;28(21):6609-6619.

2.7. Cellular uptake and internalization mechanisms

2.7.1. Cellular uptake of non-viral vectors

The uptake of the vectors by Huh-7 cells was studied two hours after the addition of the Nile Red-labelled vectors to the cells, by fluorescence microscopy and flow cytometry. SLNs were labelled with the fluorescent dye Nile Red (λ = 590 nm) as described in a previous study[29].

In order to analyze the uptake by fluorescence microscopy, cells were seeded in Millicell® EZ slides (Millipore) at a density of 1.2 x10^5 cells/well. Cells were allowed to adhere overnight and then, treated with the formulations (shRNA74 dose: 2.5 µg). Two hours later, the cells were washed three times with PBS, fixed with PFA 4% and the nuclei were dyed with the mounting media DAPI-Fluoromount-G®. Images were acquired with an Axio Observer Inverted Microscope Z.1 with ApoTome (Zeiss), using 63x magnification.

For the analysis by flow cytometry, the cells were seeded in a 24-well plate (1 x 10^5 cells/well) with complete medium, allowed to grow overnight and then, treated with the formulations (shRNA74 dose: 2.5 µg). Two hours after the addition of the vectors, the cells were washed three times with PBS, detached from plates, resuspended in PBS and directly introduced into the Gallios™ (Beckman Coulter) cytometer to be analysed at 650 nm (FL3), collecting 1x10^4 events per sample.

[29] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles for retinal gene therapy: transfection and intracellular trafficking in RPE cells. *Int J Pharm*. 2008 Aug 6;360(1-2):177-183.

2.7.2. Mechanism of internalization

2.7.2.1. Endocytosis inhibition

The effect of temperature on cell uptake was studied by incubation of Huh-7 cells at 4ºC or 37ºC for 30 min prior to the addition of the vectors (shRNA74 dose: 2.5 µg). One hour after the addition of the Nile Red-labelled vectors, the cells were collected to evaluate the vector uptake by flow cytometry, as described above.

To determine whether differences in the uptake imply differences in the silencing rate, the same temperature conditions were employed in a silencing assay. Briefly, the cells were firstly transfected, as previously described in the silencing protocol, with IRES-GFP plasmid using Transfectin™ Lipid Reagent. Two hours later, the medium was replaced and the cells were incubated with HA-SLN2 vectors (shRNA74 dose: 2.5 µg) at 4ºC or 37ºC for 1 h. Thereafter, the medium was replaced with fresh DMEM and cells were maintained at 37ºC during 48 h. Finally, GFP quantification was performed.

2.7.2.2. Endocytosis mechanisms

The endocytosis mechanisms involved in the internalization of the non-viral systems were evaluated by flow cytometry and Confocal Laser Scanning Microscopy (CLSM).

First, we evaluated the presence in Huh-7 cells of macropinocytosis, caveolae/lipid raft-mediated and clathrin-mediated endocytosis mechanisms involved in the cell uptake of nanoparticles. With this purpose, the cells were seeded in 24-well plates and grown overnight. Next, the cells were incubated for 2 h with either Alexa Fluor®488-Cholera Toxin (10 µg/ml), Alexa Fluor®488-Transferrin (50 µg/ml) or FITC-Dextran 70000 MW (dissolved in the medium at 1mg/ml), which are markers for caveolae/lipid raft-mediated endocytosis, clathrin-

mediated endocytosis, and macropinocytosis, respectively. Finally, the cells were detached from the plates and subjected to flow cytometry to detect fluorescent cells (FL1), collecting $1x10^4$ events per sample.

In order to study the endocytic mechanism involved in the uptake of the HA-SLNs, a colocalization study was also performed with Nile Red-labelled SLNs and Alexa Fluor®488-Cholera Toxin (10 µg/ml), Alexa Fluor®488-Transferrin (50 µg/ml), FITC-Dextran 70000 MW (dissolved in the medium at 1mg/ml) or the marker of lysosomes LysoTracker® (according to the manufacturer's instructions). Briefly, cells were seeded in 24-well coverslips containing plates, maintained overnight and co-incubated with Nile Red-labelled vectors and the corresponding marker for 2 h. Thereafter, the medium was removed and cells were washed twice with PBS and fixed with a 4% PFA solution. Finally, preparations were mounted with DAPI-Fluoromount-G®.

The samples were analysed by CLSM, and images were obtained with an Olympus Fluoview FV500 confocal microscope, using sequential acquisition to avoid overlap of fluorescent emission spectra. CLSM images were captured in the General Service of Analytical Microscopy and High Resolution in Biomedicine of the University of the Basque Country UPV/EHU.

Co-localization degree was estimated by means of the Mander's Overlap Coefficient (R), which indicates an actual overlap of signals, and is considered to represent the true degree of colocalization[30]. R values range from 0 to 1.0, where 0 indicates no significant overlap and 1.0 indicates complete overlap. The value of the Mander's coefficient was calculated as the mean value obtained from 7 images of each sample.

[30] Zinchuk V, Grossenbacher-Zinchuk O. Quantitative colocalization analysis of fluorescence microscopy images. *Curr Protoc Cell Biol*. 2014 Mar 3;62:Unit 4.19.1-14.

2.7.2.3. Detection of CD44 expression by immunocytochemistry

The presence of the HA specific receptor CD44 was also studied in Huh-7 cells by immunocytochemistry. For this purpose 1.2 x10^5 cells/well were seeded in a 24-well plate with cover glasses in the bottom, previously treated with poli-L-lysine.

Twenty-four h later, the cells were washed three times with PBS and fixed with 4% PFA during 10 min. Afterwards, the cells were washed three times with PBS, and 200 µl of a blocking solution (PB buffer, 0.3% Triton X-100, 10% goat serum) were added for 30 min in order to block and permeabilize the cells. An antibody solution containing the rat monoclonal CD44 antibody in PB buffer (2.5% goat serum, 0.1% Triton X-100) was then added keeping it in contact during 1 h at room temperature.

After 3 more washes with PBS, the cells were dyed with the secondary antibody Alexa Fluor 488-conjugated goat anti-rat IgG for 1 h in the dark at room temperature. Finally, the nuclei were dyed with the mounting media DAPI-Fluoromount-G®. The images were captured with an Axio Observer Inverted Microscope Z.1 with Apotome using 40x magnification.

2.7.2.4. CD44 receptor mediated cellular entry

In order to explore the involvement of the CD44 receptor in the cellular entry of the vectors, cell internalization and silencing assays were carried out after pre-incubation of the cells for 30 min with a solution of HA (3 mg/ml) mixed with normal growing media (1:3 ratio)[31]. The pre-incubation step and the subsequent incubation with the vectors were undertaken at 37ºC or 4ºC.

[31] Liu Y, Kong M, Cheng XJ, Wang QQ, Jiang LM, Chen XG. Self-assembled nanoparticles based on amphiphilic chitosan derivative and hyaluronic acid for gene delivery. *Carbohydr Polym*. 2013 Apr 15;94(1):309-316.

In the internalization study Nile Red-labelled vectors at a shRNA74 dose of 2.5 µg were incubated with Huh-7 cells for 1 h. Afterwards, the cells were collected to evaluate the uptake by flow cytometry (FL3) as described in section 2.7.1.

In the silencing assay, the cells were first transfected with IRES-GFP plasmid using Transfectin™ Lipid Reagent. Two hours later, the medium was replaced by 500 µl of HA-saturated medium for 30 min. Then, the saturated medium was replaced by fresh DMEM and the transfection was carried out at 37ºC or 4ºC for 1 h. Thereafter, cells were maintained at 37ºC for 48 h, and GFP quantification was performed.

All sets of cells pre-incubated with HA were washed twice with PBS previously to the transfection with the vectors.

2.8. Intracellular distribution of EMA-labelled DNA

In order to study the intracellular disposition of the plasmid into the cytoplasm, the cells were seeded in Millicell® EZ slides (Millipore) in a density of 1.2 x10^5 cells/well and incubated with vectors containing EMA-labelled plasmid. The labelling of the plasmid with ethidium monoazide (EMA) was carried out by Dro Byosystems S.L. (San Sebastian, Spain). Nuclei were labelled with DAPI-Fluoromount-G®. Images were captured at 4, 12 and 24 h with an Axio Observer Inverted Microscope Z.1 with ApoTome using 63x magnification.

2.9. Interaction with erythrocytes: Hemagglutination and hemolysis assay

Hemagglutination and hemolysis assays were conducted following a previously described protocol[32]. Briefly, fresh human blood was centrifuged at 4000 rpm for 5 min and the plasma and the buffy coat were discarded. Erythrocytes were washed twice by centrifugation at 4000 rpm and were diluted in PBS to a final concentration of 5% (v/v). HA-SLN2 or HA-SLN5 vectors were added to the erythrocytes suspension at 1:1 (v/v) ratio. Fifteen minutes later, samples were placed on a 96-well plate and hemagglutination was observed under an optical microscope using 40x magnification.

For the hemolysis assay, 1 h after incubation of the samples with the erythrocyte suspension at room temperature, samples were centrifuged at 4000 rpm during 5 min and the supernatants were assayed for the absorbance of released hemoglobin at 540 nm in a microplate reader. A lysis buffer was employed for the 100% hemolysis control.

2.10. Statistical analysis

The results were expressed as mean ± standard deviation. Statistical significance analysis was performed with IBM SPSS Statistics® 21. Normal distribution of samples was assessed by the Shapiro–Wilk test, and homogeneity of variance, by the Levene test. The silencing rates of the different formulations were compared with ANOVA test. A p value of < 0.05 was considered as evidence of a statistically significant difference.

[32] Delgado D, Gascón AR, Del Pozo-Rodríguez A, Echevarría E, Ruiz de Garibay AP, Rodríguez JM, Solinís MÁ. Dextran-protamine-solid lipid nanoparticles as a non-viral vector for gene therapy: *in vitro* characterization and *in vivo* transfection after intravenous administration to mice. *Int J Pharm*. 2012 Apr 4;425(1-2):35-43.

3. Results

3.1. Characterization of the nanocarriers

The characterization of the vectors prepared at two different shRNA74 to SLN ratios was performed by measuring the size and zeta potential. Table 1 features the particle size, zeta potential and polydispersity index (PdI) of the two vectors. Particle size was around 240 nm without significant difference between them. The vectors showed cationic surface charge of about 30 mV and the polydispersity index was lower than 0.24 in both cases.

Table 1. Characterization of HA-SLN vectors bearing the shRNA74 encoding plasmid. Size (nm), Zeta potential (mV) and polydispersity index represent mean ± SD (n=3).

