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REVIEW ARTICLE

Lipid-based Nanocarriers for siRNA Delivery: Challenges, Strategies and the Lessons Learned from the DODAX: MO Liposomal System

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Abstract: The possibility of using the RNA interference (RNAi) mechanisms in gene therapy was one of the scientific breakthroughs of the last century. Despite the extraordinary therapeutic potential of this approach, the need for an efficient gene carrier is hampering the translation of the RNAi technology to the clinical setting. Although a diversity of nanocarriers has been described, liposomes continue to be one of the most attractive siRNA vehicles due to their relatively low toxicity, facilitated siRNA complexation, high transfection efficiency and enhanced pharmacokinetic properties.

This review focuses on RNAi as a therapeutic approach, the challenges to its application, namely the nucleic acids' delivery process, and current strategies to improve therapeutic efficacy. Additionally, lipid-based nanocarriers are described, and lessons learned from the relation between biophysical properties and biological performance of the dioctadecyldimethylammonium:monoolein (DODAX:MO) system are explored.

Liposomes show great potential as siRNA delivery systems, being safe nanocarriers to protect nucleic acids in circulation, extend their half-life time, target specific cells and reduce off-target effects. Nevertheless, several issues related to delivery must be overcome before RNAi therapies reach their full potential, namely target-cell specificity and endosomal escape. Understanding the relationship between biophysical properties and biological performance is an essential step in the gene therapy field.

Keywords: Cationic liposomes, monoolein, siRNA delivery, DODAB, DODAC, PEGylation.

1. INTRODUCTION

The discovery of RNA interference (RNAi) mechanisms has opened a world of new opportunities in the field of gene therapy, providing alternatives to the treatment of diseases for which there are no drugs available. Although mechanisms associated with RNAi are well established, this technology still faces important challenges before it can be an effective therapeutic alternative. The delivery of nucleic acid sequences constitutes one of the most important bottlenecks to RNAi therapeutic approaches, not only concerning stability and protection of the genetic material in physiological conditions, but also regarding the ability to target specific cells and release the therapeutic payload onto their cytoplasm. Several strategies have been explored to overcome each of these barriers, including PEGylation to provide stability in physiological conditions, incorporation of target molecules to potentiate active targeting, or inclusion of stimuli-responsive molecules to improve nucleic acids endosomal escape. This review describes RNAi as a therapeutic approach, as well as the challenges faced by it, namely the nucleic acids delivery process, together with current strategies to overcome some of these challenges. Liposomes are exploited as delivery systems due to their great potential as nanocarriers [1]. The influence of the physicochemical characteristics onto the biological performance of the nanosystem is elucidated by the example of a liposomal system composed by the cationic lipids dioctadecyldimethylammonium bromide or chloride (DODAB/DODAC) and the neutral lipid monoolein (MO).

2. POTENTIAL OF RNA AS A GENE THERAPY TOOL

RNAi is a natural post-transcriptional gene silencing mechanism, by which double-stranded RNAs (dsRNAs) modulate the expression of target RNAs, in a sequence-specific dependent manner. The first time an RNAi-type of phenomenon was reported in 1990 when Napoli and Jorgen-

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sen [2] obtained white petunias when trying to generate violet ones. The authors attributed this effect to the "co-suppressing" gene introduced into the cells. In 1992, a similar phenomenon was described for *Neurospora crassa*, when the introduction of homologous RNA sequences was reported to cause "quelling" of the targeted endogenous gene [3]. The description of the same type of mechanism in animals was made by Guo and Kemphues [4], that found that both sense and anti-sense strands resulted in the degradation of *par-1* mRNA in the nematode *Caenorhabditis elegans* (*C. elegans*). It was only in 1998 that Fire and Mello provided a common explanation for the silencing of endogenous genes by "co-suppression, quelling and sense mRNA" [5]. When the authors showed, in *C. elegans*, that gene silencing was triggered by a dsRNA sequence, the term "RNA interference" was born. In 2001, the introduction of an artificial siRNA of 21 nucleotides (nt) was found to specifically block the expression of endogenous and heterologous genes in various mammalian cell lines [6], and in 2002 the therapeutic potential of siRNA was reinforced by McCaffrey *et al.,* who demonstrated effective targeting of a sequence from hepatitis C virus by RNAi *in vivo* [7]. The importance of Fire and Mello's work for the comprehension of RNAi cellular mechanisms was recognized in 2006 with the Nobel Prize in Physiology or Medicine.

The RNAi mechanism is used in the context of gene therapy with the purpose to modulate the expression of genes involved in the development of diseases. An important advantage of this loss-of-function strategy is that, irrespective to its localization, virtually every single protein can be targeted. Furthermore, antisense RNAs do not lead to genome modifications, since they are not integrated in DNA, decreasing the safety concerns associated with therapeutic applications [8]. Frequent targets of this strategy are mutant transcripts [9], viral infections [10] and molecular effectors on cancer [11-13] (Table **1**).

The potential of RNAi therapeutic approaches is demonstrated by the several clinical trials with siRNAs (Table **2**).

2.1. RNA Interference Mechanisms

(Fig. **1**) shows a representation of the small interfering RNA (siRNA) and micro RNA (miRNA) RNAi pathways. MicroRNAs are derived from the genome and function as regulators of endogenous genes [37, 38]. The transcription of most miRNAs is typically performed by RNA polymerase II (RNA Pol II) into long primary transcripts (pri-miRNAs) of at least 1000 nt, with double-stranded hairpins and single stranded 5'- and 3'-terminal overhangs [37]. The miRNA lies within the pri-miRNA double-stranded stem. A nuclear microprocessor complex crops this pri-miRNA into 65-70 nt precursor miRNAs (pre-miRNA) that, after association with the transport facilitators Exportin-5 and RanGTP, are transported into the cytoplasm. The microprocessor complex, composed by Drosha and a protein cofactor with two double-stranded RNA binding domains (dsRBDs), is responsible for the cleavage of the loop of the hairpin, and excision of the pri-miRNA into pre-miRNA [39].

Once in the cytoplasm, pre-miRNA is further processed into a mature miRNA duplex of 21-25 nt in length by the Dicer enzyme [40]. At this point, the siRNA and miRNA pathways converge, since siRNA is also processed from long dsRNAs by Dicer in the cytoplasm of the cells. Typically, 21-25 nt dsRNAs are generated by Dicer, with 2-base 3'-overhangs, hydroxyl groups at the 3'-ends, and phosphate groups at the 5'-ends. This size is long enough to offer a sequence complexity sufficient for selective binding to a specific gene in the genome [40]. The sources of siRNA are long, linear, perfectly base-paired dsRNAs, endogenously produced or introduced directly into the cytoplasm. Additionally, siRNAs can also be directly introduced into the cytoplasm of the cells or expressed from gene expression cassettes embedded in DNA plasmids or viral vector genomes [41].

Once in the cytoplasm, the resulting double-stranded siRNA or miRNA is incorporated by the minimal RNA-induced silencing complex-loading complex (RISC-loading complex) (Fig. **1**) [37, 39, 41, 42], where the two strands of dsRNA unwind, the guide strand is loaded into AGO protein while the passenger strand is discarded, and a functional RISC is generated [37, 39, 41, 42]. RISC contains only the RNA antisense guide strand complementary to the target mRNA. The strand selection is dictated by the thermodynamic stability of the duplex ends: in the miRNA and siRNA pathways, the strand preferentially loaded into AGO protein has the less stable 5′-end [39]. The miRNA strand that associates with AGO proteins is called miRNA strand, while the other is discarded (miRNA*).

Finally, nucleotides 2–6 of the guide strand, named seed sequence, initialize binding to the target mRNA. When perfect complementarity exists, the target mRNA molecule is cleaved in a very precise way: the phosphodiester linkage between the target nucleotides that are base-paired to siRNA residues 10 and 11 (counting from the 5'-end) is cleaved to generate products with 5'-monophosphate and 3'-hydroxyl termini [43]. The target dissociates after cleavage, and RISC is free to cleave additional mRNAs. While siRNA has perfect complementarity to the target mRNA, miRNA only binds imperfectly to mRNA, causing translational repression without endonucleolytic cleavage. This partial mismatched binding allows each miRNA to interact with many target mRNAs [37, 41].

3. CHALLENGES IN RNA INTERFERENCE THERAPIES – THE PROCESS OF SIRNA DELIVERY

Despite the extensive knowledge about the RNAi mechanism, there are still several challenges that must be overcome for the safe and efficient application of this loss-of-function technology as a therapeutic strategy. RNAi is an essential mechanism of cell regulation, and minor alterations in RNAi machinery can have major consequences in cellular processes. The off-target effects associated with dsRNA introduction into cells can be divided into three classes: saturation of the endogenous RNAi machinery [44]; miRNA-like off-target effects [45]; and induction of inflammatory responses due to activation of Toll-like receptors (TLRs) [46]. Other obstacles to RNAi as an effective therapeutic option include resistance to treatment [47], efficiency of the silencing effect compromised by the recognition of endosomal compartment [8, 48].

Table 1. Examples of targets used in RNAi-based approaches to cancer treatment.

Pathway	Molecular Target	Cancer Model	References
Cell cycle	Cyclin B	Prostate	[14]
	PLK1	Breast	[15, 16]
Proliferation	MAD2	Colon	[17]
	EPHA2	Ovarian	[18]
	AKT1	Prostate	[19]
	AKT2	Prostate	
	AKT3	Prostate	
	FAK	Ovarian	[20]
Cell death and survival	BCL-2	Prostate	[21]
	BCL-XL	Prostate	[22]
	MCL-1	Breast	[23]
	Survivin	Prostate	[24]
Angiogenesis	VEGF	Prostate	[25, 26]
	PAR-1	Melanoma	[27]
	CD31	Prostate	[28]
	KLF-5	Lung carcinoma	[29]
	PLX1DC	Ovarian	[20]
Cell senescence	TERT	Lewis lung tumor	[30]
Oncogenes	E6/E7	Renal	[31]
	EWS-FLI1	Ewing sarcoma	[32]
	c-RAF	Breast	[33]
	c-MYC	Melanoma	[34]
	BCR-ABL	Chronic myeloid leukemia	[35]
Resistance	P-gp	Breast	[36]

AKT1, 2, 3: protein kinase B 1, 2, 3; BCL-2: B-cell lymphoma 2; BCL-XL: B-cell lymphoma extra-large; BCR-ABL: breakpoint cluster region - abelson; CD31: cluster of differentiation 31; E6/E7: human papillomavirus oncoproteins E6 and E7; EPHA2: receptor of ephrins 2; EWS-FLI1: ewing's sarcoma–friend leukaemia virus integration 1; FAK: focal adhesion kinase; KLF-5: kruppel-like factor 5; MAD2: mitotic-arrest deficient 2; MCL-1: myeloid cell leukemia1; PAR-1: protease-activated receptor 1; P-gp: permeability glycoprotein; PLK1: Polo-like kinase 1; PLX1DC: plexin domain containing 1; TERT: telomerase reverse transcriptase; VEGF: vascular endothelium growth factor.

