

## Review

# Nanoparticle-Mediated Systemic Delivery of siRNA for Treatment of Cancers and Viral Infections

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## Abstract

RNA interference (RNAi) is an endogenous post-transcriptional gene regulatory mechanism, where non-coding, double-stranded RNA molecules interfere with the expression of certain genes in order to silence it. Since its discovery, this phenomenon has evolved as powerful technology to diagnose and treat diseases at cellular and molecular levels. With a lot of attention, short interfering RNA (siRNA) therapeutics has brought a great hope for treatment of various undruggable diseases, including genetic diseases, cancer, and resistant viral infections. However, the challenge of their systemic delivery and on how they are integrated to exhibit the desired properties and functions remains a key bottleneck for realizing its full potential. Nanoparticles are currently well known to exhibit a number of unique properties that could be strategically tailored into new advanced siRNA delivery systems. This review summarizes the various nanoparticulate systems developed so far in the literature for systemic delivery of siRNA, which include silica and silicon-based nanoparticles, metal and metal oxides nanoparticles, carbon nanotubes, graphene, dendrimers, polymers, cyclodextrins, lipids, hydrogels, and semiconductor nanocrystals. Challenges and barriers to the delivery of siRNA and the role of different nanoparticles to surmount these challenges are also included in the review.

Key words: Small interfering RNA; Nanoparticle; RNA interference; Delivery; Cancer; Virus.

## 1. Introduction

In 1998, Fire and Mello *et al.* discovered that potent and specific RNA interference can be induced by double-stranded RNA (dsRNA) in *Caenorhabditis elegans* [1]. Further investigations confirmed that similar dsRNA-triggered phenomena also exist in many other species such as plants [2], *Drosophila* [3], and mammalian cells [4, 5]. The past decade has witnessed an explosion of research on small regulatory RNAs that has yielded a basic understanding of many types of small RNAs in diverse eukaryotic species and the

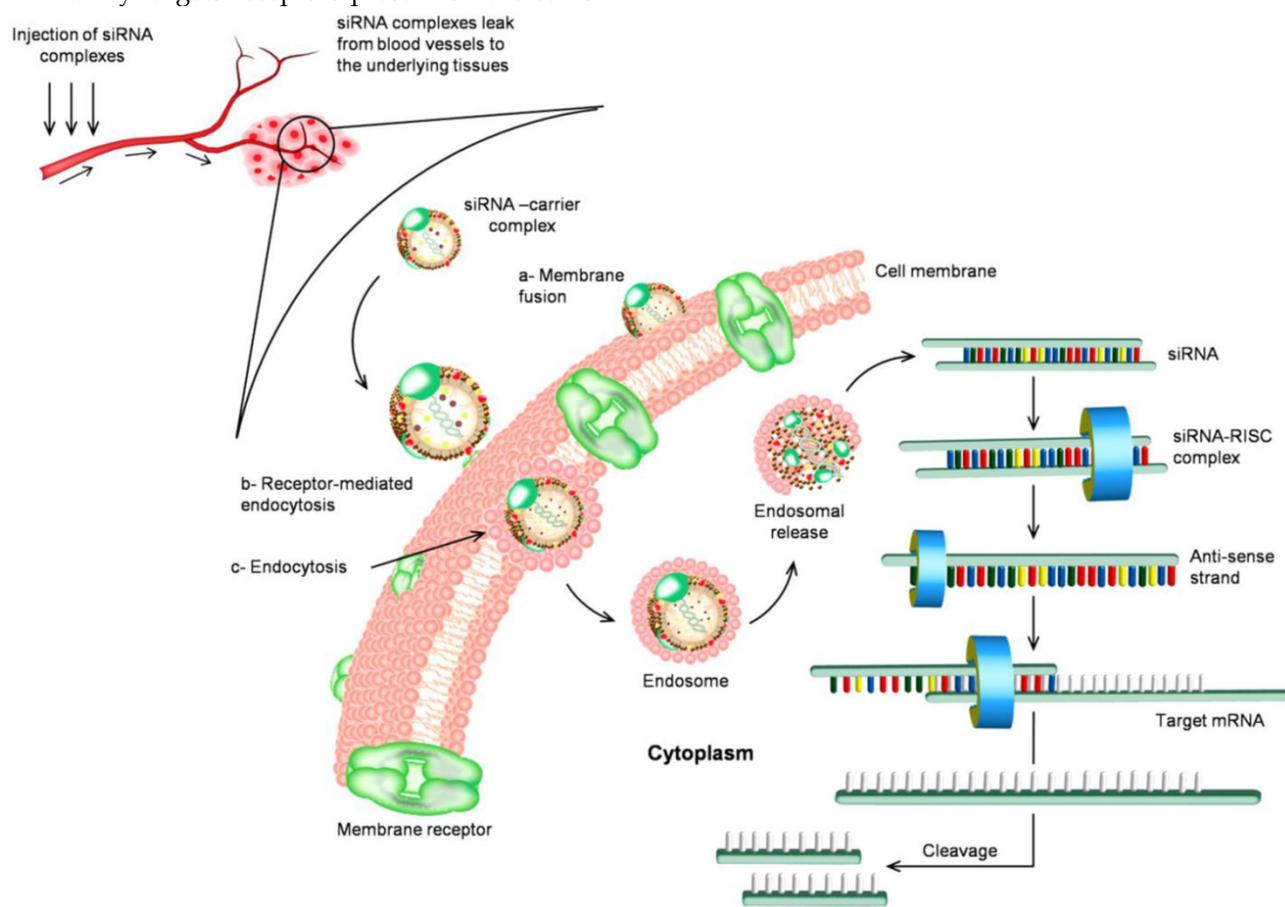
functions of key protein factors amidst the RNA silencing pathways. RNA silencing is recognized as a widespread mechanism of gene regulation in eukaryotes. The key machinery of RNAi pathway is that dsRNA molecules, experimentally or naturally occurring, can be recognized and cleaved into 21-23 nucleotide duplex termed small interfering RNA by Dicer homologues that have dsRNA binding domain and sRNaseIII-like enzyme activity, see Figure 1 [6, 7]. The siRNAs are incorporated into the multi-subunit ef-

factor complex called RNA-induced silencing complex (RISC), therefore activate the helicase activity leading to cleavage of the sense strand of siRNA [8, 9]. The remaining antisense strand recognizes the homologue region with base-pairing and degrading the target messenger RNA (mRNA) mediated by the Argonaute (Ago) family proteins with endonuclease activity, which is the catalytic core of active RISC, resulting in inhibition of gene expression [10-12]. RNAi technology has become a routine laboratory research tool for gene functional study and is making its way as a revolutionary class of therapeutics for treatment of cancers and different viral infections. This paper is focused primarily on synthetic siRNA and its delivery using nanoparticulate systems.