Vector	Size (nm)	Zeta potential (mV)	PdI
HA-SLN2	231 ± 11	+28.7 ± 0.4	0.21 ± 0.01
HA-SLN5	242 ±13	+29.4 ± 0.4	0.24 ± 0.06

Figure 1 features a TEM photograph of the HA-SLN2 vector. The image shows the spherical shape of the nanocarrier, with an external layer or corona on the surface due to the HA. The photograph confirms the particle size of the vector.

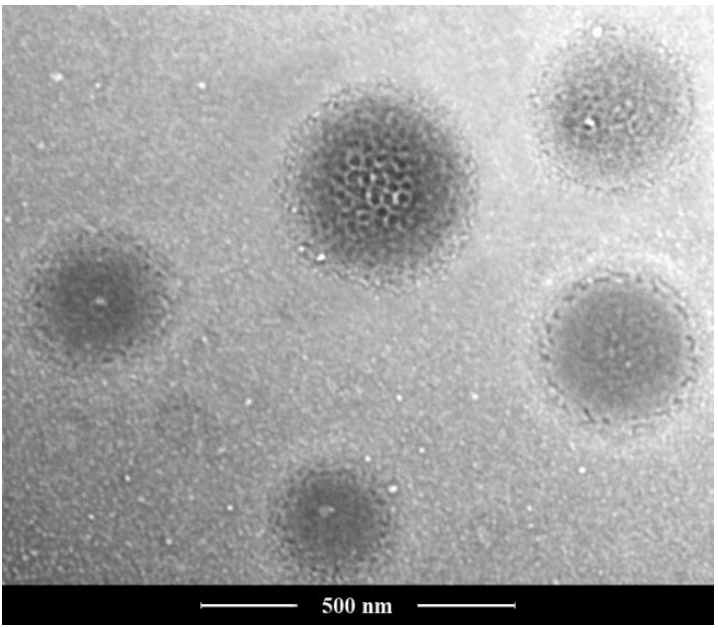

Figure 1. TEM photographs of HA-SLN2 vector, original magnification 44000x.

3.2. Inhibition of HCV IRES-GFP

Figures 2 and 3 show the ability of the vectors to silence the expression of GFP, and thus, of HCV IRES, in Huh-7 cells. When the cells were treated with shRNAscr, no inhibition of the expression of GFP was detected, and naked shRNA74 induced an inhibition level lower than 3%.

Silencing capacity of the non-viral vectors ranged from 4% to 50%, depending on the shRNA74 load on the nanoparticles and the total shRNA74 dose added to the cultured cells (Figure 3). Only with the HA-SLN2 vector at the lowest dose, silencing was not significantly different from that provided by the naked shRNA74. At each shRNA74 dose level, silencing was always higher with the HA-SLN5 vector ($p < 0.01$). Moreover, with both formulations (HA-SLN2 and HA-SLN5), silencing rates increased with the shRNA74 dose.

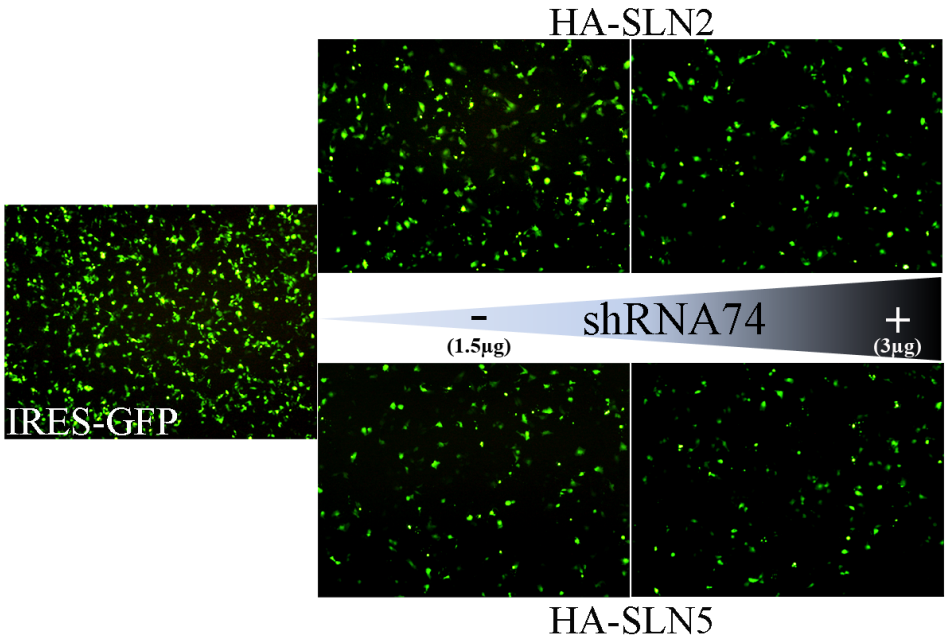

Figure 2. Fluorescence microscopy images (4x) of Huh-7 cells transfected with IRES-GFP and 48 h after the treatment with HA-SLN2 and HA-SLN5 with the lowest and the highest shRNA74 dose: 1.5 µg and 3 µg.

3.3 Cell viability

Forty-eight hours after the treatment of the Huh-7 cells with the vectors, cell viability was around 90% (Figure 3). No difference was detected depending on the vector or the shRNA74 dose ($p > 0.05$).

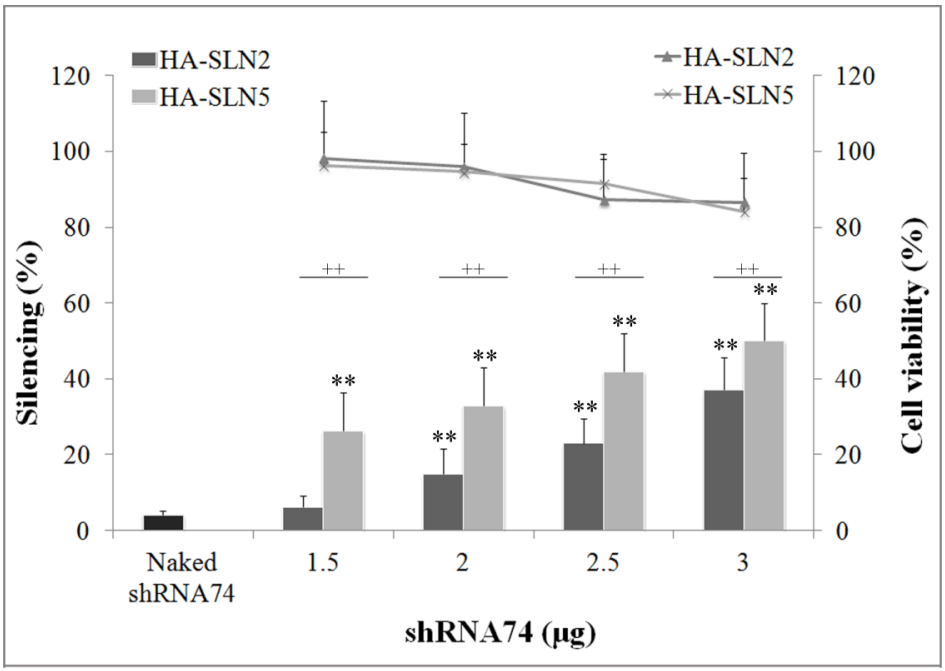

Figure 3. Silencing (bars) of the HCV IRES dependent expression in Huh-7 cells, 48 h after the silencing treatment with HA-SLN2 and HA-SLN5 vectors, at different doses of shRNA74. Error bars represent SD (n = 3).++$p < 0.01$ between formulations; **$p < 0.01$ respect to naked shRNA74. Cell viability (lines) 48 h after the treatment of Huh-7 cells with the HA-SLN vectors bearing the shRNA74 at different dose levels.

3.4 Inhibition of the HCV RNA replication

Figure 4 shows the inhibition of the HCV replication in the Huh-7 cell line supporting the stable replication of a subgenomic HCV replicon transcript (Huh-7 NS3-3') after the treatment with the vectors. The inhibition increased along with the shRNA74 dose, without significant differences between the two vectors. The highest inhibition (50%) was achieved with the highest dose level (3 µg), in line with the results obtained in the IRES-GFP silencing assay.

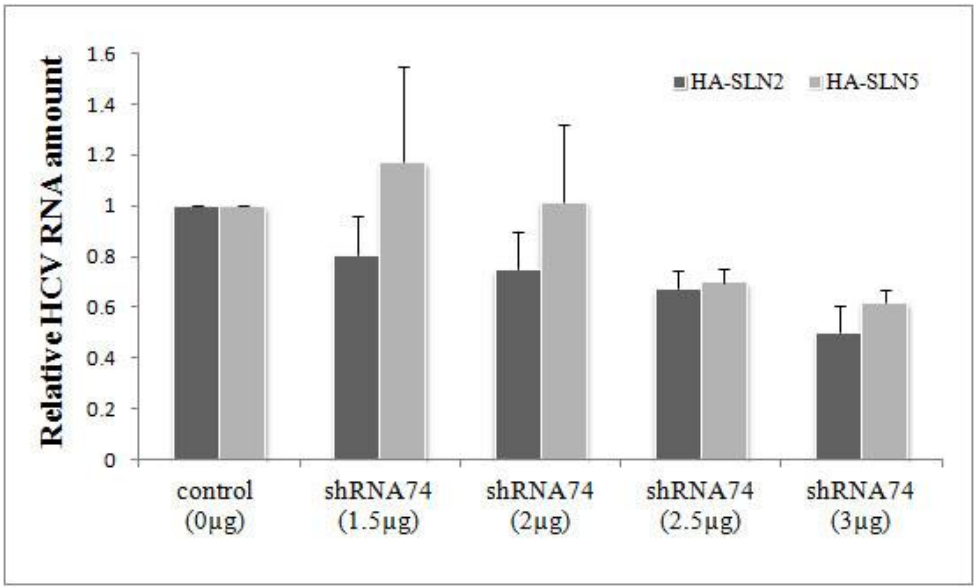

Figure 4. Relative HCV RNA amount in Huh-7 NS3-3'cells 48 h after the treatment with HA-SLN2 and HA-SLN5 vectors at increasing doses of shRNA74. Error bars represent SD (n = 3).

3.5. Cellular uptake and internalization mechanisms

3.5.1. Cellular uptake of non-viral vectors

In order to analyze the capacity of the vectors to enter the cells, we analysed the cells treated with the Nile Red-labelled vectors by fluorescence microscopy (Figure 5A) and flow cytometry (Figure 5B). As it can be seen in the figures, vectors were able to efficiently enter the Huh-7 cells, although HA-SLN5 in a higher extension.