The physicochemical properties of siRNA duplexes, namely large weight and size, high hydrophilicity and negative charge, restrict the binding to cellular membranes and do not allow naked siRNAs to enter cells by passive diffusion mechanisms [49]. Thus, endocytosis is the major route of siRNA internalization, with endosomal entrapment and lysosomal degradation important barriers for its therapeutic use. Even when siRNA escapes from endosomes, the viscosity of the cytoplasm and the presence of organelles are still obstacles for its loading into the RISC complex. Inherent physicochemical properties of nucleic acids also imply that naked siRNA is quickly degraded by serum endonucleases [50], easily filtered from the glomerulus and rapidly excreted by the kidneys, resulting in a half-life of few minutes in plasma [45, 51]. Extravasation from blood vessels to target tissues constitutes another challenge because in most tissues capillary vessel walls are impermeable to large nucleic acids. Some exceptions are tumors or inflamed tissues, where blood vessels are leaky. Furthermore, the dense network of polysaccharides and fibrous proteins of the extracellular matrix limits migration and access to target cells.

4. DEVELOPMENTS IN NANOCARRIERS FOR siRNA DELIVERY

The targeted delivery of genetic material at controlled rates is a very attractive method, and has been strongly pursued, given the importance to improve safety and efficacy. The ideal delivery method should promote high transfection efficiency, low cell toxicity, minimal effects on physiology and be reproducible [52].

Table 2. Clinical trials of siRNA-based therapeutics. Details of each trial can be found on www.clinicaltrials.gov.

Target	Disease	siRNA	Route	Phase	Sponsor	Start-end
VEGF	Macular degeneration	Bevasiranib (Cand5)	IVT	I *	OPKO Health, Inc	2004-07
VEGF	Diabetic macular edema	Bevasiranib (Cand5)	IVT	II *	OPKO Health, Inc	2006-07
VEGF	Macular degeneration	Bevasiranib (Cand5)	IVT	II *	OPKO Health, Inc	2006-07
VEGF	AMD	Bevasiranib (Cand5)	IVT	III **	OPKO Health, Inc	2007-09
VEGF	AMD	Bevasiranib (Cand5)	IVT	III wd.	OPKO Health, Inc	Nov 2009-
VEGFR1	AMD	AGB211745 (siRNA-027)	IVT	I/II *	Allergan siRNA Therap. Inc.	2004-07
VEGFR1	Choroidal neovascularization	AGB211745 (siRNA-027)	IVT	II **	Allergan siRNA Therap. Inc.	2007-09
RTP801	AMD	PF-04523655 (PF-655)	IVT	I *	Quark Pharma	2007-09
RTP801	Diabetic retinopathy, Diabetes complications	PF-04523655 (PF-655)	IVT	II **	Quark Pharma	2008-10
RTP801	Choroidal neovascularization, Diabetic retinopathy	PF-04523655 (PF-655)	IVT	II *	Quark Pharma	2009-11
RTP801	Diabetic macular edema	PF-04523655 (PF-655)	IVT	II *	Quark Pharma	2012-13
RSV-N gene	Respiratory syncytial virus infections	ALN-RSV-01	NN	II *	Alnylam Pharma	2007 Jul-Nov
RSV-N gene	Respiratory syncytial virus infections	ALN-RSV-01	NN	II *	Alnylam Pharma	2008-09
RSV-N gene	Respiratory syncytial virus infections	ALN-RSV-01	NN	IIb *	Alnylam Pharma	2010-12
P53	Injury of kidney, Acute renal failure	I5NP (QP1-1002)	IV	I *	Quark Pharma	2007-10
P53	Injury of kidney, Acute renal failure	I5NP (QP1-1002)	IV	I **	Quark Pharma	2008 - 2010
P53	Delayed graft function, Other complications of kidney transplant	I5NP (QP1-1002)	IV	I/II *	Quark Pharma	2008-14
K6A N171K	Pachyonychia congenita	TD101	I.I.	I *	Pachyonychia Congenital Project	2008 Jan-Aug
RRM2	Solid tumors	CALAA-01	IV	I **	Calando Pharma	2008-12
KSP and VEGF	Advanced solid tumors with liver involvement	ALN-VSP02	IV	I *	Alnylam Pharma	2009-11
KSP and VEGF	Advanced solid tumors with liver involvement	ALN-VSP02	IV	I *	Alnylam Pharma	2010-12
Apo B	Hypercholesterolemia	PRO-040201 (TKM-ApoB)	IV	I **	Arbutus Biopharma (ABUS)	2009-10
PKN3	Advanced solid tumors	Atu027	IV	I *	Silence Therapeutics	2009-12
PKN3	Pancreatic ductal carcinoma	Atu027	IV	I/II *	GmbH	2013-16
B2-AR	Ocular hypertension, Open-angle glaucoma	SYL040012 (Bamosiran)	Opht.	I *	Sylentis, S.A.	2009-10

(Table 2) contd….

Target	Disease	siRNA	Route	Phase	Sponsor	Start-end
B2-AR	Ocular hypertension, Open-angle glaucoma	SYL040012 (Bamosiran)	Opht.	I/II *	Sylentis, S.A.	2010-12
B2-AR	Ocular hypertension, Open-angle glaucoma	SYL040012 (Bamosiran)	Opht.	II *	Sylentis, S.A.	2012-13
B2-AR	Ocular hypertension, Open-angle glaucoma	SYL040012 (Bamosiran)	Opht.	II *	Sylentis, S.A.	2014-16
Caspase-2	Optic atrophy, Non-arteritic anterior ischemic optic neuropathy	QPI-1007	IVT	I *	Quark Pharma	2010-13
Caspase-2	Acute primary angle-closure, Glaucoma	QPI-1007	IVT	II *	Quark Pharma	2013-15
TTR	TTR-mediated amyloidosis	ALN-TTR01	IV	I *	Alnylam Pharma	2010-12
TTR	TTR-mediated amyloidosis	ALN-TIR02 (Patisiran)	IV	I *	Alnylam Pharma	2012-12
TTR	TTR-mediated amyloidosis	ALN-TIR02 (Patisiran)	IV	II *	Alnylam Pharma	2012-14
TTR	TTR-mediated amyloidosis	ALN-TIR02 (Patisiran)	IV	II *	Alnylam Pharma	2013-17
TTR	TTR-mediated amyloidosis	ALN-TIR02 (Patisiran)	IV	II *	Alnylam Pharma	2014-15
	TTR-mediated Amyloidosis	ALN-TTR02 (Patisiran)	IV	III*	Alnylam Pharma	2013 – 17
TTR	TTR-cardiac amyloidosis	ALN-TTRSC (Revusiran)	SC	I ##	Alnylam Pharma	2013-15
TTR	TTR-cardiac amyloidosis	ALN-TTRSC (Revusiran)	SC	II *	Alnylam Pharma	2013-15
TTR	TTR-cardiac amyloidosis	ALN-TTRSC (Revusiran)	SC	II *	Alnylam Pharma	2014-17
TTR	TTR-cardiac amyloidosis	ALN-TTRSC (Revusiran)	SC	III *	Alnylam Pharma	2014-17
KRAS G12D	Pancreatic ductal adenocarcinoma	siG12D LODER	EUS biopsy	I *	Silenseed Ltd.	2011-13
KRAS G12D	Pancreatic ductal adenocarcinoma	siG12D LODER	needle	II ###	Silenseed Ltd.	2017
TRPV1	Ocular pain	SYL1001	Opht.	I *	Sylentis, S.A.	2011-12
TRPV1	Dry eye syndrome	SYL1001	Opht.	I/II *	Sylentis, S.A.	2012-15
Polo-kinase-1	Solid tumors with liver involvement	TKM-080301 (TKM-PLK1)	IV	I *	National Cancer Inst.	2011-12
Polo-kinase-1	Neuroendocrine tumors, Adrenocortical carcinoma	TKM-080301 (TKM-PLK1)	IV	I/II *	Arbutus Biopharma (ABUS)	2010-15
Polo-kinase-1	Hepatocellular carcinoma	TKM-080301 (TKM-PLK1)	IV	I/II *	Arbutus Biopharma (ABUS)	2014-16
PCSK9	Elevated LDL-cholesterol	ALN-PCS02	IV	I *	Alnylam Pharma	2011-12
PCSK9	Elevated LDL-cholesterol	ALN-PCSSC	SC	I *	Alnylam Pharma	2014-15
ZEBOV L polym. VP24, VP35	Ebola virus infection	TKM-100201 (TKM-Ebola)	IV	I **	Arbutus Biopharma (ABUS)	2012 Jan-Jul
HSP47	Healthy	ND-l02-s0201	IV	I *	Nitto Denko Corp.	2013-14
HSP47	Moderate to extensive hepatic fibrosis	ND-l02-s0201	IV	I *	Nitto Denko Corp.	2014-16

(Table 2) contd….