## 2. RNAi: a potential revolutionary therapeutics

The discovery of RNAi raises the possibility to explore new approaches for many incurable and difficult to treat diseases. The advantage of siRNA as therapeutics is that siRNA can target many undruggable genes. Other than antibody-based therapeutics that mainly targets receptors present on the cell sur-

face, only a very small number of targets, mostly kinases, have been validated for traditional small molecule drugs. In addition, it is found that diseases such as cancer, genes are often deregulated by high-level amplifications [13-15]. Such genes are particularly interesting as therapeutic targets for treatment of patients that are refractory to existing therapies. However, only very few of these genes, including FGFR1, IKBKB, ERBB2, etc., are considered druggable [13]. Some malignant diseases are known to be caused by multiple gene mutations, copy number change or epigenetic changes [16, 17]. Studies show that cancers are highly heterogeneous, resulting in each patient being "unique" and requiring personalized treatment. Furthermore, cancers initially sensitive to conventional chemotherapeutics often adapt tolerance to targeted therapy by gene mutations and other mechanisms [18]. A siRNA-based drug may target any mRNAs of interest, regardless of their cellular locations or structures of the translated proteins. Therefore, siRNA therapeutics shows promises to meet these challenges and has emerged as new generation bio-drugs under intensive investigation.



**Figure 1. The circulation routine of siRNA and the biological mechanism of RNAi *in vivo*.** siRNA is associated with nanoparticles either through chemical linkage via covalent bonds or through non-covalent interactions. Nanoparticles facilitate cellular uptake of siRNA cargo the process that commonly occurs through three main pathways (a) membrane fusion, (b) receptor-mediated endocytosis, and (c) direct endocytosis. The mechanism of internalized siRNA is controlled and initiated by the interaction with RNA-induced silencing complex (RISC). The remaining antisense strand recognizes the homologue region with base-pairing and degrading the target mRNA resulting in inhibition of gene expression.

Significant progress has been made for the development of siRNA based drugs since the discovery of RNAi machinery. Currently, several potential siRNA candidates are undergoing clinical trials summarized in Table 1, such as Bevasiranib, the first siRNA based drug in clinical trials, which targets vascular endothelial growth factor (VEGF) pathway for treatment of macular degeneration; ALN-RSV01 to treat virus respiratory diseases, and CALAA-01 to

silence ribonucleotide reductase subunit 2 (RRM2) gene, which is highly overexpressed in advanced cancers. Lipid-based carriers of siRNA therapeutics can target the liver in metabolic diseases and are being assessed in clinical trials for treatment of hypercholesterolemia [19]. Phase Ib clinical trial of the first-in-human mutation-targeted siRNA Td101 against an inherited skin disorder is now completed.

**Table 1.** List of siRNA-based drugs targeting different diseases were in clinical trials.

Disease	Target	Vehicle	Drug Name	Company	Status
<b>Cancers</b>					
Solid tumor	RRM2	Cyclodextrin, Transferrin, PEG	CALAA-01	Calando Pharmaceuticals	Terminated, Phase I
Advanced solid tumors	PKN3	Liposomes	Atu027	Silence Therapeutics AG	Completed, Phase I
Pancreatic ductal adenocarcinoma	Mutated KRAS oncogene	LODER polymer	siG12D LODER	Silenseed Ltd	Active, Phase I
Metastatic melanoma	LMP2, LMP7, and MECL1	Transfection	NCT00672542	Duke University	Completed, Phase I
Chronic myeloid leukemia	Fusion genes	SV40	SV40 vectors- carrying siRNA	Hadassah Medical Organization	?
<b>Virus infections</b>					
RSV	RSV nucleocapsid	Naked siRNA	ALN-RSV01	Alnylam Pharmaceuticals	Completed, Phase II
HBV	Pre gen./Pre-C, Pre-S1, Pre-S2/S, X	Plasmid DNA	NUC B1000	Nucleonics	Completed, Phase I
	HBV conserved sequences	DPC	ARC-520	Arrowhead Research Corporation	Recruiting, Phase II
HIV	HIV Tat protein, HIV TAR RNA, human CCR5	Lentivirus	pHIV7-shI-TARCCR5R Z	City of Hope Medical Center/Benitec	Terminated, Phase 0
HCV	miR-122	Naked LNA	SPC3649 (LNA)	Santaris Pharm	Completed, Phase II
EBOV	EBOV polymerase L, VP24, and VP35 regions	SNALP	TKM-100201	Tekmira Pharmaceuticals Corporation	Terminated, Phase I
<b>Other diseases</b>					
Hypercholesterolemia	APOB	SNALP	PRO-040201	Tekmira Pharmaceuticals Corporation	Terminated, Phase I
Pachyonychia Congenita	keratin K6a	Naked siRNA	TD101	TransDerm, Inc	Completed, Phase I
Delayed graft function kidney transplant	P53	Naked siRNA	I5NP	Quark Pharmaceuticals	Active, Phase I/II
Acute renal failure	P53	Naked siRNA	I5NP	Quark Pharmaceuticals	Terminated, Phase I
Glaucoma; ocular hypertension	ADRB2	Naked siRNA	SYL040012	Sylentis, S.A.	Completed, Phase I/II
Dry eye syndrome	TRPV1	Naked siRNA	SYL1001	Sylentis, S.A.	Recruiting, Phase II
Wet AMD	VEGF	Naked siRNA	Bevasiranib	Opko Health, Inc.	Terminated, Phase III
Diabetic AMD	VEGF	Naked siRNA	Bevasiranib	Opko Health, Inc.	Completed, Phase II
Chronic optic nerve atrophy	Caspase-2	Naked siRNA	QPI-1007	Quark Pharmaceuticals	Completed, Phase I
AMD; CNV	VEGFR	Naked siRNA	Sirna-027/ AGN211745	Allergan & Sirna Therapeutics Inc.	Completed, Phase II
AMD/DME	RTP801	Naked siRNA	PF-655	Quark Pharmaceuticals & Pfizer	Completed, Phase II

Resource: <http://clinicaltrials.gov>.

CNS, central nervous system; RSV, respiratory syncytial virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HCV, hepatitis C virus; EBOV, Ebola virus; AMD, Age-Related Macular Degeneration; CNV, choroidal neovascularization; RRM2, Ribonucleotide reductase subunit 2; PKN3, protein kinase n3; KRAS oncogene, Kirsten rat sarcoma viral oncogene; LMP2, large multifunctional peptidase 2; LMP7, large multifunctional peptidase 7; MECL1, multicatalytic endopeptidase complex-like-1; HIV Tat protein, HIV-1-trans-activating protein; HIV TAR, HIV trans-activation response; CCR5, human CC chemokine receptor 5; VP24, virus protein 24; VP35, virus protein 35; APOB, apolipoprotein B; ADRB2, beta-2 adrenergic receptor; TRPV1, transient receptor potential vanilloid 1; VEGF(R), Vascular endothelial growth factor (receptor); cysteine-aspartic proteases-2 (Caspase-2); PEG, polyethylene glycol; SV40,9 Simian virus 40; DPC, dynamic polyconjugate; SNALP, stable nucleic acid-lipid particle.