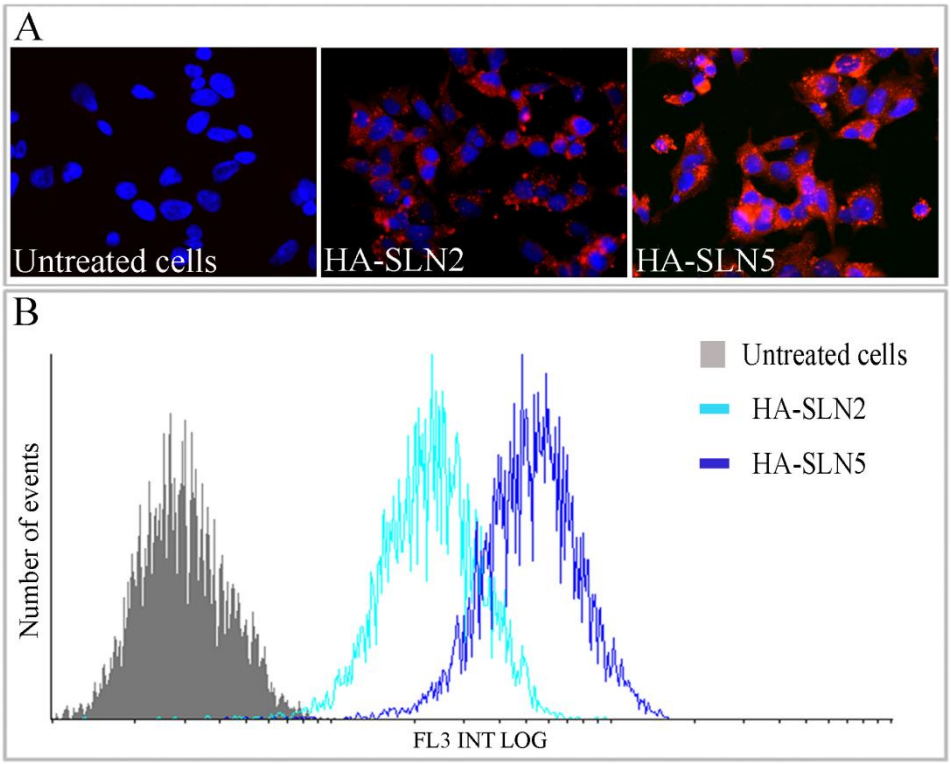

Figure 5. Cellular uptake of the vectors in Huh-7 cells 2 h after transfection with Nile Red-labelled vectors containing shRNA74 at a dose level of 2.5 µg. **(A)** Fluorescence microscopy images (40x). **(B)** Flow cytometry histograms.

3.5.2. Endocytosis inhibition

In order to study the involvement of endocytic mechanisms in the internalization of the vectors, the cell uptake and the silencing capacity at both 37°C and 4°C were compared. Histograms in Figure 6A indicates that at 4°C (light blue), cell uptake was lower than at 37°C (orange). Moreover, when endocytosis was inhibited (4°C), silencing rate significantly decreased in comparison with that obtained when the experiments were carried out at 37°C (16% vs 32%), (Figure 6B).

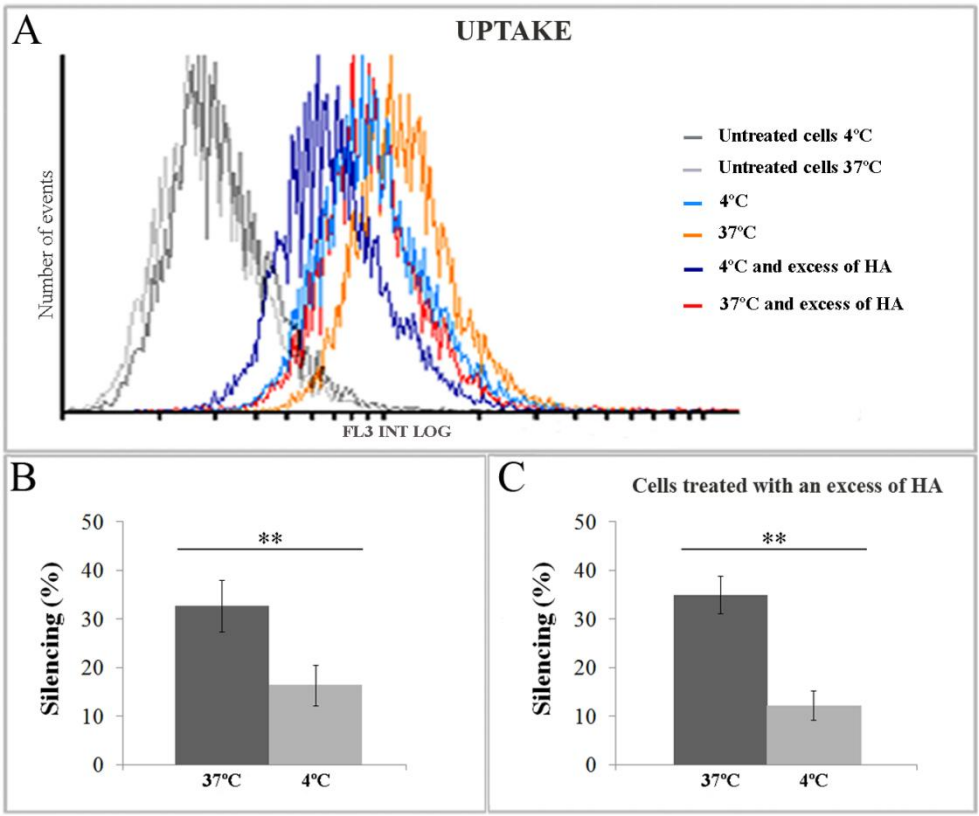

Figure 6. (A) Cellular uptake of Nile Red-labelled HA-SLN2 vectors (shRNA74 dose: 2.5 µg). Coloured lines represent uptake of the vectors after preincubation at different temperatures (4ºC and 37ºC) and in different media (no saturated vs. HA saturated) (dark grey: untreated cells at 4ºC; light grey: untreated cells at 37ºC; light blue: cells treated at 4ºC; dark blue: cells treated at 4ºC and with an excess of HA; orange: cells treated at 37ºC and red: cells treated at 37ºC and with an excess of HA). **(B)** Silencing of HCV-IRES expression in Huh-7 cells, 48 h after the silencing treatment during 1 h with HA-SLN2 vector at shRNA74 dose of 2.5 µg. **(C)** Silencing of HCV-IRES expression in Huh-7 cells, 48 h after the silencing treatment during 1 h with HA-SLN2 vector at shRNA74 dose of 2.5 µg and an excess of HA mixed in the medium. Error bars represent SD (n = 3). $^{**}p < 0.01$.

3.5.3. Internalization mechanism

The cytometry histograms of the cells treated with the endocytosis markers (FITC-Dextran 70000MW, Alexa Fluor®488-Transferrin and Alexa Fluor®488-Cholera Toxin, respectively) confirm that these mechanisms are present in the Huh-7 cells (Figure 7). Since the displacement to the right of these histograms in comparison to the histograms of untreated cells was similar for all markers, none of them dominate over the other.

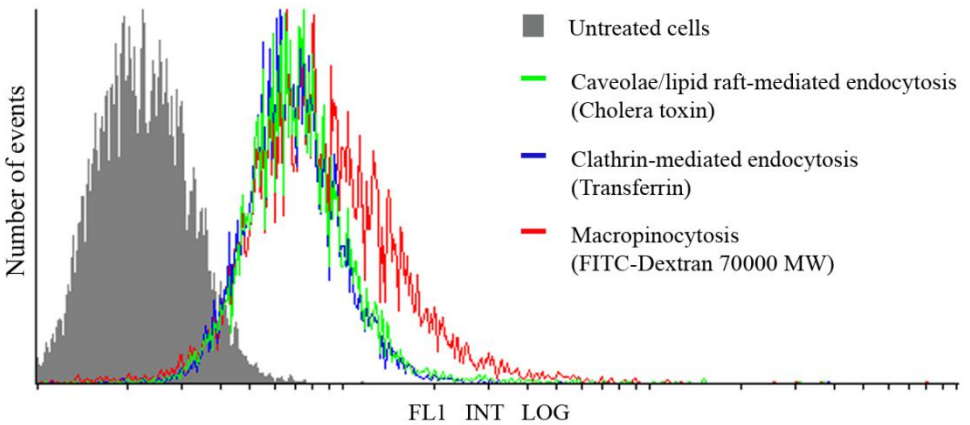

Figure 7. Clathrin-mediated endocytosis (blue), caveolae/lipid raft-mediated endocytosis (green) and macropinocytosis (red) activity in Huh-7 cells. Alexa Fluor®488-Transferrin, Alexa Fluor®488-Cholera toxin and FITC-Dextran 70000 MW were quantified using flow cytometry by measuring the fluorescence of Alexa Fluor®488 and fluorescein at 525 nm (FL1).

Figure 8 features the confocal microscopy images of Huh-7 cells after the treatment with the endocytosis markers and with the Nile Red-labelled HA-SLN2 vector. The highest co-localization level was observed with LysoTracker®, with a Mander's overlap coefficient of 0.77 (moderate co-localization). Labelled vectors colocalized with the marker of clathrin, that is transferrin (Mander's coefficient: 0.61), and with the

marker of caveolae/lipid raft-mediated endocytosis, cholera toxin (Mander's coefficient: 0.46), but the level of co-localization was lower than with LysoTracker®. The lowest colocalization was observed with the marker of macropinocytosis (Mander's coefficient: 0.10).