Target	Disease	siRNA	Route	Phase	Sponsor	Start-end
HSP47	Ebola virus infection	TKM-100802	IV	I **	Arbutus Biopharma (ABUS)	2014- 15
AT	Hemophilia A, Hemophilia B	ALN-AT3SC	SC	I *	Alnylam Pharma	2014-17
AT	Hemophilia A, Hemophilia B	ALN-AT3SC	SC	I/II #	Alnylam Pharma	2015
E3 ubiquitin ligase Cbl-b	Melanoma, Pancreatic cancer, Renal cell cancer	APN401	IV	I *	Wake Forest Univ.	2014-16
	Pancreatic Cancer, Colorectal Cancer, Solid tumors	APN401.	IV	I ##	Wake Forest Univ.	2017
DCR-MYC	Hepatocellular carcinoma	MYC	IV	I/II ##	Dicerna Pharma	2014-16
DCR-MYC	Solid tumors, Multiple myeloma, Non-Hodgkin lymphoma	MYC	IV	I ##	Dicerna Pharma	2014-15
siRNA-EphA2-DOPC	Advanced cancers	EphA2	IV	I ##	MD Anderson Cancer Center	2015
TNFR:Fc	Rheumatoid Arthritis	tgAAC94	IA	I*	Targeted Genetics Corpo-ration	2004-2005
hIFN-b	Rheumatoid Arthritis	ART-I02	IA	I##	Arthrogen	2018
GDNF	Parkinson's Disease	/AAV2-GDNF	IC	I#	National Institute of Neu-rological Disorders and Stroke (NINDS)	2012
NTN	Parkinson's Disease	CERE-120: AAV2-NTN	IC	I*	Ceregene	2005-2007
NRTN	Idiopathic Parkinson's Disease	CERE-120	IC	I/II#	Sangamo Therapeutics	2009
NRTN	Parkinson's Disease	CERE-120	IC	II*	Ceregene	2006-2008
AADC	Parkinson's Disease	VY-AADC01	IC	I#	Voyager Therapeutics	2013
AADC	Parkinson's Disease	Cohort1 Cohort2	IC	I/II##	Jichi Medical University	2015
CLN2	Batten Disease Late Infantile Neuronal Ceroid Lipofuscinosis	AAV2CUhCLN2	IC	I#	Weill Medical College of Cornell University	2004
CLN2	Batten Disease Late Infantile Neuronal Ceroid Lipofuscinosis	AAVrh.10CUCLN2	IC	I/II#	Weill Medical College of Cornell University	2010
CLN2	Batten Disease Late Infantile Neuronal Ceroid Lipofuscinosis	AAVrh.10CUhCLN2	IC	I#	Weill Medical College of Cornell University	2010
NGF	Alzheimer's Disease	CERE-110	IC	I*	Ceregene	2004-2010
CFTR	Cystic Fibrosis	Adeno-associated virus-CFTR vector	NN	I*	National Institute of Dia-betes and Digestive and Kidney Diseases	1999-2002
RPE65	Leber Congenital Amaurosis (LCA)	AAV2-hRPE65v2	Opht	I#	Spark Therapeutics	2007
RPE65	Leber Congenital Amaurosis (LCA)	AAV2-hRPE65v2	Opht	I/II #	Spark Therapeutics	2010
RPE65	Leber Congenital Amaurosis (LCA)	AAV2-hRPE65v2	Opht	III#	Spark Therapeutics	2012

(Table 2) contd….

Target	Disease	siRNA	Route	Phase	Sponsor	Start-end
GAA	Pompe Disease	rAAV1-CMV-GAA	IM	I/II*	University of Florida	2010-2015
GAA	Late-Onset Pompe Disease (LOPD)	rAAV9-DES-hGAA	IM	I##	University of Florida	2017
SMN	Spinal Muscular Atrophy Type 1	AVXS-101	IV	I*	AveXis, Inc.	2014
SMN	Spinal Muscular Atrophy Type 1	AVXS-101	IV	III##	AveXis, Inc.	2017
SMN	Spinal Muscular Atrophy	AVXS-101	IT	I##	AveXis, Inc.	2017
SMN	Spinal Muscular Atrophy	AVXS-101	IV	III###	AveXis, Inc.	2018 may
FIX	Hemophilia B	AAV8-hFIX19	IV	I**	Spark Therapeutics	2012 - 2016
FIX	Hemophilia B	AAV5-hFIXco-Padua (AMT-061)	IV	II###	UniQure Biopharma B.V.	2018
SGSH	Mucopolysaccharidosis Type 3 A Sanfilippo Syndrome	scAAV9.U1a.hSGSH	IV	I/II##	Abeona Therapeutics, Inc	2016
NAGLU	Mucopolysaccharidosis (MPS) IIIB	rAAV9.CMV.hNAGLU	IV	I/II##	Kevin Flanigan	2017
NAGLU	Sanfilippo Syndrome B	rAAV2/5-hNAGLU	I.I	I/II##	UniQure Biopharma B.V.	2013
IDS	Mucopolysaccharidosis II	SB-913	IV	I##	Sangamo Therapeutics	2017
IDUA	Mucopolysaccharidosis I	SB-318	IV	I##	Sangamo Therapeutics	2017
ARSA	Metachromatic Leukodystrophy	AAVrh.10cuARSA	I.I	I/II#	Institut National de la Santé Et de la Recherche Médicale, France	2013
GAN	Giant Axonal Neuropathy	scAAV9/JeT-GAN	IT	I##	National Institute of Neurological Disorders and Stroke (NINDS)	2015
CLN6	Batten Disease	scAVV9.CB.CLN6	IT	I/II##	Nationwide Children's Hospital	2016

AMD - age-related macular degeneration; AT – antithrombin; β2-AR - β2 adrenergic receptor; EUS - endoscopic ultrasound; K6A N171K - keratin 6AN171K mutant; KRASG12D - K-rasG12D mutant; KSP - kinesin spindle protein; LODER - local drug eluter; RRM2 - M2 subunit of ribonucleotide reductase; PCSK9 - proprotein convertase subtilisin/kexin type 9; PKN3 - protein kinase N3; RSVN gene - respiratory syncytial virus nucleocapsid gene; TTR – transthyretin; VEGF - vascular endothelial growth factor; VEGFR1 - vascular endothelial growth factor receptor 1; VP24: viral protein 24; VP35 - viral protein 35; ZEBOV L polym. - ZEBOV L polymerase. * - completed; ** - terminated; *** - suspended; # - active; ## - recruiting; ### - not yet recruiting; IVT – intravitreal; IV – intravenous injection; IT – intrathecal administration, IM- intramuscular; IA- intra-articular injection; IC – Intracranial; Opht. – ophtalmic administration;I.I.-intralesional injection; NN – nasal nebulization; wd – withdrawn.

Significant advances have been made in the development of efficient nanocarriers, such as siRNA conjugates, inorganic materials polymers and cationic lipids for siRNA delivery (Table **3**).

These systems can be categorized into viral carriers (viruses and bacteria) and non-viral carriers (polymers and cationic lipids) produced by physical methods, chemical or biological methods [52, 53]. (Table **4**) shows some advantages and disadvantages of viral and non-viral gene therapy vectors.

DNA-based expression cassettes that express short hairpin RNA (shRNA) are usually delivered to target cells *ex vivo* by viruses and bacteria, and these modified cells are then reinfused back into the patient [54]. Viral systems provide very efficient delivery and transfection to the intended target, however, they have several drawbacks, such as the potential to generate a severe immune response, as has been demonstrated in a number of non-human models and human trials. They are also limited in the size of a plasmid that they can encapsulate and by possible storage time [55, 56].

Another kind of siRNA carriers, widely used in therapeutics and biomedical engineering, are inorganic nanoparticles [68, 69]. Their attractive physicochemical properties such as: good stability and physical strength, high purity, reproducible and tunable size and morphology, and ease for surface modification, make them good candidates for siRNAs delivery [70]. In fact, recent investigations revealed that several nanomaterials are intrinsically therapeutic, since they, not only can passively interact with cells but can also actively mediate molecular processes to regulate cell functions [71]. For instance, gold nanoparticles were shown to be anti-angiogenic and with antitumor properties that interfere with cellular processes [69]. Characteristic inorganic nanoparticles are metals, metal oxides and carbon material and

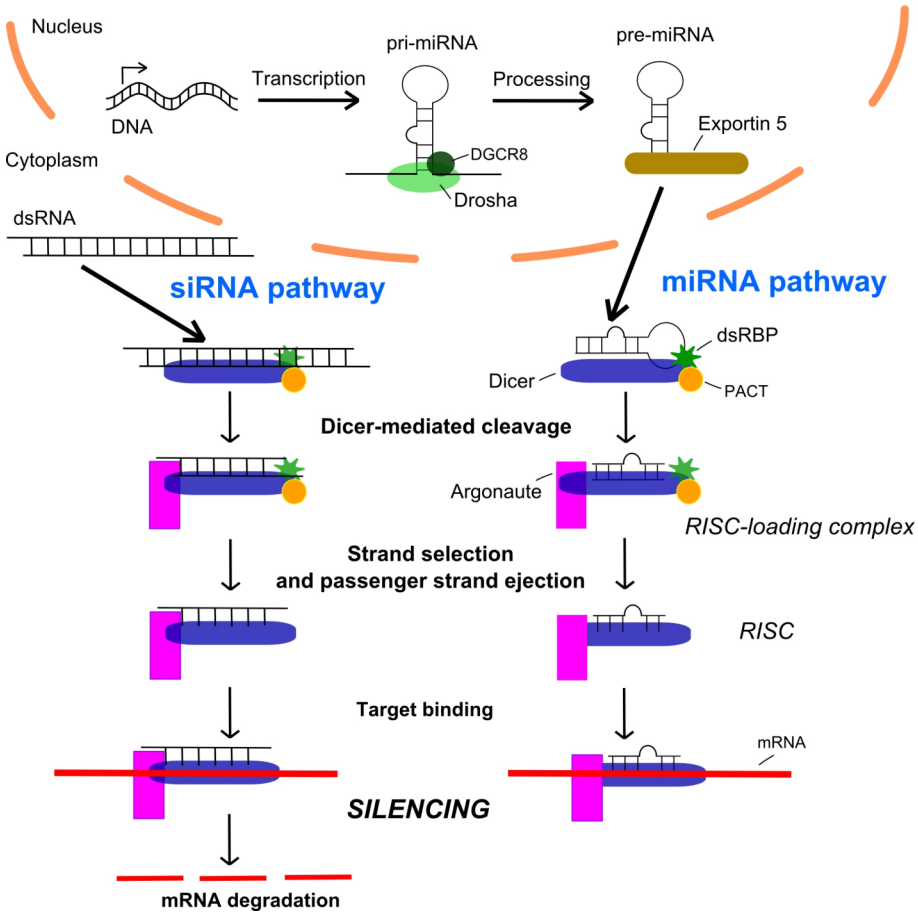

Fig. (1). Schematic representation of RNA interference mechanisms.

magnetic nanoparticles – SPIONS (Super-Paramagnetic Iron Oxide Nanoparticles). Gold nanoparticles have unique chemical and physical properties like low cytotoxicity, ease of synthesis, tunable size and morphology, ready functionalization and strong optical absorption that make them a useful scaffold for efficient recognition and delivery of biomolecules [72]. The synthesis of iron oxide nanoparticles has been intensively developed not only for its fundamental scientific interest but also for its many technological applications, such as targeted drug delivery, magnetic resonance imaging (MRI), magnetic hyperthermia and thermoablation, bioseparation, and biosensing. However, control over the shape and size distribution of magnetic iron oxide nanoparticles remains a challenge, and the different formation mechanisms of iron oxides under different conditions still need to be investigated [73]. Carbon nanotubes (CNTs) exhibit incomparable physical, mechanical and chemical properties, such as: strength, thermal conductivity, mechanical, and electrical properties. Also, could be used as additives to various structural materials [74]. Although these systems have great advantages for biomedical use, there are several concerns regarding their therapeutic use due to documented toxicity [75, 76].