### 3. Challenges and barriers to the systemic delivery of siRNA

As a therapeutic strategy, RNAi offers several advantages over small-molecule drugs, as virtually all genes are susceptible to targeting by siRNA molecules. This advantage is, however, compromised by the challenges of safe and effective delivery of oligonucleotides to diseased tissues *in vivo*, summarized in Table 2. On the top of these challenges is the targeting specificity and stability of the administrated siRNA. Any inadvertent silencing of nontargeted genes “off-target effect” may lead to problems in interpretation of data and potential toxicity. The design and selection of potent siRNAs should be carefully performed. The basic parameters for choosing siRNAs involve consideration of internal repeated sequences, secondary structure, GC content, base preference at specific positions in the sense strand, and appropriate siRNA length (19–22 bps). 2'-O-methyl ribosyl group substitution at position 2 in the guide strand could reduce silencing of most off-target transcripts with complementarity to the siRNA guide [20, 21]. In addition, the stability remains major challenge to application of siRNA *in vivo*. The naked siRNAs face rapid degradation in the extracellular environment and are not efficiently internalized into cells. The RNA backbone contains ribose, which has a hydroxyl group in the 2' position of the pentose ring instead of a hydrogen atom [22], which makes the RNA backbone very susceptible to hydrolysis by serum nucleases that cleave along the phosphodiester backbone of nucleic acids. Chemical modifications of siRNA on the sugar-phosphate backbone such as 2'-fluoro and 4'-thio modifications, incorporation of locked nucleic acids, phosphorothioation, methyl phosphonation can increase stability of dsRNA under serum-containing conditions [23]. The use of siRNA delivery vehicles is also essential for practical siRNA-mediated silencing. The proper delivery vehicles would provide protection to siRNA from degradation in the serum during circulation. On the other hand, there are multiple mechanisms by which siRNA may be recognized by receptors of the innate immune system, including both endosomal Toll-like receptors and cytoplasmic receptors [24] that can lead to systemic inflammation *in vivo* through inducing the production of type I interferons and inflammatory cytokines. This challenge of RNA-induced immunostimulation may be reduced by proper siRNA design considerations, including choices of siRNA target sequence, chemical modifications to the RNA backbone, and the delivery formulation and method. So far, two cytoplasmic receptors that have long been known to recognize long dsRNA are protein kinase R [25] and 2'-5'-oligoadenylate

synthetase [24]. A variety of siRNA backbone modification chemistries have been investigated for their capacity to suppress immune activation while maintaining gene silencing activity. Making substitutions at uridine residues with 2'-fluoro, 2'-deoxy or 2'-O-methyl groups often reduces the immunostimulatory capacity of siRNA [26, 27]. The termini (ends) of a siRNA are major determinants of immune recognition. siRNA with added 3' overhangs such as UU, can reduce immune recognition and induce more efficiently gene silencing *in vivo* [28].

**Table 2.** Challenges and barriers to the systemic delivery of siRNA.

Challenge	Solution/approach
Specificity	Well design, optimize algorithm
Stability/degradation	Chemical modification, carrier
Immune response	Chemical modification
Clearance by RES systems	Encapsulation
Targeting/biodistribution	Receptor mediated
Endosomal escaping	pH responsive release
Dissociation from carrier	Cleavable polymers for siRNA
Toxicity	Reduce off-target effect, biocompatible and biodegradable carrier

RES: reticuloendothelial system.

The systemic delivery of siRNA is further hampered by many additional anatomical and physiological defensive barriers presented by the human body, and siRNA need to overcome before to reach its site of action. The first barrier includes the renal clearance through kidney or the entrapment in reticuloendothelial system (RES) that exists in the liver, spleen, lung and bone marrow. Many delivery systems larger than ~20 nm and less than ~100 nm in diameter are thought to be optimal for avoiding both renal and RES clearance and favorably improve the passive intra-tumoral delivery due to the unique features of leaky vasculature with capillary pore size of 100–800 nm and the absence of lymphatic drainage [29, 30]. Surface modifications using hydrophilic and flexible polyethylene glycol and other surfactant copolymers, e.g., poloxamers, polyethylene oxide, are also suggested to prepare stealth delivery carriers that can remain in the systemic circulation for a prolonged period of time [31, 32]. The second barrier is the endothelial lining and extracellular matrix barrier. For successful delivery, siRNA and its carriers must be readily to extravasate and move through the complex extracellular matrix to reach the diseased cells. The normal endothelial layer lining most of tissues allows the permeation of materials through abundant small pores of about 45 angstroms diameter and relatively scarce large pores of about 250 angstroms. This small pore system restricts the permeation of materials

larger than 4 or 5 nanometers [33, 34]. Only naked siRNA oligonucleotides or that are modified with molecular conjugates should be readily permeable, while other types of formulations may not be able to efficiently reach the underlying tissues. However, this represents an opportunity for nanocarriers to specifically deliver siRNA to certain types of tumors that have fenestrated endothelia. The third barrier is bio-distribution, and it can be conquered by several approaches particularly *via* targeted delivery of siRNA [35-37]. After siRNA is successfully delivered into the cells, how it can be efficiently released from endosome also presents a big challenge. If siRNA remains inside the endosome for too long, it will inevitably be degraded. Several methods aiming to enhance endosomal escape include conjugation with lipids or peptides, pH-sensitive lipoplexes, *etc* [38]. Figure 1 presents the circulation routine of siRNA complexes and the biological mechanism of RNAi *in vivo*.

#### 4. Nanoparticles in RNAi therapeutics

Nanoparticulate systems have emerged in last few years as an alternative material for advanced diagnostic and therapeutic applications in medicine. Compared to molecular medicine, nanoparticles offer many advantages that overcome a range of challenges and barriers summarized in the previous section, particularly, bioavailability and biodistribution of therapeutic agents. The first remarkable property of nanoparticles is their superior *in vivo* retention by decreasing enzymatic degradation and sequestration by phagocytes of the reticulo-endothelial systems. This is mostly attributed to their immunochemically inert surfaces in contact with the biological environment. Increased deposition to the diseased sites *via* compromised vasculatures in the phenomenon called enhanced permeability and retention effect also contributes to their improved deposit to diseased sites

and efficacy [37]. Various other properties of nanoparticles, including size, shape, surface charge, density, composition, and surface chemistry have been investigated [39]. The accumulated data presents interesting correlations among all these properties that led to a range of outcomes. Research has been focused on windows of optimal and controllable properties that guides the design and synthesis of nanoparticle formulations [40].