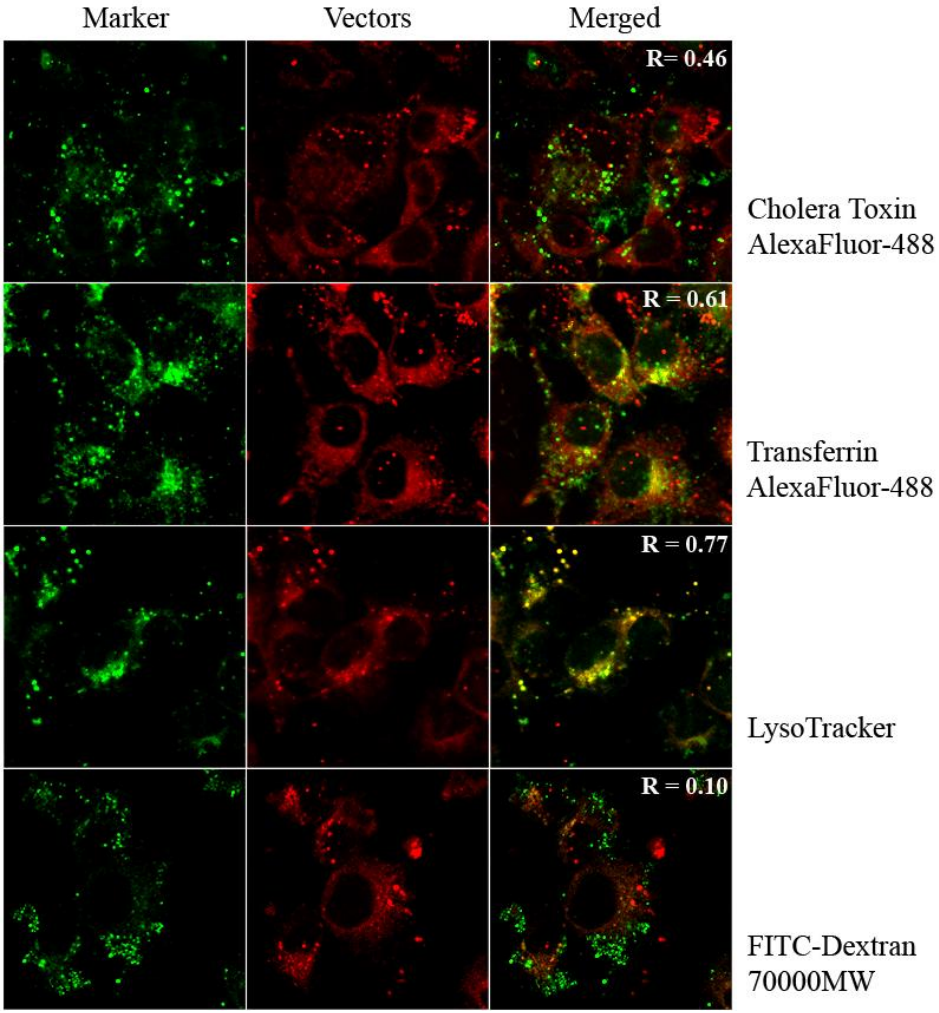

Figure 8. Fluorescence images of colocalization estudy in Huh-7 cells treated with Nile Red-labelled vectors (red) and Alexa Fluor®488-Transferrin, Alexa Fluor®488-Cholera Toxin, LysoTracker® or FITC-Dextran 70000MW (green). Colocalization results were estimated by means of the Mander's Overlap Coefficient (R).

3.5.4. Detection of CD44 expression by immunocytochemistry

Figure 9 shows the expression of the CD44 receptor (green color) in the Huh-7 cells. As can be seen, this receptor is present in this cell line, and therefore, it may be involved in the cell uptake of the vectors.

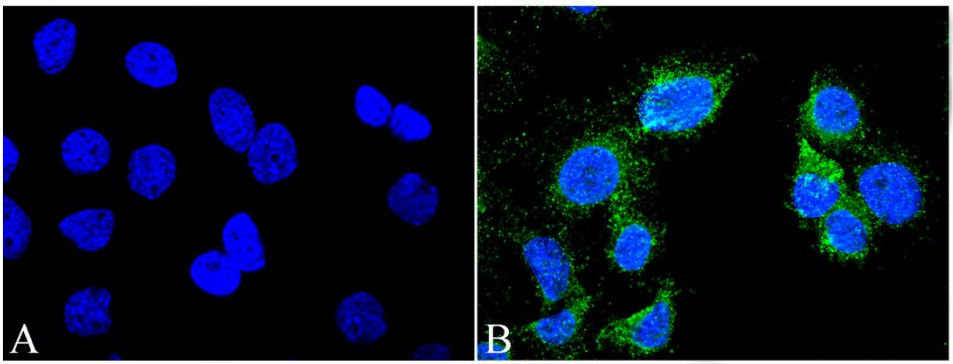

Figure 9. Fluorescence microscopy images (63x) of Alexa Fluor 488-CD44 receptor (green) in Huh-7 cell line. Cell nuclei are observed in blue. **(A)** Control sample. **(B)** Treated cells.

3.5.5. CD44 receptor mediated cellular entry

Cellular internalization of the vectors in Huh-7 cells previously treated with an excess of HA was studied by flow cytometry. One h after the addition of the nanocarriers, cell uptake was lower in the presence of HA in the culture medium at both 37°C (orange and red histograms) and 4°C (light and dark blue histograms). Even when the assay was performed at 4°C and the medium was saturated with HA, vectors were able to enter the cells. Regarding the ability of the vectors to inhibit IRES-GFP expression (Figure 6C), the addition of an excess of HA in the culture medium did not induce changes in the silencing rate, regardless of the temperature.

3.6. Intracellular distribution of EMA-labelled DNA

Figure 10 shows the images captured by fluorescence microscopy 4, 12 and 24 h after the addition of the HA-SLN vector prepared with EMA-labelled DNA. As can be seen, at 4 h the plasmid appears more compacted when the cells were treated with the HA-SLN5 formulation. Figures also show that the condensation of the plasmid decreases over time, and it approaches to the nucleus. No difference in the distribution pattern depending on the vector was detected.

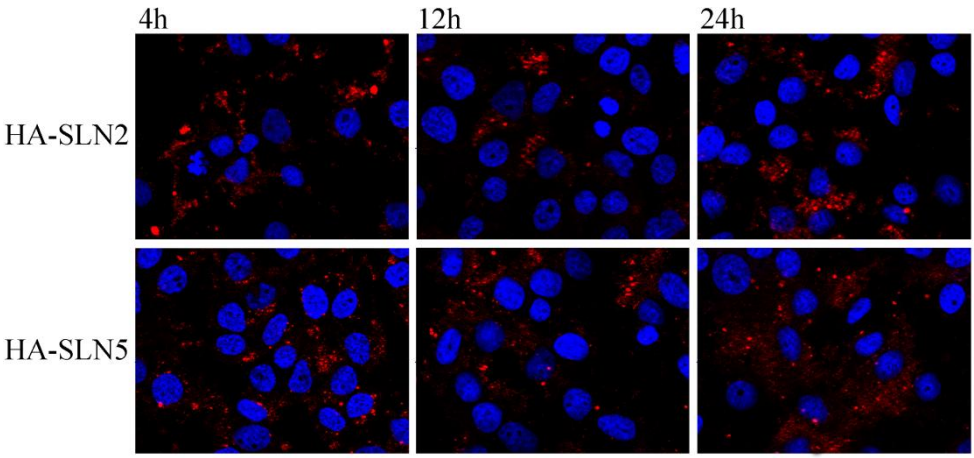

Figure 10. Fluorescence microscopy images of Huh-7 cells at 4, 12 and 24 h after treatment with HA-SLN2 and HA-SLN5 vectors. EMA-labelled DNA (red) and nuclei stained with DAPI-Fuoromont-G® (blue).

3.7. Interaction with erythrocytes

The potential agglutination capacity of the vectors was evaluated by incubating them in presence of erythrocytes. Neither HA-SLN2 nor HA-SLN5 induced agglutination of red cells (Figure 11).

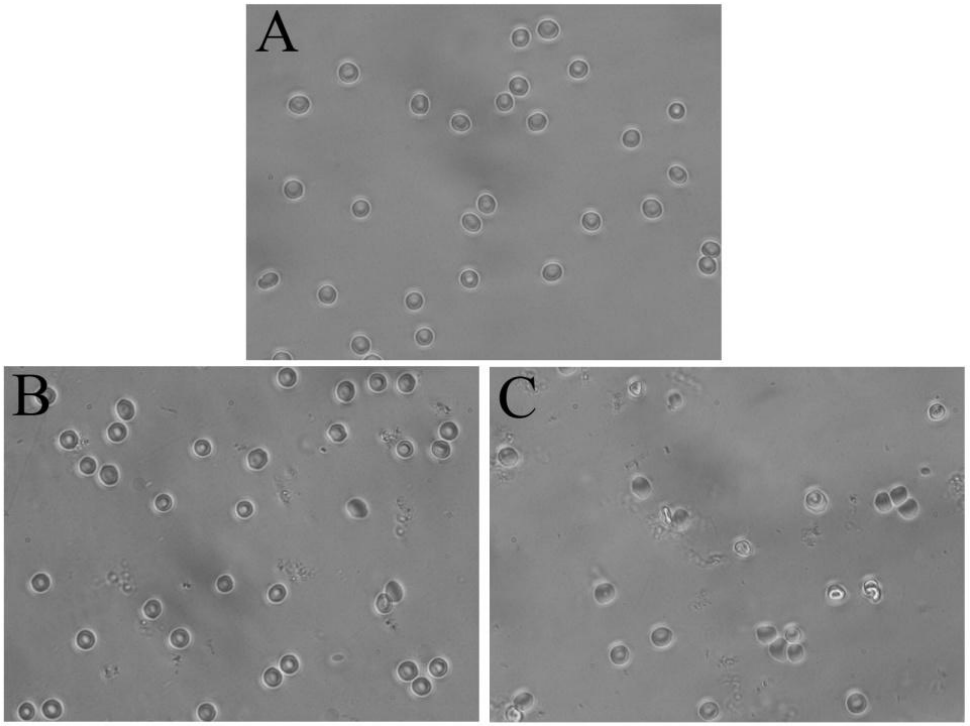

Figure 11. Lack of agglutination of erythrocytes in the presence of the vectors. **(A)** Untreated blood, **(B)** blood treated with HA-SLN2, and **(C)** blood treated with HA-SLN5.

Figure 12 shows the lack of hemolytic effect of HA-SLN2 and HA-SLN5. The level of hemolysis of the blood treated with the HA-SLN formulations was very similar to that of untreated blood.

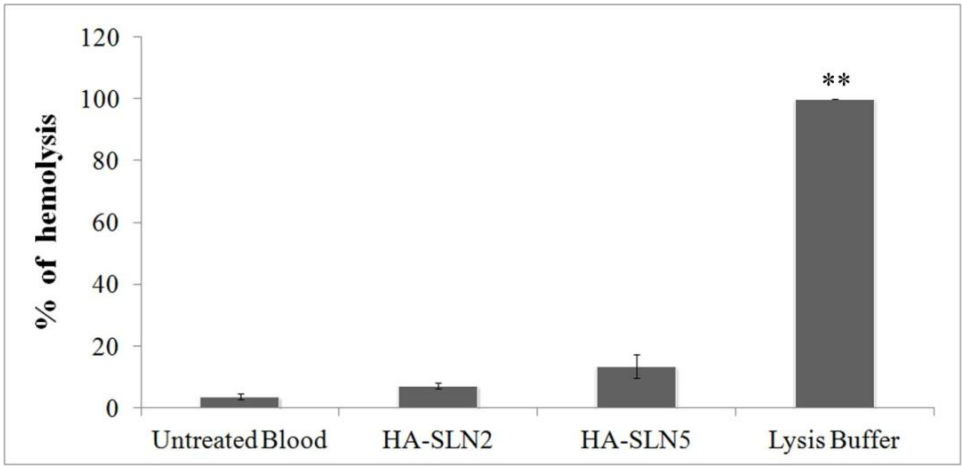

Figure 12. Hemolytic activity of untreated blood (negative control), blood treated with HA-SLN2, blood treated with HA-SLN5, and blood treated with Lysis Buffer (positive control); $^{**}p < 0.01$ respect to untreated blood and formulations.