Polymeric nanoparticles are solid, biodegradable, colloidal systems that can be classified into two major categories, natural polymers [cyclodextrin, chitosan, and atelocollagen] and synthetic polymers [polyethyleneimine (PEI), poly(dl-lactide-co-glycolide) (PLGA), and dendrimers]. These systems have been demonstrated to provide effective and efficient siRNA delivery *in vitro* and *in vivo*. However, some studies have reported inconsistent results due to discrepancies between experiments, therefore PLGA could not be applied efficiently in siRNA delivery due to the lower electrostatic interaction between PLGA and siRNA leading to less efficient endosomal escape and release of siRNA and PEI complexes have been associated with significant toxicity issues limiting their broad use in clinical trials [77].

Future studies must focus on the *in vivo* safety profiles of the different delivery systems, including undesirable immune stimulation and cytotoxicity.

Lipid-based nanocarriers will be addressed in detail as they are to be focused in this review.

4.1. Lipid-based Nanocarriers for siRNA Delivery

Liposomes were first described by Bangham in the mid-60s [78], and during the 70s the idea that they could entrap drugs and be used as drug delivery systems was further established by Gregoriadis [79]. Liposomes are composed by amphiphilic molecules that, when in aqueous solution and above the critical vesicle concentration (CVC), spontaneously self-assemble into one or multiple lipid bilayers, capable of entrapping hydrophilic substances in the inner aqueous compartments, and lipophilic compounds within the lipid layers.

Table 3. Example of nanocarriers used for siRNA delivery.

Type of System	Example
siRNA conjugates	Cholesterol [57]
	α-Tocopherol [58]
	Cell-penetrating peptides (CPPs) [59]
	Poly(ethylene glycol) (PEG) [60]
	Aptamers [61]
Inorganic materials	Gold [62]
	Iron Oxide [63]
	Carbon nanotubes (CNTs) [64]
Polymer-based nanocarriers	Poly(ethyleneimine) (PEI) [60]
	Poly-D,L-lactide-co-glycolide (PLGA) [65]
	Chitosan [66]
	Dendrimers [67]
Lipid-based nanocarriers	Liposomes [13]
	Small nucleic acids lipid particles (SNALPs) [13]

As non-viral vectors, liposomes have unique advantages, including high encapsulation efficiency, low toxicity and drug/genetic material protection against degradation factors. These vectors also reduce tissue irritation, uniformly deposit active drugs *in situ* and are biodegradable and nonimmunogenic [80]. Additionally, the liposomal membrane is composed of natural and/or synthetic lipids which are relatively biocompatible [81]. These systems have been used in the delivery of nucleic acids since the pioneering study of Felgner and colleagues, in 1987, describing the ability of the cationic lipid DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethlyl ammonium chloride) to deliver DNA to the COS-7 cell line [82]. Cationic liposomes are used frequently for non-viral gene delivery due to their positive charge, as they interact electrostatically with the negative charges of nucleic acid phosphate groups, resulting in a nano-complex where the genetic material is entrapped – a process that leads to the formation of lipoplexes [83]. Also, it facilitates interactions with the negatively charged components of cell membranes, together with a good tolerability, high transfection activity and good pharmacokinetic properties. Cationic lipids used for gene therapy are composed of three basic domains: a positively charged headgroup, a hydrophobic chain, and a linker which joins the polar and non-polar regions. The nature of the cationic headgroup influences the ability to condense and protect nucleic acids, the cytotoxicity associated with the liposomes, and the overall transfection efficiency [84]. The influence of the hydrophobic chain on toxicity has not yet been adequately addressed but it is known that the hydrophobic lipid anchor helps to maintain the self-aggregating lipid organization, and its length and saturation influences the biological activity of the cationic lipids [84]. The nature of the linker group (for example ethers, esters, carbamates or amides) influences the conformational flexibility, degree of stability and biodegradability of the amphiphile. Although more efficient in the transfection process, usually ethers cause higher toxicity, since they form chemically more stable but non-biodegradable linkers when compared to esters. Esters links are biodegradable and less toxic but not so stable, which can thus affect the transfection outcome [85, 86].

Table 4. Advantages and disadvantages of viral and non-viral gene therapy vectors.

	Viral Vectors	Non-viral Vectors
Advantages	High transduction efficiency	Low immunogenicity and antigenicity
		No risk of chromosomal insertion
		Ease of production
	Capacity to infect many types of cells	Possibility of functionalization for targeted delivery and endosomal escape
		Low toxicity
	Optimized endosomal escape	Drug/genetic material protection against degradation factors
	Efficient delivery	Reduce tissue irritation
Disadvantages	Strong immune response	Low transfection efficiency
	Possibility of chromosomal insertion and proto-oncogene activation	High doses are toxic
	Difficult production	Lack of intrinsic tropism
	Possibility of contamination with live virus	Lack of intrinsic mechanism for endosomal escape
	Limited plasmid size	

Not only monomeric cationic lipids but also dimeric amphiphiles with two hydrophobic tails and two polar headgroups linked by a covalently bonded spacer, known as Gemini amphiphiles, are explored for siRNA therapy applications, apart from anionic lipids (Table **5**).

Neutral *(helper)* lipids like DOPE, cholesterol or monoolein are usually included in the formulations to help nucleic acids escape endosomes [87, 88], aid nucleic acids complexation by allowing a closer contact and packing of their helices [89], decrease toxicity associated with the cationic lipids [90, 91], or confer more favorable properties in terms of bilayers fluidity [92]. DOPE is one of the most widely used *helper* lipids, as it is believed to improve transfection efficiency due to its tendency to undergo a transition from a lamellar to a non-lamellar structure (hexagonal configuration), under acidic pH, facilitating fusion with lipid bilayers and allowing lipoplex endosomal escape [87]. The presence of DOPE in the formulation can also decrease the charge ratio of lipid to DNA required to achieve maximum transfection *in vitro*, thus reducing the toxicity associated with an excess of lipid. Nevertheless, when in low serum levels or its absence, the aggregate instability imposed by helper lipid DOPE is advantageous in contact with serum proteins, as the dissociation of lipoplexes followed by aggregation often leads to precipitation and results in the loss of efficient transfection [93].

While many studies have demonstrated that lipoplex structure and function can be compromised in the presence of serum, little attention has been paid to the adsorption of specific proteins and how this might be affected by formulation parameters. After systemic administration, nanomaterials are exposed to various physiological fluids, mostly blood. The adsorption of proteins on NPs can modify the diverse physicochemical properties of NPs such as size, surface charge, surface composition, and functionality, hence giving NPs a new biological identity - protein corona (nanoparticle-protein complex) which is complex and unique to each nanomaterial and NP [94, 95]. The advantages of including cholesterol in the formulations have been associated with a higher protection of nucleic acids from nuclease degradation [96], with effects on liposome bilayer fluidity [92], and with reduced binding of serum proteins to liposomes [97]. Also, Batker *et al.* characterized changes that occur in the protein corona when DOTAP-based lipoplexes are formulated with different amounts of cholesterol and have demonstrated that increased cholesterol contents mitigate the amount of protein that binds to lipoplexes, and the number of proteins that adsorb was also reduced in formulations containing ≥ 67 mol% cholesterol [95].

Table 5. Example of liposomal systems used for siRNA delivery.

Type of Liposomes	Lipid Composition	Purpose of Investigation	Ref
Cationic	DOTAP:DOPE	Luciferase silencing on MCF-7 cells stably expressing the luciferase protein	[110]
	DOTAP:Chol	Luciferase silencing on MCF-7 cells stably expressing the luciferase protein	[110]
	DODAB/C:MO	eGFP silencing on H1299 cells stably expressing the eGFP	[100]
	DHDEAC:Chol:DSPE-PEG	Tumor suppression and gene silencing in SK-OV-3 xenograft mouse model	[111]
	DOPE	Silencing gene expression in activated human macrophages	[112]
Gemini	1,5-bis(1-imidazolilo-3-alkoxymethyl) pentane dichloride dicationic gemini surfactants	Complexation of different DNA and RNA sequences; cytotoxic evaluation	[113]
	bis-quat conventional and serine-derived Gemini surfactants	Delivery efficiency of anti-survivin siRNA; Effect of the combination of chemotherapeutics with survivin gene silencing	[114]
Anionic	DOPG:DOPE + Ca^{2+} ions	eGFP silencing on MDA-MB-231 cells stably expressing the eGFP	[115]
PEGylated	DODAB:MO:PEG-ceramide	BCR-ABL silencing on K562 cells	[116]
Targeted	Chol:DSPC:DODAP:PEG-ceramide:Trf-coupled PEG-DSPE	BCR-ABL silencing on K562 cells	[13]
Immunoliposomes	DOTAP:Chol:DSPE-PEG-mal:anti-EGFR antibody	Luciferase silencing on SMMC-7721, LM3 and Hep3B cells stably expressing the luciferase protein	[117]

Chol – cholesterol; DODAB - 1,2-dioleoyl-3-dimethylammonium-bromide; DODAC - 1,2-dioleoyl-3-dimethylammonium-chloride; DODAP - 1,2-dioleoyl-3-dimethylammonium-propane; DOPE - 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOPG - 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol); DOTAP - 1,2-dioleoyl-3-trimethylammonium-propane; DSPC - 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine; DSPE–PEG - 1,2-dioctadecanoyl-sn-glycero-3-phosphoethanolamine (polyethylene glycol); DSPE-PEG-Mal - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol); eGFP - enhanced green fluorescence protein; EGFR - epidermal growth factor receptor; MO – monoolein; PEG - poly(ethylene glycol); Trf- transferrin receptor.

Monoolein has also been suggested as a *helper* lipid [98, 99] not only due to its ability to fluidize and stabilize liposomal structures, but also due to its ability to form non-lamellar inverted cubic phases, known to be intermediates of fusion processes [98, 100, 101], discussed in more detail in section 6.