The properties that have been validated in chemotherapeutics are also exploited for siRNA packaging and delivery [41-46]. The effort began with stable association of siRNA molecules with the nanoparticles and their retention in circulation. Methods of conjugating siRNAs with other inert and biocompatible molecules, such as cholesterol and long-chain fatty acids have been reported [47, 48]. Complexation, encapsulation, and non-covalent association of siRNA into several nanoconstructs are reported. Success has been limited to date and there are still numerous challenges associated with many stages along the delivery process especially several recent reports on the toxicity and instability of some siRNA-nanoparticle complexes *in vivo* [49-51]. Different nanoparticle systems offer various advantages and disadvantages based on their composition, physical, and chemical characteristics, thus leading to a range of effectiveness when associated with siRNA. It has been found that unique challenges are associated with siRNA as many relatively successful technologies for oligonucleotides and DNA delivery did not translate to expected results for siRNA. An example is cationic lipid-gene complexes that are widely adapted for transfection and yet the release of siRNA during the intracellular pathways remains a major hurdle. Here we reviewed the main types of nanoparticle systems, and discuss their advantages, disadvantages, and the current state of development, summarized in Table 3 and Table 4.

**Table 3.** Types of nanoparticle systems used in siRNA delivery.

Nanoparticle system <sup>a</sup>				Target gene <sup>b</sup>	Silencing (%) <sup>c</sup>	Delivery route <sup>d</sup>	Ref
Type	Shape	Size	ζ potential				
<b>Silica, silicon-based nanoparticles</b>							
MSNPs	Spherical	~ 220 nm	ND	Bcl-2	~80%	<i>In vitro</i> , A2780/AD cells	[54]
	Spherical	>130 nm	29-38 mV	GFP	55-60%	<i>In vitro</i> , HEPA-1 cells	[55]
	Spherical	832 nm	25.4 mV	Pgp	80 or 90%	<i>In vitro</i> , KBV1 cells	[56]
	Spherical	>50 nm	ND		>50%	<i>In vivo</i> , i.v. injection in mice with MCF-7 cells	[57]
<b>Metal, metal oxides nanoparticles</b>							
MNPs	Irregular/spherical	≤156.2 nm	26-46 mV	GFP	54.8%	<i>In vitro</i> , PC-3 cells	[64]
	Spherical	70-150 nm	2±2 mV	GFP	21.5%	<i>In vitro</i> , SHEP cells	[65]
	Spherical	~ 60 nm	-2.6 mV	GFP	49.2%	<i>In vitro</i> , C6 glioma cells	[66]
	Spherical	75 nm	-30 mV	GFP	20%	<i>In vitro</i> , MDA-MB-435 and A549 cells	[67]
	Irregular/spherical	100 nm	-2-40 mV	Luc	30%	<i>In vitro</i> , 4T1 cells	[68]
	Irregular/spherical	120 nm	~ 40 mV	Luc	~75%	<i>In vitro</i> , 4T1 cells	[70]
	AuNPs	Spherical	100 nm	ND	GFP	73.5	<i>In vitro</i> , PC-3 cells

Nanoparticle system <sup>a</sup>				Target gene <sup>b</sup>	Silencing (%) <sup>c</sup>	Delivery route <sup>d</sup>	Ref	
Type	Shape	Size	ζ potential					
	Spherical	26.8 nm	ND	EGFP	72%	<i>In vitro</i> , K1 Cells	[77]	
	Spherical	~110 nm	30±9 mV	β-gal	48%	<i>In vitro</i> , SVR-bag4 cells	[79]	
	Spherical	18.2 nm	~12 mV	GFP	57.8%	<i>In vitro</i> , MDA-MB-435 cells	[80]	
	Rod	L = 46.5 nm D = 17.3 nm	ND	Galectin-1	~83%	<i>In vitro</i> , MDM cells	[81]	
<b>Carbon-based materials</b>								
CNTs	SWNTs	D = ~1-3 nm; L = ~200 nm	ND	CXCR4 /CD4	50-60%	<i>In vitro</i> , human T cells and PBMCs	[91]	
	SWNT	ND	ND	TERT	>80%	<i>In vitro</i> , HeLa cells	[92]	
	SWNTs	L = 50-300 nm	ND	Lamin A/C	>40 %	<i>In vitro</i> , HeLa cells	[93]	
	SWNTs	D = 1-1.4 nm L = 0.1-1 μm	ND	ERK	75%	<i>In vitro</i> , cardiomyocytes	[94]	
	SWNT	L = 50-300 nm	ND	cyclin A2	31%	<i>In vitro</i> , K562 cells	[95]	
	MWNT	D = 20-30 nm L = 0.5-2 μm	ND	siTOX	50%	<i>In vitro</i> , A549 cells <i>In vivo</i> , i.t. injection in mice with Calu 6 cells	[96]	
	MWNT	D = 9.5 nm; L = 1 μm	-64 mV	Luc	60-90%	<i>In vitro</i> , H1299 cells	[209]	
Graphene	Sheet-like	~ 200 nm	55.5 mV	Bcl-2	30-60%	<i>In vitro</i> , HeLa cells	[106]	
<b>Dendrimers</b>								
PAMAM	Spherical	72-165 nm	ND	siGLOred	ND	<i>In vitro</i> , A2780 cells	[112]	
	Spherical	~150 nm	ND	Bcl2	22-84%	<i>In vitro</i> , A2780 cells	[113, 114]	
	Spherical	120-180 nm	ND	Bcl2	70-50%			
<b>Polymers</b>								
Chitosan	Spherical	200 nm	ND	POSTN, FAK, PLXDC1	>51%	<i>In vitro</i> , SKOV3ip1, HeyA8, A2780, A2780ip2 and MOEC cells <i>In vivo</i> , i.v injection in mice	[123]	
	ND	ND	ND	Src/Fgr	81.8%	<i>In vitro</i> , SKOV3ip1 and HeyA8 cells <i>In vivo</i> , i.v. injection in mice	[124]	
	ND	<500 nm	51 mV	HPV16 E7	~31%	<i>In vitro</i> , CaSki cells	[125]	
	Spherical	400-500 nm	32-45 mV	HPV16 E6	~58%	<i>In vitro</i> , SiHa cells	[126]	
Dextran	Spherical	100-300 nm	-15.9 mV	Luc	60%	<i>In vitro</i> , PC-3 cells	[148]	
Polycations	ND	7-40 nm	ND	Luc	Up to 90%	<i>In vitro</i> , Neuro 2A cells	[136]	
				VEGF	~66%	<i>In vitro</i> , HepG2 cells	[143]	
				GFP	~80%			
	Spherical	100-300 nm	1.54 mV	RFP	80%	<i>In vitro</i> , B16F10 cells	[144]	
	ND	100 nm	ND	GFP	76±14%	<i>In vitro</i> , primary human glioblastoma cells	[145]	
	ND	200 nm	-2.7 mV	VEGF	ND	<i>In vitro</i> , PC-3, KB, HeLa, A2780, and A549 cells	[146]	
	Spherical	314 ±15 nm	-6.0 mV	RFP	ND	<i>In vitro</i> , B16F10 cells	[147]	
	ND	1-5 μM	ND	VEGF	60%	<i>In vitro</i> , A2780 cells	[149]	
	Micelles	ND	75-85 nm	>3.3 mV	PLK-1	ND	<i>In vitro</i> , OSRC-2 cells	[49]
		Star-shaped	56±3 nm	ND	EGFP	74±1.5%	<i>In vitro</i> , HeLa cells <i>In vivo</i> , i.v. injection in mice	[138]
<b>Cyclodextrin</b>								
	ND	~70 nm	ND	RRM2	77%	In clinical trial, i.v. injection, advanced solid tumors	[153]	
	ND	~60-100 nm	5-10 mV	RRM2	50%	<i>In vitro</i> , Neuro2A-Luc cells	[155]	
	ND	~70 nm	ND	RRM2	ND	<i>In vivo</i> , i.v. injection in monkeys	[157]	
	ND	~80 nm	10 mV	Luc	50%	<i>In vitro</i> , <i>In vivo</i> , Neuro2A-Luc cells	[158]	
<b>Lipid-based nanoparticles</b>								
Liposomes	Spherical	184 nm	42.9 mV	siTOX	50%	<i>In vitro</i> , A549 cells <i>In vivo</i> , i.t. injection in mice with Calu 6 cells	[96]	
	Spherical	190 nm	37.8 mV	Luc/ GFP	70%	<i>In vitro</i> , HT1080 cells <i>In vivo</i> , i.v. injection in mice	[160]	
	ND	80-100 nm	ND	HBV	40-50%	<i>In vitro</i> , Huh7 liver-derived cells <i>In vivo</i> , i.v. injection in mice	[172]	
	ND	ND	ND	EphA2	50%	<i>In vivo</i> , i.p. injection in mice	[176]	
	ND	ND	ND	IL-8	95%	<i>In vitro</i> , HeyA8 or SKOV3ip1 cells	[177]	
	Spherical	85-90 nm	ND	HCV IRES	32-48%	<i>In vitro</i> , HeyA8 and SKOV3ip1 cells <i>In vivo</i> , i.p. injection in mice	[177]	
	ND	81-85 nm	ND	ZEBOV Lpol., VP24, VP35	90%	<i>In vivo</i> , i.v. injection in mice	[178]	
	ND	81-85 nm	ND	ZEBOV Lpol., VP24, VP35	66%	<i>In vivo</i> , bolus intravenous infusion, monkeys	[179]	
	ND	ND	ND	MARV VP24, VP35, VP40, NP, Lpol	60-100%	<i>In vitro</i> , HepG2 cells <i>In vivo</i> , i.v. injection in mice	[180]	
	<b>Hydrogels</b>							
	Spherical	7-8 μm	20-30mV	EGFP	ND	<i>In vitro</i> , HUH7 cells	[182]	
	Spherical	Rz ~54 nm	<0.3 mV	EGFR	~35%	<i>In vitro</i> , Hey, BG-1 cells	[183]	
	ND	~100 nm	ND	EGFR	ND	<i>In vitro</i> , Hey cells	[184]	
	ND	ND	ND	GFP	80%	<i>In vitro</i> , HEK293 cells	[189]	