4. Discussion

In recent years, SLN-based carriers have become one of the most interesting non-viral gene delivery vectors due to safe and cost-effective concerns[33,34,35]. In this study, SLNs have been combined with protamine (P), a cationic peptide which condenses DNA, presents nuclear localization signals and improves the transcription[36], and with hyaluronic

[33] Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Gascón AR, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. *Int J Pharm*. 2014 Apr 25;465(1-2):413-426.

[34] Ruiz de Garibay AP, Delgado D, Del Pozo-Rodríguez A, Solinís MÁ, Gascón AR. Multicomponent nanoparticles as nonviral vectors for the treatment of Fabry disease by gene therapy. *Drug Des Devel Ther*. 2012;6:303-310.

[35] Delgado D, Gascón AR, Del Pozo-Rodríguez A, Echevarría E, Ruiz de Garibay AP, Rodríguez JM, Solinís MÁ. Dextran-protamine-solid lipid nanoparticles as a non-viral vector for gene therapy: *in vitro* characterization and *in vivo* transfection after intravenous administration to mice. *Int J Pharm*. 2012 Apr 4;425(1-2):35-43.

[36] 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 Nov;79(3):495-502.

acid (HA), a biocompatible, biodegradable and nontoxic polyanion used for pharmaceutical and biomedical applications[37]. The resulting HA-SLN vector presented features that facilitate cell internalization of the genetic material: particle size in the nanometer range, and cationic surface charge. Moreover, it has previously demonstrated capacity to effectively bind the shRNA74 expression plasmid and protect it from nuclease degradation[38].

HCV preferentially replicates and propagates in hepatocytes (it has been suggested that liver cells may supply additional factors such as proteins and RNA, essential to the viral cycle)[39]; therefore, we evaluated the antiviral activity of the vectors in the human hepatoma-derived cell line Huh-7.

Silencing studies in Huh-7 cells showed the ability of the vectors to inhibit the HCV IRES function in a shRNA74 dose-dependent manner, with HA-SLN5 being more effective than HA-SLN2. Naked shRNA74 hardly induced silencing activity, which confirms the relevance of the delivery system for an efficient transfection.

In the present study, the vectors also showed capacity to inhibit the HCV replication in the Huh-7 NS3-3' hepatoma-derived cell line containing a subgenomic HCV replicon transcript[40]. The inhibition of the HCV replicon, approximately 50% at the highest concentration tested did not depended on the shRNA74 to SLN ratio. A single dose was not able to completely clear the HCV replicon from the cells, which suggests that

[37] de la Fuente M, Seijo B, Alonso MJ. Novel hyaluronic acid-chitosan nanoparticles for ocular gene therapy. *Invest Ophthalmol Vis Sci*. 2008 May;49(5):2016-2024.

[38] Torrecilla J, del Pozo-Rodríguez A, Apaolaza PS, Solinís MÁ, Rodríguez-Gascón A. Solid lipid nanoparticles as non-viral vector for the treatment of chronic hepatitis C by RNA interference. *Int J Pharm*. 2015 Feb 1;479(1):181-188.

[39] Romero-López C, Berzal-Herranz A. The functional RNA domain 5BSL3.2 within the NS5B coding sequence influences hepatitis C virus IRES-mediated translation. *Cell Mol Life Sci*. 2012 Jan;69(1):103-113.

[40] Lohmann V, Körner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science*. 1999 Jul 2;285(5424):110-113.

either treating cells with a higher amount of shRNA74, or delivering multiple doses, could be more successful in accomplishing knock-down of all the replicon viral particles from the cells. Limitations of maintaining the cells in culture for longer periods of time made it difficult to study whether the long-term treatment with the shRNA74 formulations could eradicate HCV completely. However, as a proof-of-principle, and to the best of our knowledge, this study shows, for the first time, the SLN-based vector bearing the shRNA74 as an intracellular therapeutic approach to specifically block HCV replication. Inhibition of IRES through siRNA74 is effective against the two genotypes of HCV that frequently develop resistance to interferon and rivabirin combination therapy, 1a and 1b genotypes[41]. Consequently, our delivery system could be an interesting therapeutic strategy against these two HCV genotypes.

Intracellular delivery of shRNA is a major challenge due to the inability of large and negatively charged molecules to cross the cell membrane; therefore, the design of a vector for gene therapy relies on a comprehensive understanding of internalization and intracellular trafficking pathways in the target cells.

As Figure 5 shows, cell uptake is higher when the vector is prepared with a lower shRNA74 load (HA-SLN5), and it may explain the higher silencing efficacy of the vector in comparison with HA-SLN2. These results show that, in this case, correlation between the cell uptake and silencing effect exists.

One of the most important mechanisms for cell uptake of non-viral vectors is endocytosis[42], which is an energy-dependent process that occurs at around 37°C. This process, which involves different mechanisms, participates in the internalization of our vectors, since both

[41] Prabhu R, Garry RF, Dash S. Small interfering RNA targeted to stem-loop II of the 5' untranslated region effectively inhibits expression of six HCV genotypes. *Virol J*. 2006 Nov 27;3:100.

[42] Khalil IA, Kogure K, Akita H, Harashima H. Uptake pathways and subsequent intracellular trafficking in non-viral gene delivery. *Pharmacol Rev*. 2006 Mar;58(1):32-45.

cell uptake and silencing rate were lower at 4°C than at 37°C (Figure 6). We have shown the presence of clathrin-mediated endocytosis, caveolae/lipid raft-mediated endocytosis and macropinocytosis in Huh-7 cells. As co-localization study revealed (Figures 7 and 8), clathrin-mediated endocytosis and caveolae/lipid raft-mediated endocytosis participated in the internalization of our nanocarriers, as previously showed in other cell lines[43,36]. In Huh-7 cells, the contribution of the clathrin-mediated entry was higher than that of the caveolae/lipid raft-mediated route. The co-localization with Lysotraker®, which indicates the presence of the nanocarriers in the lysosomes, is line with the participation of endocytic mechanisms. In addition to clathrin- and caveolae/lipid raft-mediated endocytosis, macropinocytosis could also contribute to the internalization of our vectors since it has been shown to play a role in the uptake of SLN based non-viral vectors in HeLa cells[44], and because it has been suggested that HA induces macropinocytosis[45]. However, the co-localization study indicated that this mechanism does not participate in the entry of the vectors, although it is present in Huh-7 cells.

Inhibition of endocytosis not only led to a decrease in cell internalization, but also to a decrease in the silencing rate. However, silencing level decreased in a higher extension than the uptake, which seems to indicate that endocytosis is a mechanism highly productive for transfection, and therefore for silencing. At 4°C transfection was not completely inhibited; accordingly, other mechanisms different from endocytosis, such as energy-independent mechanisms must be also

[43] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles for retinal gene therapy: transfection and intracellular trafficking in RPE cells. *Int J Pharm*. 2008 Aug 6;360(1-2):177-183.

[44] Ruiz de Garibay AP, Solinís Aspiazu MÁ, Rodríguez Gascón A, Ganjian H, Fuchs R. Role of endocytic uptake in transfection efficiency of solid lipid nanoparticles-based non-viral vectors. *J Gene Med*. 2013 Nov-Dec;15(11-12):427-440.

[45] Greyner HJ, Wiraszka T, Zhang LS, Petroll WM, Mummert ME. Inducible macropinocytosis of hyaluronan in B16-F10 melanoma cells. *Matrix Biol*. 2010 Jul;29(6):503-510.

involved in the cell uptake. One of these mechanisms that can be involved in the internalization of nanoparticulate systems is receptor-mediated uptake. CD44, also present in Huh-7 cells, is the primary cell surface receptor for HA internalization[46] and it has also shown to be involved in the cell uptake of different nanocarriers[47,48,49,50]. Therefore, the presence of HA in our vectors may facilitate the interaction with the cell surface, inducing the uptake. Actually, when we blocked the CD44 receptor with an excess of HA in the culture medium, the uptake of the vectors decreased. Since the HA-receptor binding proceeds at 4°C and 37°C, the effect of the excess of HA was detected at both temperatures, which confirms that CD44 receptor participates in the cell internalization. Curiously, in spite of the decrease of cell uptake in the presence of an excess of HA in the medium, the silencing efficacy did not decreased, either at 37°C or at 4°C. These results would indicate that this mechanism of cell uptake is not productive for transfection of our nanocarriers. However, it is important to note that when a route of internalization is blocked, other mechanisms, including any other more productive for transfection, may be activated and may compensate the effect of the blockage[51,52].

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[46] Knudson W, Chow G, Knudson CB. CD44-mediated uptake and degradation of hyaluronan. *Matrix Biol*. 2002 Jan;21(1):15-23.

[47] Yamada Y, Hashida M, Harashima H. Hyaluronic acid controls the uptake pathway and intracellular trafficking of an octaarginine-modified gene vector in CD44 positive- and CD44 negative-cells. *Biomaterials*. 2015 Jun;52:189-198.

[48] de la Fuente M, Seijo B, Alonso MJ. Bioadhesive hyaluronan-chitosan nanoparticles can transport genes across the ocular mucosa and transfect ocular tissue. *Gene Ther*. 2008 May;15(9):668-676.

[49] Wojcicki AD, Hillaireau H, Nascimento TL, Arpicco S, Taverna M, Ribes S, Bourge M, Nicolas V, Bochot A, Vauthier C, Tsapis N, Fattal E.. Hyaluronic acid-bearing lipoplexes: physico-chemical characterization and *in vitro* targeting of the CD44 receptor. *J Control Release*. 2012 Sep 28;162(3):545-552.

[50] Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Gascón AR, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. *Int J Pharm*. 2014 Apr 25;465(1-2):413-426.

[51] Islam MA, Firdous J, Choi YJ, Yun CH, Cho CS. Regulation of endocytosis by non-viral vectors for efficient gene activity. *J Biomed Nanotechnol*. 2014 Jan;10(1):67-80.