4.2. Mechanism of siRNA-lipoplexes Formation

Since both siRNA and DNA are double-stranded nucleic acids with anionic phosphodiester backbones, they interact electrostatically with cationic lipids and spontaneously form supramolecular assemblies - the so-called lipoplexes. The type of lipid, lipid composition (cationic lipid:neutral lipid molar fraction), and ratio between the positive charges of cationic lipids and the negative charges of nucleic acids (charge ratio, C.R.) determine lipoplex morphology. Lipoplexes composed of ODNs and siRNA show morphologies similar to that of DNA lipoplexes [102], with nucleic acids observed between lipid layers, forming multilayered structures. Nevertheless, the different size and structure of pDNA and ODNs/siRNA makes the lipoplex formation process slightly different for the two types of molecules. Weisman *et al.* [103] proposed a model where negatively charged single-stranded ODN molecules act as bridges between cationic membranes, stabilizing the condensed lamellar phases. The ODN-mediated adsorption of lamellae, one by one, is followed by the restructuration of the lipids, explaining the presence of lamellar defects. Although very similar to the mechanism of liposome reorganization proposed for DNA-lipoplexes [104], this model does not suggest the existence of lipid mixing. Other studies have nevertheless reported fusion among vesicles during ODN-lipoplex formation [105].

Additional differences between ODN and DNA-lipoplexes include a smaller aqueous thickness associated with ODN than with DNA or less organization of ODNs between the lipid layers when compared to DNA [103]. Another aspect to consider is that pDNA has several kilo base pairs (bp), while siRNA usually has 21 to 23 bp, affecting the electrostatic interactions with cationic liposomes. Moreover, the hydroxyl group in the 2'- position of the ribose makes RNA much less stable that the deoxyribose of DNA.

The different sizes of siRNA and pDNA also influence the number of positive charges needed to achieve the same complexation efficiency. When using the same polycation, the fact that pDNA has a higher molecular weight and higher negative charge than siRNA can result in the formation of more stable complexes for pDNA than siRNA. Therefore, unpacking of pDNA could be more difficult than the release of siRNAs from the complex [106].

5. STRATEGIES TO OVERCOME THE BARRIERS FOR EFFICIENT siRNA DELIVERY IN A THERAPEUTIC STRATEGY

siRNAs can only exert their silencing effect once they are incorporated into the RISC complex in the cytosol of the cells. Nevertheless, before siRNAs reach their site of action, numerous barriers must be overcome that depend not only on the targeted organs but also on the siRNA administration route used. Although local delivery of siRNA poses less challenges when compared to systemic administration, in many cases systemic delivery is the only way to reach certain disease sites.

The general steps that siRNA has to overcome, from the site of administration to the site of action, include: distribution through the organs blood circulation; transport from the blood vessels within the organ to the interstitium; transport across the interstitial space to the target cells; and internalization by the target cells. Endocytosis is the major route of siRNA internalization and siRNA endosomal entrapment and lysosomal degradation are additional barriers for its therapeutic use. Moreover, even when siRNA escapes from endosomes, the viscosity of the cytoplasm and the presence of organelles are still obstacles to its loading into the RISC complex.

(Fig. **2**) represents some of the main barriers faced by nanocarriers after systemic administration: (I) aggregation and interaction with blood components; (II) internalization of the nanocarriers by the targeted cells; (III) nucleic acids escape from endosomes.

5.1. PEGylation to Improve Nanocarrier Stability in Physiological Conditions

Initial strategies to improve liposome circulation time were based on mimicking erythrocyte membranes, by performing modifications with gangliosides like monosialoganglioside (GM1) [107]. Later, hydrophilic polymers like poly(ethylene glycol) (PEG) were introduced with the same purpose [108, 109].

Nowadays nanocarriers are usually coated with hydrophilic materials in order to form a protective hydrophilic layer around them. The polymers' flexible chains occupy the space near the liposomal surface, preventing other macromolecules from being in the same space, decreasing the binding of opsonins and thus uptake by macrophages.

As a result, these long-circulating liposomes can take advantage of the enhanced permeability and retention effect (EPR), accumulate in the interstitial space of solid tumors [118], which is a useful approach when the target tissue is a tumor.

Although different long hydrophilic polymer chains and non-ionic surfactants were developed and used as shielding groups, such as polysaccharides, polyacrylamide, poly(vinyl alcohol), poly (N-vinyl-2-pyrrolidone), PEG and PEG-containing copolymers, PEG is still the most widely used material to achieve steric stabilization [119]. PEG is a linear polyether diol with several properties that make it attractive for biomedical applications [120], such as good biocompatibility, very low toxicity, immunogenicity and antigenicity, good excretion kinetics and solubility in both aqueous and organic media. Moreover, PEG is highly hydrated in water, forming a large excluded volume where the hydrophilic chains are in rapid motion [121].

A common method to graft PEG onto the surface of liposomes (a process called PEGylation) is to use cross-linked lipids to anchor the polymer to the liposomal membrane [120]. PEG linked to distearoylphosphatidylethanola-

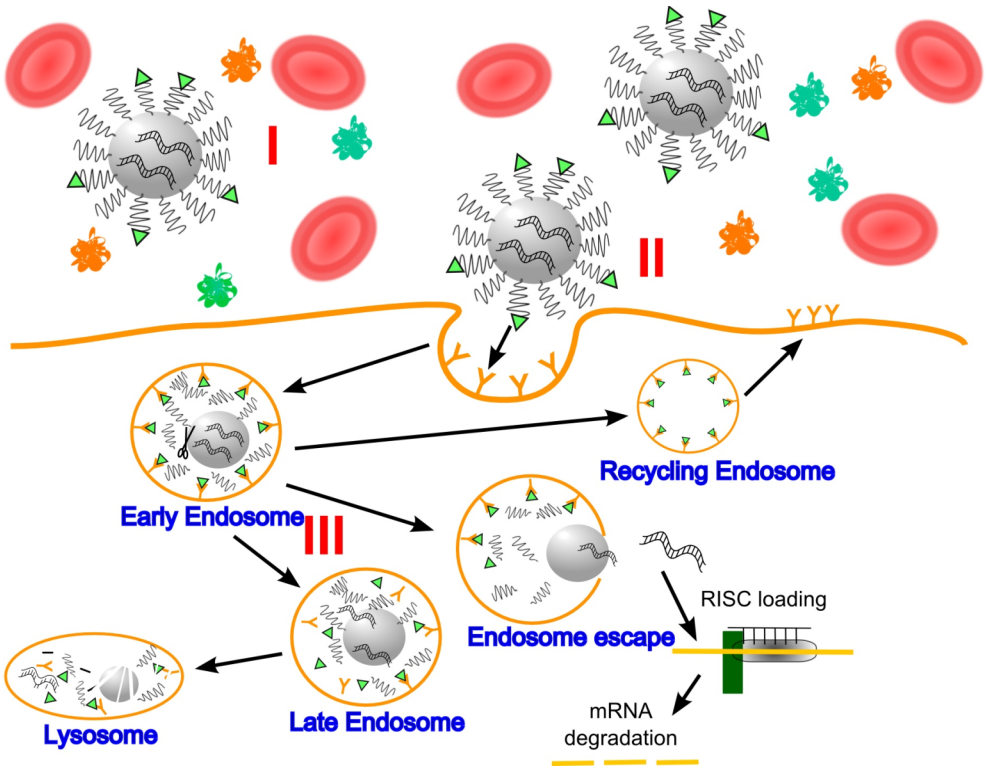

Fig. (2). Common strategies to overcome the barriers to systemic, non-viral delivery of siRNA. **I.** PEGylation of nanocarriers to avoid aggregation and unspecific interactions with blood components, *e.g.* cells and proteins. **II.** Attachment of targeting ligands to improve nanocarriers' cellular uptake by receptor-mediated endocytosis. **III.** Use of "intelligent" materials to allow dissociation of PEG chains from the surface of nanocarriers to promote endosomal escape. The components in the figure are not represented at scale.

mine (DSPE) (DSPE-PEG), is a well-known example [108]. Physical adsorption of PEG, or covalently attachment of reactive groups onto the surface of liposomes, can also be used [120]. Depending on graft density, PEG can assume different conformations at the surface of liposomes (mushroom or brush). Although some opsonization is always inevitable, it can be minimized by the presence of a near perfect PEG shell surrounding the liposomes. However, excessive percentages can lead to the formation of micelles composed of PEG-lipids that may act as liposome destabilizing agents [122]. Dar *et al.* investigated different strategies for preparing liposomes with three different PEG amounts (2.5 mol %, 5 mol % and 8 mol %) to identify the best possible formulation that leads to efficient *in vivo* gene silencing. Intravenous administration of SEV-5 (Vesicles containing 5 mol % of DSPE-PEG-2000) at 5 mol % PEG in ovarian cancer xenograft mouse model confirmed the stability and nontoxic nature of the formulation and the delivery of therapeutic siRNA mediated by these system led to significant tumor tropism and efficient gene silencing [111]. Therefore, good stealth liposomes include a high – but not excessive– density of PEG-lipids at their surface.

PEGylation of liposomes not only extends circulation times in blood but it also improves stability by avoiding aggregation. Braeckmans *et al.* [123] used fluorescent Single Particle Tracking (fSPT) to analyze if PEGylation actually suppressed aggregation of liposomes in blood. The authors found that the presence of 10 % DSPE-PEG significantly reduced liposomes aggregation when compared to the non-PEGylated liposomes. Dakwar and coworkers studied the colloidal stability of liposomes in mouse intraperitonial fluid, plasma from a healthy patient and ascites fluid from a patient diagnosed with peritoneal carcinomatosis [124]. The authors found that the inclusion of PEG improved liposome stability in the different fluids.

However, PEG's presence can be a disadvantage for some phases of the delivery process: PEGylated nanocarriers might not adequately encapsulate and protect nucleic acids from nuclease activity, and PEG can inhibit nanocarriers cellular internalization or difficult siRNA endosomal release [125, 126]. These evidence clearly suggest a "PEGylation dilemma", since there is a need for a stable PEG coating when nanocarriers are in blood circulation, to avoid recognition by the immune system and prevent aggregation, but simultaneously PEG chains can become an obstacle for cellular internalization and endosomal escape. The presence of PEG chains on the liposome surface can avoid the release of genetic material from endosomes by more than one mechanism: (i) stabilization of the lipoplexes lamellar organization, impairing the structural reorganization required for endosomal membrane destabilization [126]; (ii) inhibition of the contact between lipids from liposomes and from endosomal membranes, that is essential for membrane destabilization and nucleic acids release [127]. Therefore, there is the need for so-called smart materials, able to respond at the appropriate time and place and provide PEG chains association/dissociation accordingly.