Nanoparticle system <sup>a</sup>		Target gene <sup>b</sup>	Silencing (%) <sup>c</sup>	Delivery route <sup>d</sup>	Ref	
Type	Shape	Size	ζ potential			
	ND	ND	ND	IL-10	80%	<i>In vitro</i> , APCs [190]
	ND	ND	ND	TG2	72-92%	<i>In vivo</i> , s.c. injection in mice [191] <i>In vitro</i> , A375SM and MDA-MB231 cells
	Irregular	200-500 nm	ND	GFP	66±8.2%	<i>In vitro</i> , HCT-116 cells [192]
	ND	6 nm	ND	mTOR	72%	<i>In vitro</i> , NIH 3T3 cells [193]
	Spherical	111±15 nm	36.7mV	GFP	ND	<i>In vitro</i> , MDA-MB-435 cell [194]
	ND	100-400 nm	ND	GFP/VEGF	53%	<i>In vitro</i> , HeLa cells and PC-3 cells [195]
<b>Semiconductor nanocrystals</b>						
	Ellipsoid	ND	ND	EGFP	29%	<i>In vitro</i> , HeLa cells [199]
	Spherical	16±1 nm	~21 mV	Her-2	65%	<i>In vitro</i> , SK-BR-3 cells [203]
	Spherical	17 nm	8.5 mV	Human CYPB	98.19	<i>In vitro</i> , MDA-MB-231 cells [204]
	Spherical	200 nm	ND	VEGF	29.7±3%	<i>In vitro</i> , PC-3 cells [205]

<sup>a</sup>Values are estimated for nanoparticle delivery system (NP and siRNA) by different techniques, including TEM, SEM, AFM, and DLS for shape and size, while zeta potential for surface charge. ND, not determined; MSNPs, mesoporous silica nanoparticle; MNP, magnetic nanoparticles; MNCs, magnetic nanoclusters; AuNPs, gold nanoparticles; CNTs, carbon nanotubes; PAMAM, polyamidoamine; SWNT, single-walled nanotubes; MWNT, multi-walled nanotubes; G4, generation 4; L, length; D, diameter; PEL, polyethylenimine; PLL, poly-L-lysine; Rz, z-average radii; PEG, poly(ethylene glycol); PPD, PEG-peptide-dioleoylphosphatidyl ethanolamine;

<sup>b</sup>Bcl-2, B-cell lymphoma 2; Pgp, P-glycoprotein; GFP, green fluorescent protein; Luc, luciferase; EGFP, enhanced green fluorescent protein; β-gal, β-galactosidase; ERK, extracellular regulated kinase; CXCR4, chemokine receptor type 4; CD4, cluster of differentiation 4; TERT, telomerase reverse transcriptase; VEGF, vascular endothelial growth factor; RFP, red fluorescent protein; periostin; FAK, focal adhesion kinase; PLXDC1, plexin domain containing 1; Src, Src family tyrosine kinase; EphA2, EPH receptor A2; HPV16 E7, human papilloma virus 16 E7 gene; PLK-1, polo-like kinase 1; RRM2, ribonucleotide reductase M2; EphA2, EPH receptor A2; HBV, hepatitis B virus; HCV IRES, hepatitis C virus internal ribosome entry site; ZEBOV, Zaire ebolavirus; Lpol, L polymerase; VP, viral protein; MARV, Marburg virus; IL-8, interleukin 8; IL-10, interleukin 10; TG2, tissue transglutaminase; mTOR, mammalian target of rapamycin; Her-2, human epidermal growth factor receptor 2; Human CYPB, human cyclophilin B.