[52] del Pozo-Rodríguez A, Pujals S, Delgado D, Solinís MA, Gascón AR, Giralt E, Pedraz JL. A proline-rich peptide improves cell transfection of solid lipid nanoparticle-based non-viral vectors. *J Control Release*. 2009 Jan 5;133(1):52-59.

At intracellular level, 4 h after the treatment of the cells with the vectors, the EMA-labelled plasmid appeared highly condensed and, as expected, the higher the plasmid load in the nanocarrier, the lower the condensation degree. Moreover, as time went by, the plasmid appears less condensed and closer to the nuclear membrane. The process of decondensation of the plasmid is due, on the one hand, to the effect of HA and, on the other hand, to the lysosomal activity; both had previously been shown to facilitate the release of the plasmid from the nanoparticle, where it is highly condensed due to the cationic lipids and the protamine[50,53].

As mentioned above, the liver is the main target for HCV infection, and we had previously demonstrated that SLN-based vectors intravenously administered to mice efficiently induced transgenic expression in the liver[54,55]. The lack of hemolytic activity and capacity to agglutinate red cells of our vectors is indicative of its biocompatibility, condition that is necessary for any gene therapy delivery system. HA is a polyanion with stealth properties, and probably contributes to avoid the interaction of the vector with blood components.

[53] Delgado D, Gascón AR, Del Pozo-Rodríguez A, Echevarría E, Ruiz de Garibay AP, Rodríguez JM, Solinís MÁ. Dextran-protamine-solid lipid nanoparticles as a non-viral vector for gene therapy: *in vitro* characterization and *in vivo* transfection after intravenous administration to mice. *Int J Pharm*. 2012 Apr 4;425(1-2):35-43.

[54] del Pozo-Rodríguez A, Delgado D, Solinís MA, Pedraz JL, Echevarría E, Rodríguez JM, Gascón AR. Solid lipid nanoparticles as potential tools for gene therapy: *in vivo* protein expression after intravenous administration. *Int J Pharm*. 2010 Jan 29;385(1-2):157-162.

[55] Delgado D, del Pozo-Rodríguez A, Angeles Solinís M, 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 Nov 20;50(3-4):484-491.

5. Conclusion

The results obtained in this study indicate that the shRNA74 incorporated in a delivery system based on SLN, HA, and P is a promising and feasible therapeutic strategy for the treatment of chronic HCV infection. The silencing rate was related to the capacity of the vectors to enter the cells, with the energy-dependent mechanisms being the most productive. The hematological studies predicted the biocompatibility of the vectors after systemic administration, property that is a requisite to further address the ability of the system to clear HCV infection using a small animal model, and to assess whether this approach could be therapeutically useful in human.

Acknowledgments

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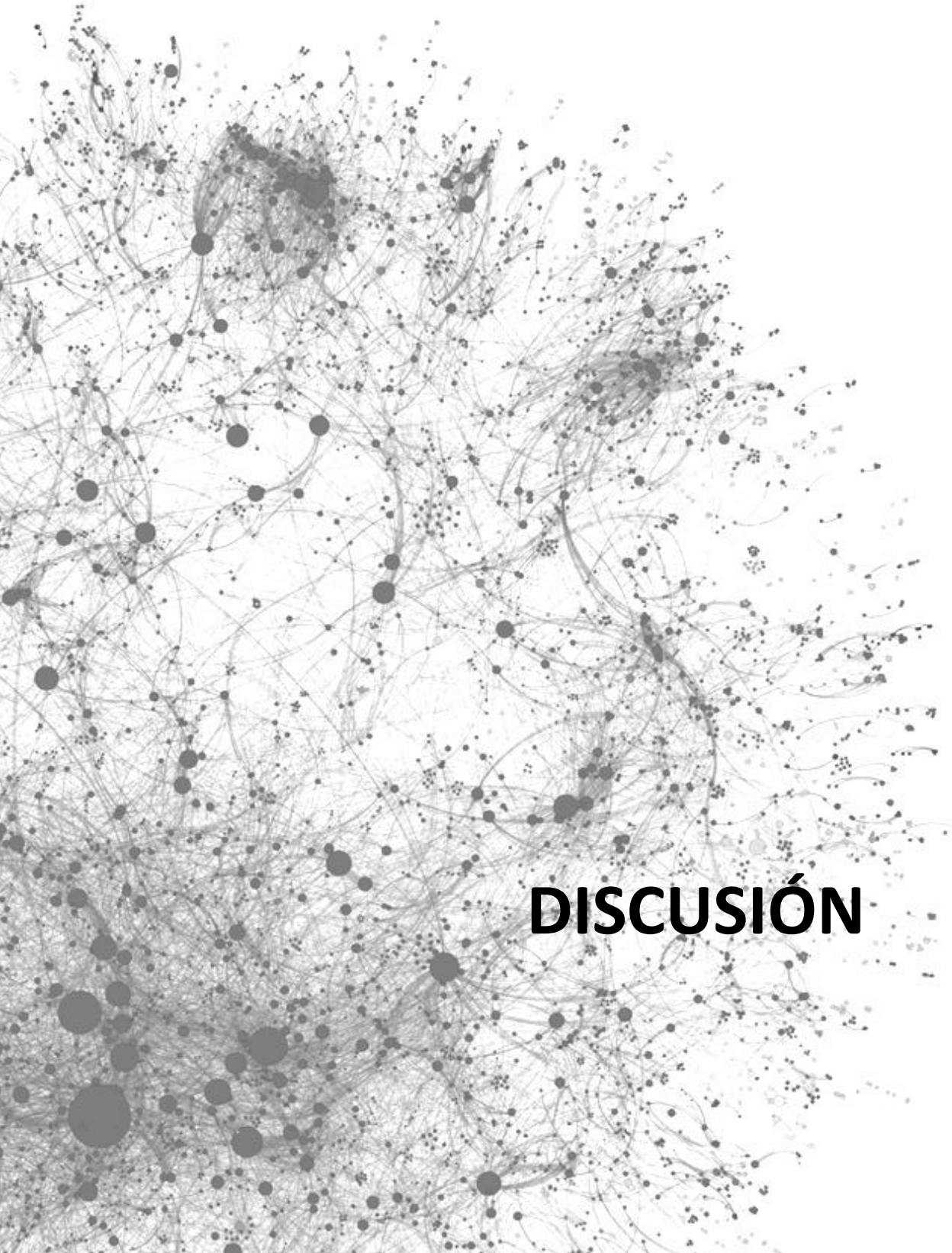

DISCUSIÓN

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Desde el descubrimiento del ARN de interferencia (RNAi)[1], muchos estudios han demostrado su potencial como estrategia terapéutica, y actualmente representa una interesante herramienta para el control y/o tratamiento de diferentes enfermedades, como el cáncer[2], enfermedades neurodegenerativas[3], o enfermedades infecciosas[4,5,6]. Entre las enfermedades infecciosas que pueden ser potencialmente tratadas con RNAi, destacan las infecciones debidas al virus de la inmunodeficiencia humana, al virus de la hepatitis B o al virus de la hepatitis C (HCV).

El genoma de HCV consta de una sola cadena de ARN (+), que se replica en el citoplasma celular, y por tanto, este virus puede ser un buen candidato para el tratamiento con RNAi, mediante el silenciamiento de secuencias específicas de su ARN.

A la hora de diseñar una terapia basada en RNAi, tiene gran importancia la adecuada selección de la secuencia diana del virus. En el presente trabajo de tesis se seleccionó la secuencia denominada "Sitio Interno de Entrada al Ribosoma" (IRES), que se encuentra en el extremo 5' de la región no traducida (UTR) y que es necesaria para la síntesis

[1] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*. 1998 Feb 19;391(6669):806-811.

[2] Huang PI, Lo WL, Cherng JY, Chien Y, Chiou GY, Chiou SH. Non-viral delivery of RNA interference targeting cancer cells in cancer gene therapy. *Curr Gene Ther*. 2012 Aug;12(4):275-284.

[3] González-Alegre P. ARN de interferencia terapéutico para enfermedades neurodegenerativas. *Rev Neurol*. 2008 Dec 16-31;47(12):641-647.

[4] 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

[5] Chandra PK, Kundu AK, Hazari S, Chandra S, Bao L, Ooms T, Morris GF, Wu T, Mandal TK, Dash S. Inhibition of hepatitis C virus replication by intracellular delivery of multiple siRNAs by nanosomes. *Mol Ther*. 2012 Sep;20(9):1724-1736.

[6] Romero-López C, Sánchez-Luque FJ, Berzal-Herranz A. Targets and tools: recent advances in the development of anti-HCV nucleic acids. *Infect Disord Drug Targets*. 2006 Jun;6(2):121-145.

proteica y por tanto, para la replicación del virus[7,8]. Trabajos previos han demostrado que el uso de esta secuencia como diana da lugar a la inhibición de la replicación del HCV[9,10,11]. Más específicamente, la secuencia seleccionada para nuestro trabajo ha sido el dominio II del IRES-HCV, ya que es activa en los genotipos 1a y 1b del HCV. Es importante tener en cuenta que el genotipo 1 del HCV es más agresivo y más resistente al tratamiento con interferón y rivabirina que los genotipos 2 y 3[12]. Además, los subtipos 1a y 1b son los más comunes tanto en Europa como en Estados Unidos[13].

Entre los diferentes tipos de RNAi, los shRNAs o "short hairpin RNA" son vectores de ADN que generan horquillas de ARN de doble hebra que reconocen el ARNm diana e inducen su degradación, inhibiendo la producción de la proteína clave en el desarrollo del proceso patológico. Esta estrategia presenta importantes ventajas frente a otros tipos de RNAi, como es una mayor estabilidad y la producción de un efecto más duradero. Sin embargo, presenta una desventaja debido a la necesidad de llegar al núcleo de la célula para desencadenar el proceso de silenciamiento, con las dificultades que ello implica. Por ello, es esencial el diseño de un sistema de administración adecuado que, además de

[7] Moon JS, Lee SH, Kim EJ, Cho H, Lee W, Kim GW, Park HJ, Cho SW, Lee C, Oh JW. Inhibition of Hepatitis C Virus in Mice by a Small Interfering RNA Targeting a Highly Conserved Sequence in Viral IRES Pseudoknot. *PLoS One*. 2016 Jan 11;11(1):e0146710.

[8] Komar AA, Hatzoglou M. Exploring Internal Ribosome Entry Sites as Therapeutic Targets. *Front Oncol*. 2015 Oct 20;5:233.

[9] Hamazaki H, Ujino S, Abe E, Miyano-Kurosaki N, Shimotohno K, Takaku H. RNAi expression mediated inhibition of HCV replication. *Nucleic Acids Symp Ser (Oxf)*. 2004;(48):307-308.