Different environment-responsive PEG-derivatives have been developed to overcome the "PEGylation dilemma": pH-

sensitive linkers between PEG and the lipid anchors, that are stable at neutral pH but hydrolyzed in acidic environment [119]; removal of PEG triggered by the action of enzymes [128]; use of conjugates of lipids and hydrophilic polymers as exchangeable moieties, which can dissociate from the liposomes with different kinetics. PEG-ceramides (PEG-Cer) form one such example of semi-stable coatings, able to offer good protection and provide stability in blood while maintaining good cellular uptake and transfection activity [129]. Other PEG-lipid conjugates include PEG-phosphatidylethanolamine (PEG-PE), PEG-diacylglycerol, PEG-dialkyloxypropylamine or PEG-(*N*-methyl-4-alkylpyridinium chlorides) (PEG–SAINT) conjugates [119]. The structure, length and saturation of the PEG-lipid conjugates define the anchorage strength of PEG to the lipid bilayer and the kinetics of diffusion [119]. Usually, shorter acyl chain groups (for instance 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) (C14) > (1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine) DPPE (C16) > DSPE (C18)) and unsaturated anchors diffuse faster than longer, and saturated, acyl chain groups. This usually results in improved transfection efficiencies *in vitro*, but reduced circulation times *in vivo*. Thus, a compromise between prolonged circulation times, protection of nanocarriers, interaction with target cells and delivery of the payload must be reached to design efficient nanosystems.

An additional important factor to consider when designing a nanocarrier system is the preparation method. The simplest way to form PEGylated siRNA lipoplexes is by directly mixing the nucleic acids with the PEGylated liposomes. However, for high PEG densities, siRNA becomes bound to the outer surface of the liposomes, making them susceptible to premature release into the blood stream [125]. An alternative method is to form the siRNA-lipoplexes with non-PEGylated cationic liposomes and then proceed to the PEG grafting of the formed lipoplexes – the post-PEGylation alternative [116]. This allows a better protection of the siRNA inside the lipid bilayers while taking advantage of the PEG shielding effect necessary for systemic administration. Other methods consist in hydrating a lipid film which already includes PEG, with a siRNA aqueous solution, or using the ethanolic dilution method for the preparation of siRNA-lipoplexes [130].

5.2. Targeted Delivery to Improve the Effectiveness

It is thought that the predominant route for nanocarrier internalization is endocytosis, triggered by non-specific electrostatic interactions between the positive nanocarriers and negative proteoglycans at the surface of the cells [131]. Nevertheless, nanocarriers can also be tailored for receptor-mediated endocytosis (Table **6**). In fact, by using targeted delivery, the intracellular drug concentration can be increased, more effective tumor targeting can be achieved, non-specific toxicity can be reduced and the overall therapeutic effect can be enhanced [132].

Several studies indicate that nanocarriers enter cells *via* clathrin-dependent endocytosis (or clathrin-mediated endocytosis (CME)), caveolae-mediated endocytosis and/or macropinocytosis and that these pathways of entry are not mutually exclusive. More importantly, not all internalization pathways result in an effective release of the nanocarriers' payload into the cytoplasm. When receptor-mediated endocytosis is not used, internalization of nanocarriers depends on their size, surface charge and shape, as well as on the cell type [133-135].

5.3. Stimuli-sensitive Molecules to Improve Endosomal Escape

Escape of nucleic acids from endosomes implies that nanocarriers are able to induce a perturbation on the lamellar structure of endosome membranes. The mechanism by which cationic liposomes escape from endosomes can be either by induction of a fusion event between lipoplex and endosome membranes or by a local and transient membrane perturbation that leads to the formation of pore-like structures. These mechanisms can be explained by the displacement of anionic lipids from the cytoplasm-facing monolayer into the interior of the endosome, by a flip-flop mechanism. During the process, anionic lipids of the endosomal membrane laterally diffuse into the lipoplexes, resulting in the formation of an "ion pair" between anionic phospholipid headgroups and cationic lipids, charge neutralization, and reduction in the headgroup area that will favor the inverted conformation according to geometrical restrictions [136]. At the same time, the cationic charge neutralization results in the dissociation of the nucleic acid from the lipoplex and escape into the cytosol [137]. For instance, replacement of DOPE by 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), a structural analog of DOPE with no activity to form inverted hexagonal phases under acidic pH, resulted in no *helper* activity and no transfection efficiency [138]. The fact that DOPC is more strongly hydrated than DOPE can decrease the proximity of interaction nanocarrier-intracellular membranes and affect escape from endosomes [131]. Lipoplexes can adopt a large variety of non-lamellar phases, like micellar and cubic phases, all able to perturb the bilayer structure of endosomes [131].

Apart from the inclusion of *helper* lipids in the liposomal formulations, other approaches have been investigated to potentiate the release of nucleic acid from lipoplexes into the cytosol of the target cells. For example, pH-responsive liposomes containing synthetic glutamic acid-based zwitterionic lipids showed improved fusogenic potential at acidic pH [139]. Cationic liposomes can also be modified in order to perform a proton sponge effect similar to polyethylenimine (PEI). Kumar *et al.* [140] reported the synthesis and transfection efficiency of novel histidylated cationic amphiphiles containing a single endosome-disrupting histidine head group, which facilitated the release of DNA into the cytoplasm of the cells. Other alternative can be the use of cell-penetration peptides (CPPs), cationic and/or amphipathic 10-30 amino acid sequences able to cross the plasma membrane or enter cells *via* endocytosis and induce endosomolytic activity. CPPs can form complexes with nucleic acids, through electrostatic interaction, or can be incorporated into more complex delivery systems like liposomes of polymer nanocarriers. The development of these peptides was inspired by the endosomal disruptive properties of fusogenic sequences of viral fusion proteins. It is well-known that endosomal escape of the influenza virus is driven by the presence of hemagglutinin subunit HA2, a fusogenic peptide with a short chain of N-terminal amphiphilic anionic peptides [141].

Table 6. Examples of targeting molecules explored to achieve specific nucleic acids/drug delivery.

	Target/targeting Molecule	Endocytosis Pathway	Overexpression	Example	Ref.
Proteins	Transferrin receptor (TfR); transferrin (Tf)	CME	TfR overexpressed in malignant cells	Tf-liposomes to silence BCR-ABL in leukemic cells	[13]
	Integrin $\alpha_v\beta_3$; arginine-glycine-aspartic acid (RGD) peptide	Caveolae	$\alpha_v\beta_3$ overexpressed in angiogenic endothelium	RGD-liposomes loaded with doxorubicin	[142]
Antibodies	Tyrosine kinase MET; scFv antibody binding MET	CME	MET involved in growth, invasion and metastasis in cancer	scFv-PEG-liposomes loaded with doxorubicin	[143]
Small molecules	Folate receptor (FR); folate (fol)	Caveolae and clathrin-independent endocytosis; CME	FR-α: malignant tissues of epithelial origin. FR-β: patients with CML and AML	fol-nanocarriers to silence Her-2 in human KB cells.	[144]
Aptamers	Nucleolin; AS1411 (specifically binds to nucleolin)	dynamin-independent; more than one pathway	Nucleolin overexpressed on the surface of cancer cells	AS1411-PEG-liposomes to silence BRAF in melanoma	[145]

CME - clathrin-mediated endocytosis; scFv - human single chain variable fragment.

The N-terminus peptide undergoes a conformational change induced by pH alterations, which trigger fusion of the viral membrane with endosomal membranes, leading to viral genome leakage to cytosol. GALA (glutamic acid-alanine-leucine-alanine) is an example of a synthetic amphipathic pH-sensitive endosome-disruptive peptide. A drop in pH promotes a conformational change from a random coil to an amphipathic α-helix, leading to disruption of lipid membranes and release of their content. The incorporation of GALA with transferrin-containing lipoplexes significantly increased luciferase gene expression in COS-7 cells [146].

5.4. Chemical Modifications of Nucleic Acids

The direct use of naked siRNA is limited to local delivery and to specific sites such as the eye, nose and lungs and systemic application of siRNA therapeutics requires the use of safe and efficient delivery systems, including direct chemical modification of siRNA and/or optimization of delivery systems (ex: liposomal formulations, nanoparticle conjugation and antibodies that target cellular moieties) [147].

Both DNA and RNA oligonucleotides can be modified chemically to alter several features which are important for successful delivery to cells (examples in Table **7**). RNA is much more susceptible to nuclease activity, therefore, its modifications are particularly relevant. Chemical modifications of the RNA backbone have been developed in order to increase resistance to nucleases, biodistribution, thermal stability improvement, specificity for target mRNA and blood lifetime extension without altering the nucleic acid sequence or interfering with silencing efficiency.

Transfection efficiency of mRNA has been greatly improved and the half-life of mRNA has been intensely increased, ranging from a few minutes to several hours by chemical modifications. The advantage of using drugs with short half-lives is that they can be rapidly removed from the patient should adverse reactions develop. However, the therapy must usually be administered either at relatively high concentration and/or frequency to maintain an effective dose. In this regard, it is important to guarantee that repeated administrations are safe and feasible [148]. Specific sequence motifs can be recognized by Toll-like receptors (TLRs) and induce cellular immune responses, which constitutes another challenge [149]. Base modifications can reduce immune activation, and the addition of modified nucleotides into siRNA suppresses unwanted immunostimulation [150]. Nevertheless, recent studies reported that, in certain circumstances, immune stimulation could be beneficial and may represent an alternative treatment strategy against cancers and viral infections [151].

Common modifications to the ribose ring include fluorine (2'-F), methoxy (2'-OMe), locked nucleic acids (LNA) and unlocked nucleic acids (UNA). Phosphorothioate, boranophosphate modifications, uncharged nucleic acid mimics or linkage of hydrophobic ligands (*e.g.*, cholesterol) are other examples [49, 152-154]. In fact, some of the ongoing clinical trials use naked siRNA, although chemically modified siRNAs, which are delivered locally, reduce the risk of RNA degradation and systemic immune activation associated with systemic delivery. Alnylam Pharmaceuticals has several siRNA drugs undergoing clinical trials. Their most advanced drug, also one of the most advanced siRNA therapeutics, Patisiran, is a Lipid Nanoparticle (LNP) containing siRNA against mutant transthyretin for the treatment of transthyretin amyloidosis [155].