<sup>c</sup>from its control value

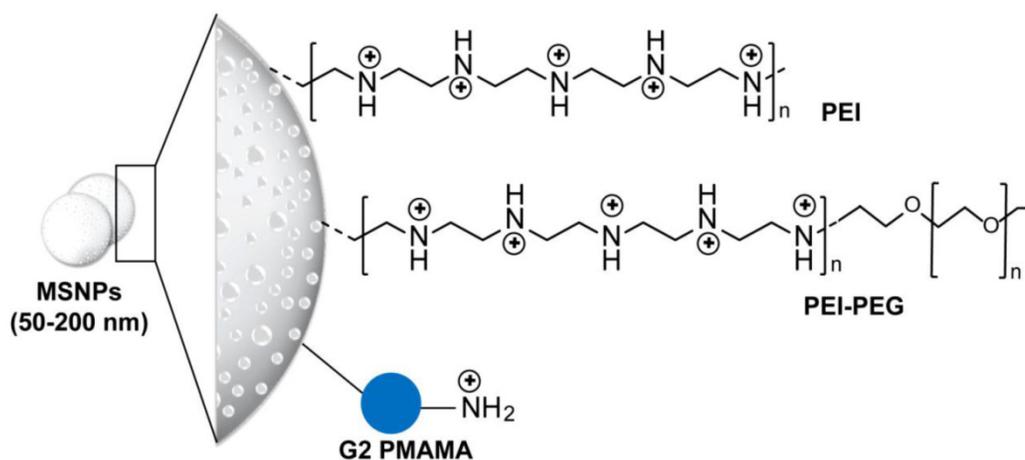
<sup>d</sup>SVR bag4 is an endothelial cell line; A2780, A2780/AD, A2780ip2, SKOV3ip1, HeyA8 and BG-1 are human ovarian cancer cell lines; KBV1 is human epidermoid carcinoma; SK-BR-3, MCF-7, MDA-MB-435 and MDA-MB231 are human breast cancer cell lines; HEPA-1 is mouse liver cancer cell line; HeLa, CaSki and SiHa cells are human cervical cancer cell line; SHEP is human neuroblastoma cell line; A549, Calu 6 and H1299 are lung cancer cell lines; 4T1 is murine breast cancer cell line; PC-3, human prostate carcinoma cell line; K1 is a Chinese hamster ovary cell line; MDM is human monocyte-derived macrophage cells; PBMCs are peripheral blood mononucleated cells; K562 is chronic myelogenous leukemia cell line; HUH7 and HepG2 are human liver cancer cell lines; B16F10 is murine melanoma cell line; KB is human oral cavity epidermal cancer cell line; MOEC is mouse endothelial cell line; Neuro2A is mouse neural crest-derived cell line; HT1080, human fibrosarcoma cell line; HCT-116 is colonic epithelial cell line; HEK293, human embryonic kidney 293 cell line; APCs are antigen-presenting cells; A375SM is metastatic human melanoma cell line; NIH 3T3 is mouse embryonic fibroblast cells; i.v., intravenous; i.t., intratumorally; i.p., intraperitoneal; s.c., subcutaneous.

**Table 4.** Advantages and disadvantages of different nanoparticles to siRNA delivery.

Nanoparticle	Advantages	Disadvantages	Ref
MSNPs	Large surface area Stability Biocompatibility Tuned biodegradability Controllable porosity, allows multifunctional and sequential delivery Surface reactivity, and easy functionalization	<i>In vivo</i> toxicity	[52, 53, 58-63]
MNPs	Large surface area Small in size, allows longer circulation and improved tissue penetration High magnetization for remotely-controlled and fast delivery Controlled clustering Potential for multimodal applications (e.g. targeting, diagnostic, and therapy)	Poor colloidal stability Limited biocompatibility and cytotoxicity Non-biodegradability	[64, 71-73, 83]
AuNPs	Large surface area Easy synthesis, modification and bioconjugation Rational stability and biocompatibility Potential for multimodal applications (e.g. targeting, diagnostic, and therapy)	High cost of large scale production Stickiness and limited <i>in vivo</i> stability Non-biodegradability	[74, 82, 83]
CNTs	Large surface area Ultra-high functionalization and loading capacities High penetration capacity to biological barriers Ultimate electrical and thermal conductivities and mechanical strength Encapsulation and storage functions in delivery of molecules	Difficulty in production and handling Non-biodegradability Unresolved toxic properties	[84-86, 108]
Graphene	Large surface area Facile synthesis Colloidal stability Easy surface functionalization Good electrical and mechanical properties	High cost and difficulty of massive production Non-biodegradability Increased biosafety concerns	[100, 102, 107]
Dendrimers	Very precise size and shape controllability Water solubility and biocompatibility Elicit negligible immune response Easy electrostatic interaction with nucleic acid and protection from nuclease activity	Non-specific cytotoxicity Limited release of the associated bio-actives Rapid clearance	[109, 110, 115]
Polymers	Easy and cheap production Fine tenability of structure and properties Simplicity for loading and complexation with nucleic acid by electrostatic interaction It can be tailored for a wide range of molecular weights Natural polymers are nontoxic, biocompatible, and biodegradable.	Limited stability Synthetic polymers cause cellular necrosis and apoptosis	[23, 50, 117, 125, 144]
Cyclodextrin	Low toxicity Act as molecular containers that can help to enhance biological properties of loaded molecules Lack of immune stimulation	High cost production Concerns regarding their safety and limited solubility	[150, 151, 159]

Liposomes	In vivo stability due to the absence of enzyme degradation in humans		
	Biocompatibility Rapid cellular uptake The flexibility of synthesis, modification and formulation Targeting and controlled release Easy conjugation and functionalization with components such as targeting, contrast agents, probes, and fluorophores.	High production cost Limited instability and leakage of loaded materials Low solubility Rapid clearance	[160, 161, 175]
Hydrogels	Tenable synthesis and physicochemical properties Selective surface-functionalization High degree of porosity and high loading capacity Controlled and sustained release into the target tissues Biocompatibility and biodegradability	High cost production Instability	[181, 185, 188]
	Quantum dots	Size and structure-based tunable emission High molar extinction coefficient High photo and chemical stability Potential for synergistic diagnostic and therapeutic applications	Potential toxicity Particle aggregation, degradation and removal

MSNPs, mesoporous silica nanoparticles; MNPs, magnetic nanoparticles; MNCs, magnetic nanoclusters; AuNPs, gold nanoparticles; CNTs, carbon nanotubes.