[10] Ilves H, Kaspar RL, Wang Q, Seyhan AA, Vlassov AV, Contag CH, Leake D, Johnston BH. Inhibition of hepatitis C IRES-mediated gene expression by small hairpin RNAs in human hepatocytes and mice. *Ann N Y Acad Sci*. 2006 Oct;1082:52-55.

[11] Kanda T, Steele R, Ray R, Ray RB. Small interfering RNA targeted to hepatitis C virus 5' nontranslated region exerts potent antiviral effect. *J Virol*. 2007 Jan;81(2):669-676.

[12] Prabhu R, Garry RF, Dash S. Small interfering RNA targeted to stem-loop II of the 5' untranslated region effectively inhibits expression of six HCV genotypes. *Virol J*. 2006 Nov 27;3:100.

[13] Zein NN. Clinical significance of hepatitis C virus genotypes. *Clin Microbiol Rev*. 2000 Apr;13(2):223-235.

facilitar la internalización celular, favorezca la entrada del shRNA al núcleo celular.

Entre los sistemas de administración no virales utilizados en terapia génica, las nanopartículas sólidas lipídicas (SLNs) han demostrado tener un gran potencial. Entre sus ventajas frente a otro tipos de vectores no virales, destacan la biocompatibilidad, seguridad y facilidad de producción a gran escala[14,15,16,] y la posibilidad de incorporar en su superficie ligandos específicos para favorecer la internalización celular o la distribución selectiva a tejidos y órganos concretos, lo que redunda en un mejor perfil de eficacia y seguridad.

Teniendo todo ello en cuenta, en la primera parte de esta tesis se llevó a cabo el diseño y caracterización de vectores no virales basados en SLNs con el plásmido que codifica un ARN interferente frente al dominio II de la secuencia IRES-HCV (shRNA74).

Se diseñó un vector compuesto por SLNs, protamina y un polisacárido (dextrano o ácido hialurónico). La protamina es un péptido que posee una gran capacidad para condensar los ácidos nucleicos, lo que favorece su protección frente a la degradación por nucleasas, y además facilita el paso del material genético al núcleo, y también se ha descrito que mejora la transcripción[17,18]. Por otro lado, el dextrano (DX) y el ácido hialurónico (HA) son polisacáridos biocompatibles que han

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[18] Masuda T, Akita H, Harashima H. Evaluation of nuclear transfer and transcription of plasmid DNA condensed with protamine by microinjection: the use of a nuclear transfer score. *FEBS Lett*. 2005 Apr 11;579(10):2143-2148.

demostrado previamente su capacidad para mejorar la eficacia de transfección de las SLNs[19,20,21], e incluso en el caso del DX, se ha observado que su incorporación a las SLNs hace que éstas permanezcan más tiempo en circulación sistémica tras su administración intravenosa a ratones, ya que se reducen las interacciones de las nanopartículas con componentes sanguíneos que inducen su opsonización y eliminación del organismo[22].

Para este estudio preparamos 4 vectores, con HA y con DX y con distinta carga de shRNA74 (proporción ADN:SLN 1:2 o 1:5). Las interacciones entre los componentes de la formulación condicionan tanto la estructura final como las características físico-químicas del vector. Cuando los vectores se prepararon con HA, se observó una capa (también llamada corona) rodeando la superficie, atribuida a la disposición de las cadenas helicoidales del HA alrededor de la nanopartícula[23]. Esta capa no se observó en los vectores que se prepararon con DX, por lo que queda demostrado que la utilización de un polisacárido u otro condiciona las características superficiales del complejo final.

Todos los vectores que preparamos presentaron características que facilitan la internalización celular del material genético, como es el tamaño de partícula en el rango nanométrico (200-240 nm) y carga

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[23] Apaolaza PS, Del Pozo-Rodríguez A, Torrecilla J, Rodríguez-Gascón A, Rodríguez JM, Friedrich U, Weber BH, Solinís MA. 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 Nov 10;217:273-283.

superficial positiva. La proporción de shRNA74 en el vector no afectó al tamaño de partícula, independientemente del polisacárido utilizado. Cuando los vectores se prepararon con HA, no se observaron diferencias significativas en la carga superficial dependiendo del contenido de shRNA74; sin embargo, en el caso de los vectores preparados con DX, la carga superficial fue mayor cuando la proporción de SLNs es mayor (DX-SLN5), y hay por tanto, un mayor contenido en cargas positivas debidas a las nanopartículas. El hecho de que la proporción shRNA74:SLN afecte o no a la carga superficial del vector dependiendo del polisacárido utilizado se puede explicar porque se trata de la carga superficial neta de los vectores, y cambios en el número de cargas no se traducen necesariamente en cambios a nivel superficial.

Además de las características físico-químicas de los vectores, la eficacia de los vectores va a depender del equilibro entre la capacidad de condensar el material genético, directamente relacionada con la capacidad de protección frente a la degradación (principalmente debido a nucleasas), y la capacidad de liberarlo en el citoplasma de la célula diana. En este sentido, todos los vectores mostraron un comportamiento similar, con un alto grado de condensación del plásmido, capacidad para protegerlo de la acción de la DNasa (aunque fue ligeramente superior en el caso del vector preparado con HA), y capacidad para liberarlo.

El HCV se replica y acantona en el interior de los hepatocitos a lo largo de su ciclo de vida; por ello, los estudios de eficacia de silenciamiento se llevaron a cabo en una línea celular de carcinoma hepático de origen humano (células HepG2).

Previamente a la evaluación de la eficacia de transfección, es importante conocer la capacidad de los vectores para ser internalizados por las células, ya que podría ser un paso limitante para la transfección. Hay que tener en cuenta que el proceso de internalización se inicia con la interacción entre los vectores y la superficie de la célula, en la cual juega

un papel muy importante la carga superficial del vector, ya que las cargas positivas de éste interaccionan con cargas negativas de la superficie celular.

En los experimentos que se llevaron a cabo, se comprobó que todos los vectores eran captados de forma rápida y eficiente, independientemente del polisacárido empleado, aunque fue mayor cuanto menor era la carga de shRNA74 en el vector, es decir, cuando para la misma dosis de shRNA74, la cantidad de nanopartículas añadidas a las células es mayor (DX-SLN5 y HA-SLN5). La diferencia en la captación de los vectores en función de la carga shRNA74 puede explicar, al menos en parte, la diferencia observada en el porcentaje de silenciamiento obtenido, con un mayor porcentaje de silenciamiento con los vectores DX-SLN5 y HA-SLN5, frente a DX-SLN2 y HA-SLN2, respectivamente. Los resultados de silenciamiento también mostraron que éste depende de la dosis de shRNA74, alcanzándose los niveles más altos (aproximadamente un 60%) con la dosis de 3 µg de shRNA74 incorporada en el vector HA-SLN5.

La internalización celular no explica, sin embargo, la mayor capacidad de silenciamiento del vector preparado con HA en comparación con el vector preparado con DX. La mayor eficacia del vector que contiene HA podría deberse, en parte, a la mayor capacidad para proteger el ADN (tal como demostró el estudio de protección frente a la DNasa). Sin embargo, otras razones podrían también justificar esa diferencia. Por ejemplo, en un estudio previo[24], se demostró que el HA es capaz de facilitar la descondensación del plásmido a nivel intracelular, lo que facilitaría la transfección. También habría que tener en cuenta los posibles mecanismos de internalización celular, que podrían ser diferentes dependiendo de la composición del vector, y que condicionan

[24] Apaolaza PS, Delgado D, del Pozo-Rodríguez A, Gascón AR, Solinís MÁ. A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases. *Int J Pharm*. 2014 Apr 25;465(1-2):413-426.

la disposición intracelular del material genético a nivel intracelular, y por tanto la eficacia de transfección. En general, las SLNs son internalizadas por mecanismos de endocitosis, y dependiendo de la línea celular y de la composición, pueden utilizar la endocitosis mediada por clatrinas o mediada por caveolas/lipid raft. En las células HepG2 estos mecanismos están presentes y los vectores podrían utilizar estas dos vías. Sin embargo, el vector preparado con HA, también podría ser captado por las células tras la interacción con el receptor CD44 (también presente en las células HepG2), ya que el HA es un sustrato de este receptor. El hecho de que el vector HA-SLN utilizara, al menos en parte, esta vía de entrada y el vector DX-SLN no, podría justificar también las diferencias en la eficacia de silenciamiento.

Una vez que los vectores demostraron su capacidad para silenciar la secuencia IRES-HCV en las células HepG2, el siguiente paso fue demostrar su eficacia en la línea celular de hepatoma humano Huh-7 NS3-3'[25] que porta un replicón subgenómico del virus. Previamente se comprobó que la eficacia de los vectores para silenciar el IRES-HCV era similar en la línea celular Huh-7. Para este estudio se seleccionaron los vectores preparados con HA, ya que resultaron más eficaces para silenciar el IRES-HCV. Tras la administración de una única dosis de los dos vectores (HA-SLN2 y HA-SLN5), se consiguió un silenciamiento del replicón de hasta el 50% con la dosis más alta evaluada (3 µg de shRNA74). El silenciamiento fue dependiente de la dosis, pero no hubo diferencias dependiendo de la carga de shRNA74 en el complejo. Estos resultados confirman la potencial utilidad de estos vectores en el tratamiento de la hepatitis C crónica.

Debido a la importancia que tienen los mecanismos de internalización celular sobre la disposición del plásmido a nivel intracelular, y por tanto en la capacidad de transfección, el objetivo de la

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siguiente etapa del proyecto fue la evaluación de los mecanismos de captación celular de los vectores, lo que podría servir para una mejor optimización de la formulación.

Como se ha comentado anteriormente, uno de los principales mecanismos de entrada de los vectores no virales es la endocitosis[26], que es un proceso dependiente de energía y que se inhibe a 4°C. Los estudios de internalización celular revelaron que efectivamente, la endocitosis está implicada en la entrada de los vectores en la célula, ya que a 4°C se inhibió tanto la entrada como el nivel de silenciamiento del IRES-HCV; sin embargo, una pequeña reducción en la captación dio lugar una importante disminución del silenciamiento (aproximadamente a la mitad). Esto indicaría que la endocitosis es una vía de entrada muy eficiente desde el punto de vista de la transfección. Los estudios de co-localización con los marcadores de los principales mecanismos de internalización por endocitosis (transferrina para la endocitosis mediada por clatrinas, y toxina del cólera para la endocitosis mediada por caveolas/lipid raft) revelaron que ambos mecanismos están implicados en la entrada del vector, aunque la entrada mediada por clatrinas en mayor grado. La co-localización con LysoTracker®, marcador de lisosomas, estaría en línea con estos mecanismos de entrada, que finalmente conducen al vector a los lisosomas.