Phase I and II studies of siRNA therapeutics in the past 2 years have demonstrated potent (as high as 98%) and durable, for weeks, gene knockdown in the liver, with some signs of clinical improvement and without unacceptable toxicity [153] and, recently, announced positive Phase III data on their therapy for hereditary ATTR amyloidosis. (ClinicalTrials.gov. NCT01960348: APOLLO - The study of an investigational drug, patisiran (ALN-TTR02), for the treatment of transthyretin (TTR) - mediated amyloidosis https://clinicaltrials.gov/ct2/show/NCT01960348 Accessed 12 October 2017). Other companies have also invested in the application

Table 7. Examples of chemically modified antisense oligonucleotides.

Modification	Features
DNA, RNA (R=H, OH)	Easily degraded by nucleases Poor pharmacokinetics Unstable
Phosphorothioate (PS) DNA	One of the non-bridging phosphate oxygens is replaced by a sulfur atom Increases resistance to nuclease degradation Allows RNase H activity Improves pharmacokinetics Supported in all antisense mechanisms
Phosphorodiamidate morpholino oligonucleotides (PMOs)	Ribose (RNA) or deoxyribose (DNA) is replaced by a morpholine ring and the phosphorothioate or phosphodiester (RNA) groups are replaced by phosphorodiamidate groups Neutral molecule Increases resistance to nuclease degradation Does not support RNase H activity Used for translation arrest or splicing alterations
2'-O-Methoxyethyl (2'-MOE) and 2'-O-methyl (2'-OMe)	Modifications to the 2'-position Increases resistance to nuclease degradation Supports RNase H activity
Locked Nucleic Acids (LNA)	Bicyclic system with the 4'-carbon tethered to the 2'- hydroxyl group Improves hybridization properties Improves nuclease resistance Supports RNase H activity

of lipoplex-based siRNA drugs due to their advantages in the genetic therapy field [156].

6. LESSONS LEARNED FROM THE DODAB/C:MO LIPOSOMAL SYSTEM

Over the past years, siRNA nanocarriers of increased complexity have been developed, incorporating a variety of molecules to improve stability, provide fusogenicity and allow active targeting by the system. However, the basic understanding of the characteristics driven by the physicochemical properties of the nanosystem is sometimes overlooked, delaying the progression into the clinical setting. In this section, we demonstrate how important the full characterization of a liposomal system can be for the understanding of its biological performance and to provide information for a successful, rational design of a siRNA nanocarrier.

Our research group has been working with a formulation composed by the cationic lipids dioctadecyldimethylammonium bromide/chloride (DODAB/C) and the neutral lipid monoolein (MO) in the past years for nucleic acids/drug delivery [98-100, 116, 157-164]. DODAB and DODAC are composed by a hydrophobic group formed by two 18 carbon long acyl chains (C18:0), linked to a stable quaternary ammonium headgroup. The positively charged monovalent counter-ions bromide (Br^-) or chloride (Cl^-) form DODAB and DODAC lipids, respectively (Fig. **3**).

DODAB/C vesicles can be prepared by sonication or extrusion [165], ethanol injection [159], or by simply dissolving the lipid powder in water, above the lipids' transition temperature (T_m) [166]. DODAB and DODAC CVC are very low, allowing the formation of bilayer structures at concentrations as low as 10 μM [167]. Their phase behavior has been extensively studied, and the lipids found to form bilayer structures when dispersed in aqueous media and above T_m, although with different the thermotropic behavior for DODAC and DODAB [168]. The hydrated Cl^- is larger than the hydrated Br [169], implying that Cl^- ion cannot be as proximal to the cationic headgroup as Br^-, being less competent in the neutralization of its positive charges. Thus, DODAC has a less ordered polar region due to stronger repulsive interactions between the lipid headgroups. Although DODAB forms bilayers more densely packed at the headgroup region than DODAC [167, 169], it exhibits lower T_m values and forms bigger vesicles than DODAC.

1-Monoolein (1-(cis-9-octadecenoyl)-rac-glycerol, MO) is a neutral lipid composed by an unsaturated hydrocarbon chain attached to a glycerol backbone by an ester bond (Fig. **3**). MO is biodegradable since it can be degraded by esterase activity in different tissues[172]. Its biocompatibility and non-toxicity made MO extensively used in different areas, ranging from pharmaceuticals, food, cosmetics, and agriculture, to protein crystallization [171, 172]. The monoolein/water phase diagram (Fig. **3**) shows that MO can form several different phases according to temperature and water content, including lamellar (L_α), inverted hexagonal (H_{II}), and bicontinuous cubic phases (type G (gyroid, Q_{II}^G) and cubic phase D (diamond, Q_{II}^D) [173]. An unusual feature of the water/monoolein system is the excess-water phase separation region. MO has the particularity of forming two inverted bicontinuous cubic phases in excess water, consisting of two intertwined but not interpenetrating water channels separated by a lipid bilayer surface [171, 173]. MO cubic phases can contain up to 40 % (w/w) of water.

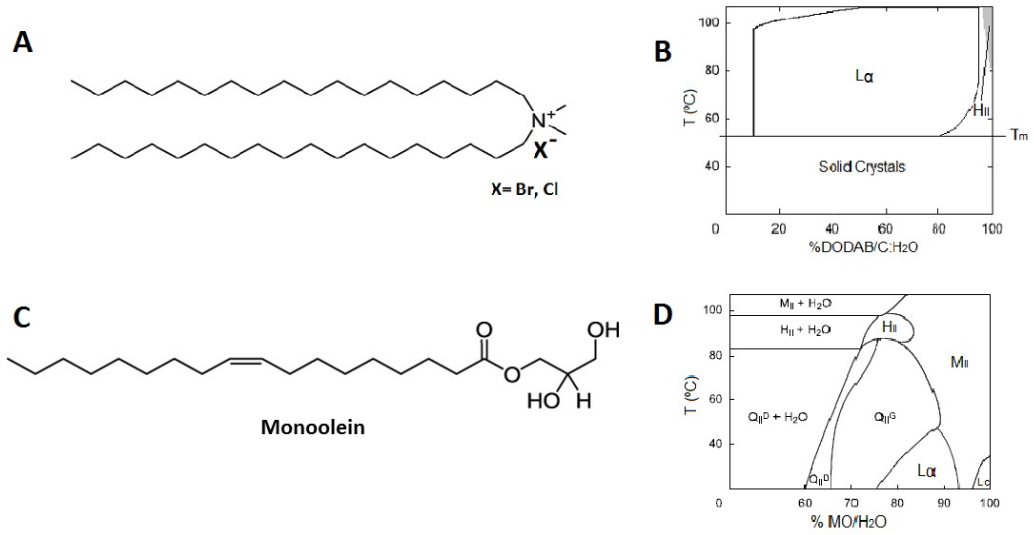

Fig. (3). Representation of the chemical structure of dioctadecyldimethylammonium lipids (**A**) and phase diagram of DODAB (adapted from [170]) (**B**), chemical structure of monoolein (MO) (**C**) and phase diagram of MO (**D**) 157.171. When X = Br dioctadecyldimethylammonium bromide (DODAB) is formed, while for X = Cl, dioctadecyldimethylammonium chloride (DODAC) is formed.

The presence of lipid and aqueous domains in MO cubic phases allows the solubilization of both hydrophilic, lipophilic and amphiphilic compounds, which makes this molecule very interesting for drug delivery purposes. Not only can it accommodate high payloads, but it also allows the combination of molecules with different hydrophilicity like nucleic acids and drugs in one single nanocarrier. Moreover, MO can help to overcome one of the most important bottlenecks in nucleic acids delivery – endosomal escape - due to its fusogenicity.

The combination of two lipids that form such different lyotropic phases provides a rich panel of structural organizations that can be tailored according to specific applications. A detailed physicochemical characterization of the DODAB:MO system revealed that two different aggregation structures could be obtained, depending on the cationic:neutral lipid molar fraction [159] (Fig. **4**).

When DODAB is in excess (χDODAB $\geq$ 0.5), bilayer-based structures are predominantly observed, with size and fluidity dependent on the exact molar fraction and temperature. When χDODAB < 0.5, densely packed cubic-oriented particles are mainly observed. The same dual behavior was seen for pDNA lipoplexes [98, 99]: for χDODAB $\geq$ 0.5, multilamellar structures of lipid bilayers alternating with DNA monolayers were formed; for χDODAB < 0.5, there is the formation of high-curvature zones, where lipid bilayers cross each other with DNA monolayers stacked between them. These are presumed to be MO-rich domains alternating with DODAB-rich domains (with multilamellar organization), supporting the existence of inverted structures in the liposomal system.

The formulation DODAB:MO was first proposed as a non-viral gene delivery system in 2010 by Real Oliveira *et al.* [174], and more recently DODAB:MO and DODAC:MO liposomes were also validated for siRNA delivery [100, 116, 164] MO-based formulations form small sized siRNA-lipoplexes with positive surface charge, were highly internalized by the cells and able to silence expression of the model protein eGFP [100]. Nevertheless, the different DODAX:MO membrane properties strongly influenced liposomes' bio-interface, defining stability and interaction with cellular models (Fig. **4**).

DODAB:MO and DODAC:MO liposomes present different lipid organization driven by the presence of different counter-ions on the cationic lipids: bromide and chloride define the mode MO is integrated into DODAB and DODAC systems [100]. For formulations prepared at cationic:neutral lipid molar fraction (2:1), a homogeneous integration of MO into DODAC bilayers is observed, inducing higher disorder to this system comparing to what is observed for the DODAB system. The higher membrane packing determined for DODAB:MO (2:1) formulation is explained by the formation of DODAB-rich and MO-rich domains, which results in a lower disturbing effect of MO when compared to when it is homogeneously integrated throughout the membrane [100]. The effects of MO on the transition temperature and enthalpy of DODAB/C:MO liposomes also pointed to a more homogeneous incorporation into DODAC- than into DODAB-bilayers [100]. MO's more uniform distribution in DODAC:MO bilayers resulted in a slightly greater ability to fuse with model endosomal membranes when compared to DODAB:MO bilayers [100]. Nevertheless, this aspect also compromised stability and DODAC:MO (2:1) liposomes released almost all of their siRNA content after 1 h incubation in physiological conditions. This strongly reduced DODAC:MO lipoplexes efficiency, leading to lower cellular internalization and ability to silence the eGFP protein when compared to DODAB:MO liposomes (Fig. **4**).