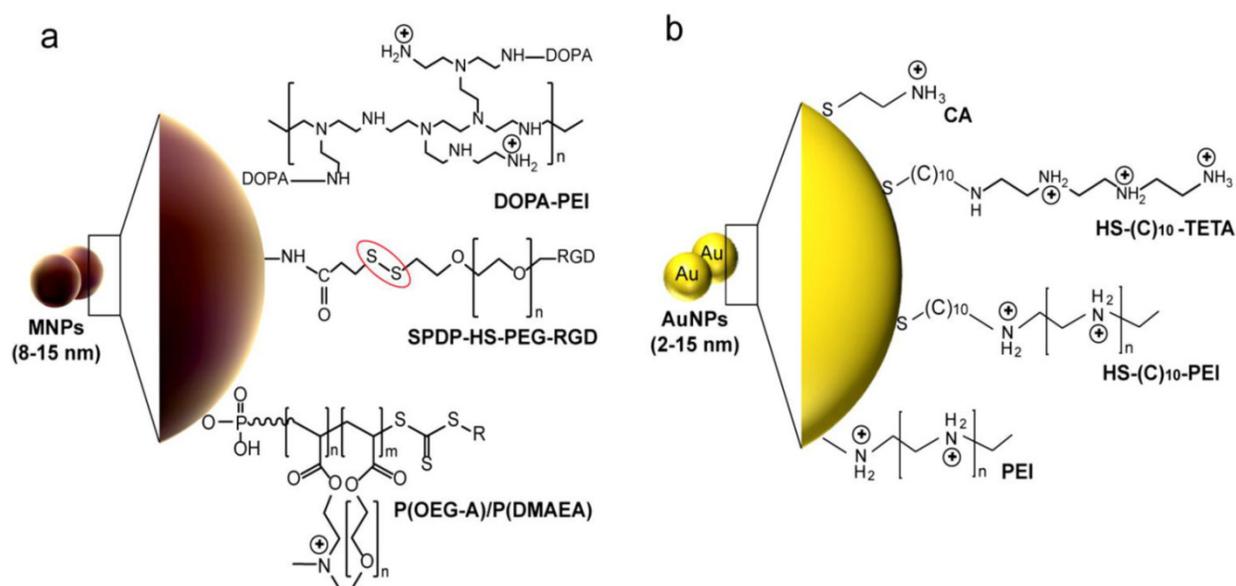


**Figure 2. Structure of mesoporous silica nanoparticles applied for delivery of siRNA.** Phosphonate-modified mesoporous silica nanoparticles (MSNPs) are further modified using electrostatic attachment of a polyethylenimine (PEI) polymer, which was used for subsequent covalent attachment of poly(ethylene glycol) (PEG) or electrostatic-based loading of siRNA [56, 57]. In addition, 3-isocyanatopropyltriethoxysilane (ICP) is utilized for the preparation of generation 2 (G2) amine-terminated polyamidoamine (PAMAM) dendrimers-modified MSNPs. The terminal amino groups of PAMAM are covalently reacted with ICP functional groups on the MSN surface. These dendrimer-modified MSNPs can efficiently complex with siRNAs through electrostatic interaction [54].

#### 4.1 Silica and silicon-based nanoparticles

Silica and silicon-based delivery systems have emerged in drug delivery applications due to their controllability in nanopore formation and surface modifications. This enabled a multi-component and multi-functional bio-delivery systems that can possibly perform a sequence of functions at various stages of the delivery route [52, 53]. Several spherical mesoporous silica nanoparticles (MSNPs) with core size range from 50 to 200 nm and modified with polyethylenimine (PEI) or PEI-polyethylene glycol (PEG) or polyamidoamine (PAMAM) are applied for siRNA, see Figure 2 for more details on particle structure and surface modifications. This surface layer of cationic polymer or quaternized dendrimers was added to permit the electrostatic loading of the siRNA, while the presence of protective polymer of PEG was showed to improve the cellular uptake and delivery of MSNPs-siRNA conjugates. For example, PAMAM dendrimer-modified MSNPs carrying both doxorubicin (Dox) and B-cell lymphoma 2 (Bcl-2) gene silenc-

ing siRNA has been demonstrated to enhance the cytotoxicity of Dox by 64-fold due to the suppression of membrane pumps that mediate drug resistance [54]. Cationic PEI-MSNPs were particularly efficient for transducing mouse hepatoma HEPA-1 cells with a siRNA construct that was capable of knocking down green fluorescent protein (GFP) expression with minimal or no cytotoxicity [55]. Recently, Meng *et al.* have demonstrated that encapsulation of Dox by mesoporous silica nanoparticles could enhance and restore Dox sensitivity in drug resistant cancer cell lines by codelivery of a siRNA that knockdown P-glycoprotein drug exporter [56, 57]. It is worth noting that the application of silica nanoparticles are additionally promoted by its intrinsic biocompatibility [55, 58], biodegradability [59], and its efficient bio-elimination *in vivo* [60, 61]. However, several observations indicating disadvantageous metabolic changes and toxicity especially *in vivo* have pronouncedly been recorded. These observations were mainly correlated to the particle surface functionalization and the presence of active silanol groups [62, 63].



**Figure 3. Common surface modifications of metal and metal oxides-based nanoparticles reported in siRNA delivery.** (a) Magnetic nanoparticles (MNPs). Branched PEI polymer is utilized for preparation of magnetite nanoparticle clusters (MNCs) [64, 68]. PEI is directly reacted with MNPs to form stable nanocomplexes or indirectly anchored through the covalent binding to 3,4-dihydroxy-L-phenylalanine (DOPA). N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) is applied to activate bovine serum albumin (BSA)-MnMEIO nanoparticles. The SPDP-activated MnMEIO nanoparticles are then treated with thiolated poly(ethylene glycol) (PEG) functionalized with a cyclic Arg-Gly-Asp (RGD) peptide, or thiolated siRNA at the distal end (red circle) [67]. MNPs can be coated with two different polymers of poly(oligoethylene glycol) methyl ether acrylate (P(OEG-A)) and poly(dimethylaminoethyl acrylate) (P(DMAEA)). P(DMAEA) forms an internal layer with a slight positive charge for electrostatic immobilization of siRNA and P(OEG-A) forms an outer antifouling shell for long circulation *in vivo* [65]. (b) Gold nanoparticles (AuNPs). Amine functionalized AuNPs are directly prepared by surface modification with cystamine hydrochloride (CA) that carries amine-derived positive charge [75]. AuNPs modification with a positive charged polymer layer of PEI [77] or triethylenetetramine (TETA) [79] are reported for the simple electrostatic conjugation and delivery of siRNA. These modifications are commonly accomplished with Au-thiol non-covalent binding through thiol-terminated linkers such as 11-mercaptoundecanoic acid [75, 79].