Otro mecanismo de entrada dependiente de energía es la macropinocotisis, que en estudios previos se ha demostrado que también puede estar implicado en la captación de nanopartículas. Sin embargo, nuestros vectores no co-localizaron con el marcador de macropinocitosis (dextrano de alto peso molecular), por lo que descartamos que este

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mecanismo esté implicado, a pesar de que se ha descrito que el HA puede inducir la entrada a través de macropinocitosis[27].

Incluso a 4°C, el vector fue capaz de inhibir el IRES-HCV, lo que indica que otros mecanismos no dependientes de energía estarían también implicados en la internalización. Como se sugirió anteriormente, el HA es un ligando del receptor CD44[28], presente en las células Huh-7, y por tanto podría participar en la captación de los vectores. Para comprobarlo, realizamos los estudios de captación y silenciamiento bloqueando dicho receptor añadiendo en el medio de cultivo un exceso de HA. Los resultados mostraron que aunque el bloqueo del receptor CD44 redujo la captación, no dio lugar a una disminución del silenciamiento; esto indicaría que esta vía de entrada es menos eficiente que la endocitosis. En cualquier caso, es importante tener en cuenta que al bloquear un mecanismo de entrada se pueden activar otros[29], y además, dada la complejidad de los sistemas biológicos, pueden intervenir simultáneamente diferentes mecanismos de entrada, produciéndose competencia entre ellos[30], lo que puede dificultar la interpretación de los resultados.

Una vez en el interior de la célula, la disposición intracelular del material genético es otro aspecto clave para la transfección. El plásmido tiene que ser capaz de liberarse del vector y acceder al núcleo. Tras el tratamiento de las células Huh-7 con el vector, se observó la descondensación del plásmido en el interior de la célula a lo largo del

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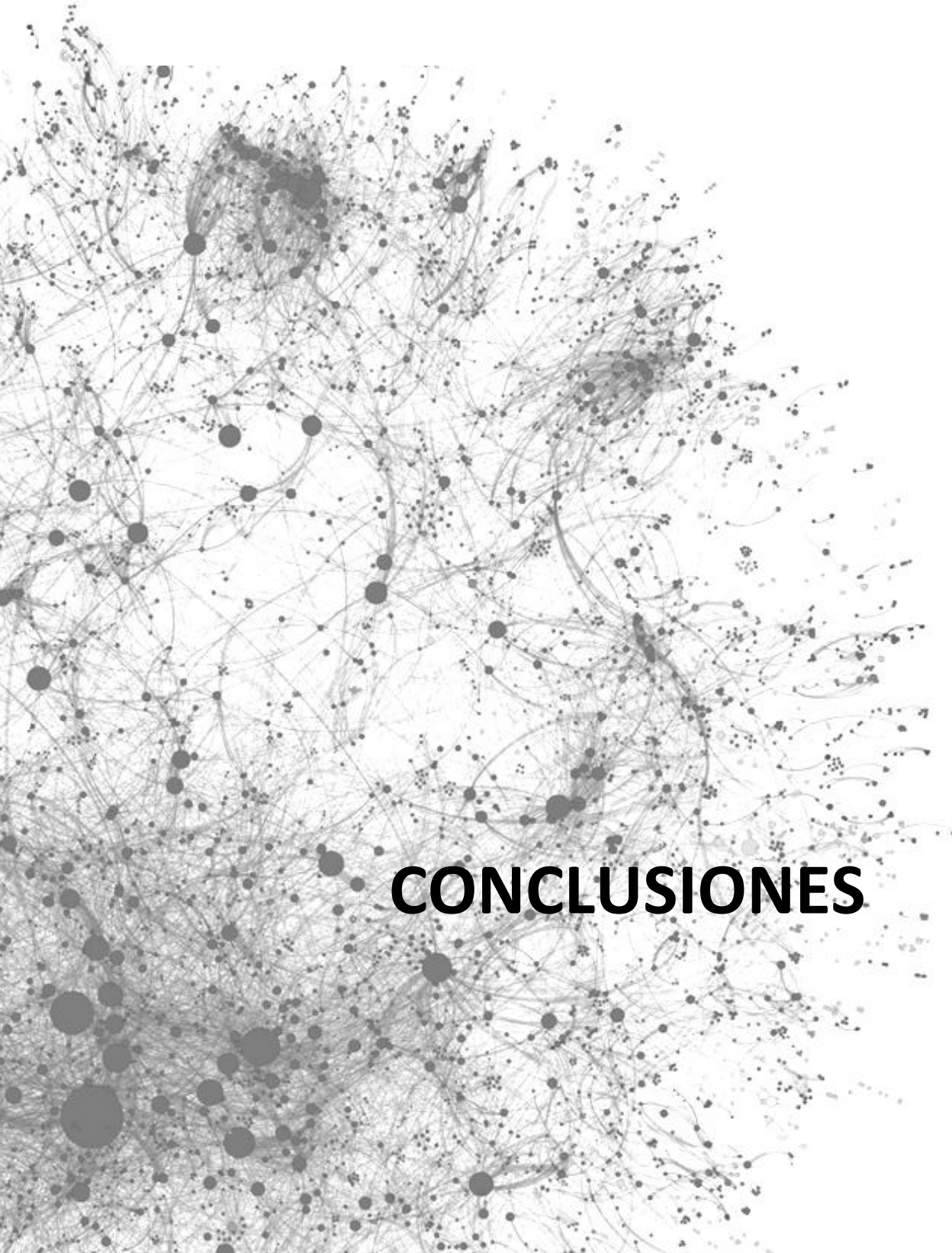
tiempo, que podría está relacionada tanto con la capacidad del HA para descondensar el plásmido, como con la actividad lisosomal.

Respecto a la potencial toxicidad de los vectores, los estudios de viabilidad celular en todos los modelos celulares utilizados confirman la inocuidad de los vectores a este nivel. Puesto que el dato de viabilidad celular por sí solo no es indicativo de la potencial toxicidad *in vivo*, y teniendo en cuenta una posible administración parenteral, evaluamos el efecto del vector HA-SLN a nivel hematológico midiendo el grado de hemólisis y la capacidad de aglutinación de eritrocitos. El vector no mostró actividad hemolítica ni tampoco indujo la aglutinación de eritrocitos. Es importante tener en cuenta que al HA se le atribuye la capacidad de reducir la interacción de las nanoparatículas con componentes sanguíneos debido a un impedimento estérico. Estos resultados confirmarían la adecuación del vector para una siguiente evaluación *in vivo* en animales de experimentación.

En conclusión, esta tesis ha demostrado la capacidad de los vectores no virales basados en SLNs, P y HA, como sistemas de administración del plásmido shRNA74, para inhibir *in vitro* la replicación del HCV. Los resultados aquí presentados sirven como prueba de concepto de la utilidad de los vectores basados en SLNs como una nueva estrategia terapéutica para el tratamiento de las infecciones crónicas debidas al HCV, que podría ser utilizada como alternativa o como complemento de los actuales tratamientos de las infecciones crónicas resistentes a ellos. En cualquier caso, son necesarios más estudios, especialmente estudios *in vivo* en modelos animales de infección por HCV.



CONCLUSIONES

CONCLUSIONES

1. El plásmido shRNA74 incorporado en los vectores no virales compuestos por SLNs, protamina, ácido hialurónico (HA-SLN) o dextrano (DX-SLN) es capaz de silenciar en cultivos celulares de hepatocitos HepG2 la expresión de la secuencia denominada Sitio Interno de entrada al Ribosoma (IRES) del virus de la hepatitis C, necesaria para la replicación del virus.

2. Los vectores HA-SLN y DX-SLN presentan una alta capacidad de internalización en las células HepG2, sin que se detecten diferencias en función del polisacárido utilizado.

3. La eficacia de silenciamiento y la viabilidad celular dependen del polisacárido utilizado y de la carga de plásmido en las nanopartículas. Con los dos vectores, la eficacia de silenciamiento aumenta al aumentar la dosis de plásmido. Los vectores preparados con HA resultaron ser más eficaces que los preparados con DX; sin embargo, la viabilidad celular a altas dosis fue mayor con el vector DX-SLN.

4. El plásmido shRNA74 incorporado en el vector HA-SLN es capaz de inhibir el replicón subgenómico del virus de la hepatitis C en el modelo celular de hepatocitos Huh-7 NS3-3'.

5. La eficacia de silenciamiento del vector HA-SLN en las células Huh-7 depende del mecanismo de entrada y de la consiguiente disposición intracelular del mismo. El vector es internalizado tanto por endocitosis

mediada por clatrinas como mediada por caveolas/lipid raft, siendo la vía endocítica eficiente desde el punto de vista de la transfección. El receptor CD44 también está implicado en la captación del vector, siendo esta vía menos eficiente que la endocítica. La macropinocitsis no parece estar involucrada en la internalización de los vectores en esta línea celular.

6. A nivel hematológico, el vector HA-SLN no mostró actividad hemolítica ni tampoco indujo la aglutinación de eritrocitos, lo que probablemente está relacionado con la capacidad del HA de reducir la interacción de las nanopartículas con componentes sanguíneos debido a un impedimento estérico.

7. Este trabajo muestra por primera vez la potencial utilidad de los vectores no virales basados en SLNs para el tratamiento de las infecciones crónicas debidas al virus de la hepatitis C. Esta nueva estrategia terapéutica podría ser utilizada como alternativa o como complemento de los actuales tratamientos, por lo que son necesarios estudios adicionales que corroboren los resultados obtenidos hasta el momento, entre ellos estudios en modelos animales de la infección.

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La infección por el virus de la hepatitis C sigue siendo hoy en día un problema de salud pública a nivel mundial para el que los tratamientos existentes no siempre resultan del todo efectivos, debido a la diversidad de genotipos virales y la aparición de resistencias por presión farmacológica. Por lo tanto, es necesario desarrollar estrategias terapéuticas alternativas a los tratamientos actuales.

Puesto que el virus de la hepatitis C es un virus de una sola cadena de ARN (+) con capacidad para replicarse en el citoplasma de las células que infecta, el uso del ARN de interferencia puede ser una buena estrategia terapéutica capaz de inhibir su replicación. El material genético debe entrar de manera eficaz en las células diana. En este sentido, las nanopartículas sólidas lipídicas (SLNs) son un sistema de administración adecuado debido a su biocompatibilidad, seguridad y facilidad de producción. La posibilidad de incorporar en su superficie ligandos con el fin de lograr, entre otros, un incremento de la internalización celular o una distribución selectiva a tejidos y órganos concretos, redunda en una mayor eficacia y/o menores efectos adversos.