For formulations prepared at cationic:neutral lipid molar fraction of (1:2), the counter-ions' effects on membrane organization are not so discernible, and DODAB:MO (1:2) and DODAC:MO (1:2) systems seem more alike, resulting in similar biological responses (Fig. **4**). The incorporation of a higher amount of MO into the bilayers disrupts DODAB-rich

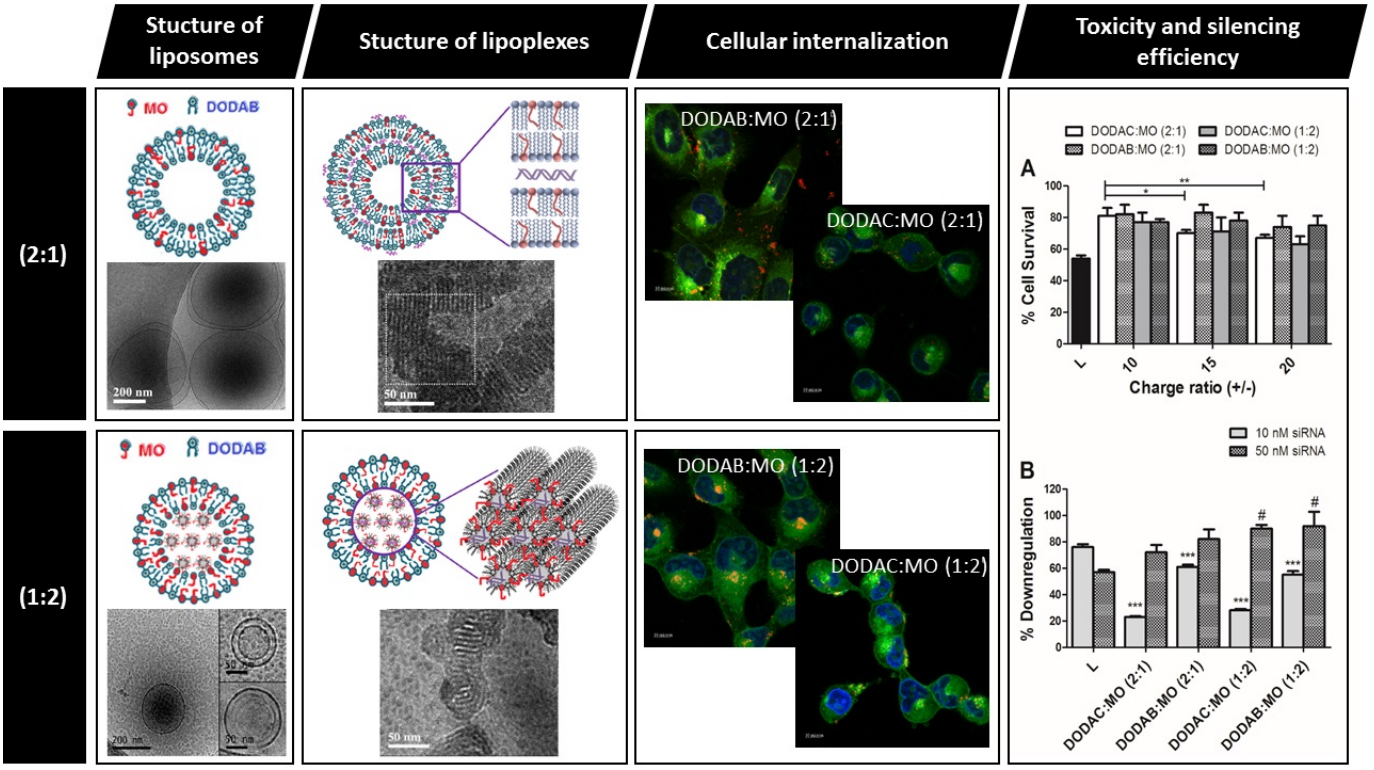

Fig. (4). Representation of the DODAX:MO liposomes and lipoplexes structures, lipoplexes cellular internalization, cytotoxicity and silencing efficiency, according to the cationic:neutral lipid molar fraction. In DODAX:MO (2:1) the lamellar phase is predominant and MO is within the DODAB lamellar phase. In DODAX:MO (1:2), MO is organized in inverted non-lamellar structures limited by DODAX lamellar phases. Adapted from [100, 158, 159, 162].

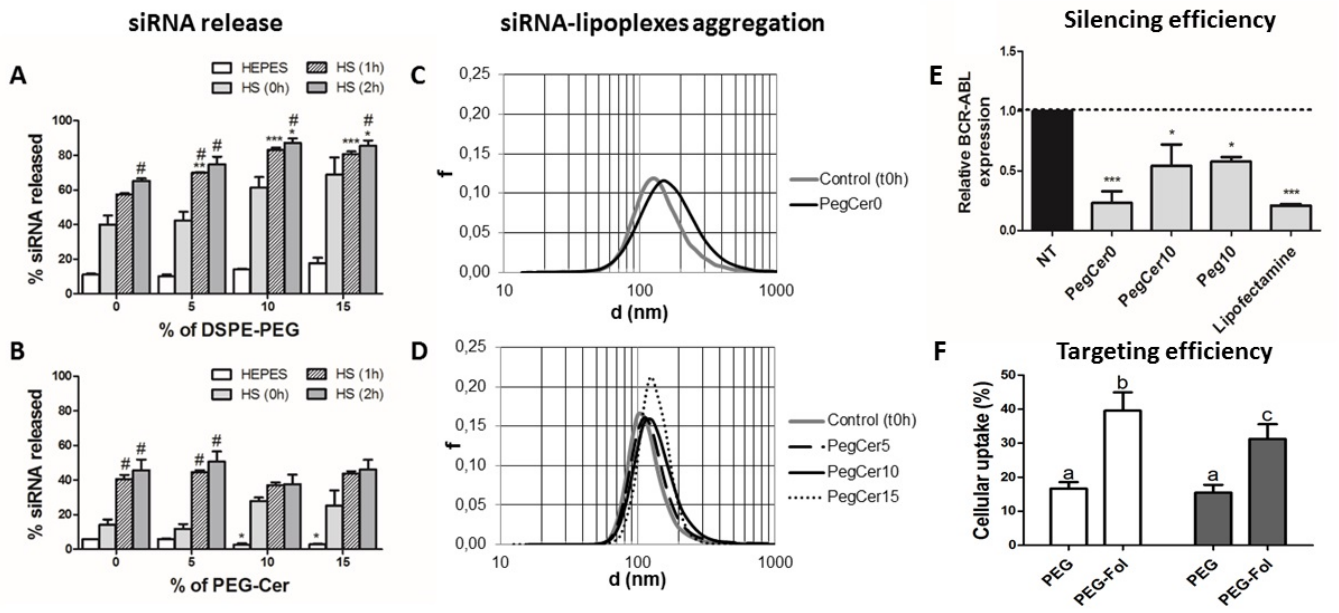

Fig. (5). Representative results obtained during the optimization of the DODAX:MO lipoplexes PEGylation for systemic siRNA administration. Results show the lipoplexes ability to retain siRNA after incubation in human serum (**A** and **B**), their ability to avoid aggregation (**C** and **D**), their capacity to downregulate BCR-ABL fusion gene (**E**), and finally cellular internalization after incorporation of folate as a targeting molecule (**F**). Adapted from 116.164.

domains. In fact, after a certain percentage, MO is probably excluded from the bilayer membranes, forming MO-rich inverted non-lamellar structures in the core of the liposomes (Fig. **4**).

DODAB:MO (2:1) was further optimized for systemic siRNA delivery (Fig. **5**). Different pegylation strategies were tested (pre-pegylation with DSPE-PEG and post-pegylation with PEG-ceramide), and the best system was validated in a leukemia cell line (K562 cell line) that expresses the pathogenic BCR-ABL fusion protein [116].

Both the PEGylation method and type of PEG moiety (DSPE-PEG or PEG-ceramide) influenced lipoplexes performance in physiological conditions [116]. Pre-PEGylation of DODAB:MO (2:1) siRNA-lipoplexes resulted in lower cellular internalization and silencing efficiency. The presence of DSPE-PEG decreased cellular internalization, probably affecting endosomal escape, which compromised siRNA-lipoplexes silencing efficiency outcome. The looser post-PEGylation with PEG-ceramide was beneficial in these aspects (siRNA protection, siRNA-lipoplexes internalization and silencing efficiency), but it still provided enough stability to avoid massive aggregation in human serum and decrease binding of serum proteins. PEG-ceramide nanocarriers better-retained siRNA in human serum (Fig. **5A** and **5B**), avoiding massive aggregation (Fig. **5C** and **5D**), and effectively silencing the BCR-ABL gene (Fig. **5E**), to an extent sufficient to affect K562 cell survival [116].

In a different work, a targeting approach was also followed to improve delivery efficiency (Fig. **5F**). Functionalized DODAC:MO:PEG-folate were prepared and found to be better internalized by FR-α positive breast cancer cells when compared with FR-α negative cells and to systems lacking PEG-folate [164].

These works show the potential of MO-based liposomes for siRNA delivery and reinforce the importance of a careful optimization and selection of lipid components before *in vivo* application. Nevertheless, a good clinical response is expected for DODAB:MO (2:1) siRNA-lipoplexes, since DODAB:MO liposomes were already demonstrated to be well tolerated by mice [160] in terms of toxicity and recognition by the immune system.

CONCLUSION

The fact that liposomes have been used in the clinic for more than 20 years, especially for chemotherapy, together with the simplicity by which they form complexes with negatively charged nucleic acid molecules, makes lipid-based nanosystems as one of the most attractive siRNA delivery vehicles for gene therapy. Nevertheless, there are still important obstacles to overcome before RNAi constitutes an effective therapeutic approach, particularly related to the nucleic acids delivery process. Several strategies can be used to improve delivery efficiency, namely concerning nanocarrier stability in biological fluids, their ability to actively target specific cells, as well as to take advantage of different physiological stimuli to carry siRNA into the cytoplasm of the cells. This review covers some of these strategies, explaining the mechanisms behind each approach and giving examples of liposomal-based systems optimized to overcome specific barriers to the delivery process. An efficient delivery of siRNA to target cells will allow the use of lower doses and decrease off-target effects associated with RNAi therapeutic approaches. Understanding the relationship between physicochemical properties and biological effects is the first step in the development of a successful nanocarrier, as exemplified by the lessons learned from the liposomal system DODAB/C:MO.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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