## 4.2 Metal and metal oxides nanoparticles

Different kinds of metal nanoparticles have so far been tested for gene delivery purpose. Among them, magnetic nanoparticles (MNPs) have received increasing attentions owing to their unprecedented capabilities to achieve remotely-controlled transfection by applying an external magnetic field. This process of magnetic-based transfection, referred as magnetofection, is one of the most powerful tools recently reported to enhance gene transfection efficiency. It reduces transfection time *via* magnetic field and force cellular transport processes [64]. Generally, magnetic nanoparticle intracellular siRNA delivery systems comprise surface polymer layers in order to enhance the siRNA-particle complex formation and their total cellular uptake (Figure 3a). Iron oxide nanoparticles modified with two different polymers, *i.e.* poly(oligoethylene glycol) methyl ether acrylate and poly(dimethylaminoethyl acrylate) were efficiently applied in siRNA transfection into human neuroblastoma SHEP cells both in the presence and in the absence of a magnetic field [65]. This potential magnetic nanoparticle-based transfection has further been expanded into several multifunctional systems suitable for concurrent targeting, diagnostic and therapeutic applications. A nanovector for siRNA comprised of iron oxide magnetic nanoparticle core coated with PEI, siRNA, and chlorotoxin and possesses dual tar-

geting specificity and dual therapeutic effects has also been described for targeted cancer imaging and therapy [66]. Lee *et al.* developed a cancer-cell-targeted gene silencing system prepared from a magnetic-nanoparticle platform on which a fluorescent dye, siRNA, and a Arg-Gly-Asp (RGD)-peptide targeting moiety were attached [67]. These multifunctional magnetic nanovectors exhibited significant targeting, intracellular uptake and gene silencing effects in cancer cells. On the other hand, magnetic nanocrystal clusters (PMNCs)-cross-linked with PEI were developed to magnetically trigger intracellular delivery of siRNA [64, 68, 69]. Such new nanocomposite carriers are highly controlled clustering structures with multi-nanoparticles cores surrounded by a shell of alkylated PEI [64, 68, 70]. The clustering approach in these composites greatly improves their magnetic responsiveness compared to individual nanoparticles, which provide more enhanced, site-specific and retained release of siRNA [64]. In addition, the presence of PEI shells, with favorable transfection properties of PEI, enables rapid and improved conjugation and transduction efficiency of siRNA molecules. PEI is highly cationic helping condensed electrostatic conjugation of anionic siRNA forming compact polyelectrolyte complexes with siRNA. Also, its role in nucleic acid protection and rapid endocytosis enhances the total delivery efficiency of these systems [71-73]. Park *et al.* reported

that the gene silencing effect of siRNA mediated clustering magnetic nanocrystals was considerably higher than that of siRNA complexed with single magnetic nanoparticles [64]. Recently, Chen group validated both magnetic iron oxide [68] and magnesium oxide [70] nanoparticles-based cluster systems for the delivery of siRNA at *in vitro* and *in vivo* levels. Their initial results for luciferase siRNA delivery to luciferase transfected 4T1 murine breast cancer (fLuc-4T1) cells, and to a fLuc-4T1 xenograft model concluded the efficiency of the systems to cause significant knockdown of luciferase and demonstrated efficient protection of the loaded siRNA [68, 70].

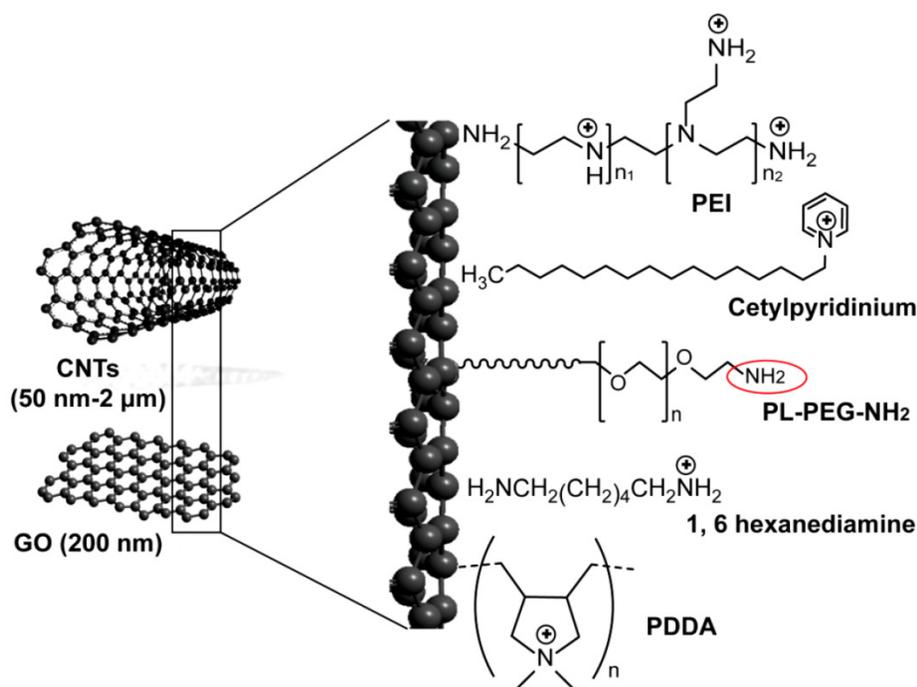
Other classes of metal nanoparticles for delivering siRNA have been proposed, including the use of gold nanoparticles (AuNPs). Due to their easy synthesis, amenable modification and bioconjugation, and rational stability and biocompatibility, AuNPs have recently been considered as excellent siRNA delivery systems [74]. Thus far, the strategies developed to deliver siRNA by AuNPs can be classified into two categories: 1) siRNA conjugated directly to the AuNP surface *via* a gold-thiol bond or electrostatic interaction, and 2) siRNA adhered to the AuNP surface modified with polymer layers [75-77]. As shown in Figure 3b, both strategies incorporate PEG, PEI or other passivating molecules to assist in stabilization and/or promoting endosomal escape into the cytoplasm [75, 77, 78]. The polyvalent siRNA/AuNPs conjugates showed a greater half-life and prolonged gene knockdown compared to free RNA duplexes [75-77, 79, 80]. Positively charged AuNPs electrostatically complexed with negatively charged siRNA-PEG conjugates were described by Lee *et al.* to successfully suppress the expression of GFP within the human prostate carcinoma cells [75]. PEI-capped AuNPs/siRNA that target endogenous cell-cycle kinase, an oncogene polo-like kinase 1 (PLK1), displayed a significant gene expression knockdown in MDA-MB-435s cells, whereas it was not obvious when the cells are treated with PLK1 siRNA using PEI as the carrier [80]. Elbakry *et al.* proposed layer-by-layer (LbL) assembly of the oppositely charged siRNA and PEI on the surface of AuNPs. The resultant LbL-coated nanoparticles were able to reduce the cellular enhanced green fluorescent protein (EGFP) production to about 28% in CHO-K1 cells [77]. Also, dendronized PEI AuNPs suppressed  $\beta$ -gal expression by ~50% with minimal toxicity [79]. Recently, Reynolds *et al.* utilized gold nanorods-galectin-1 siRNA complexes to inhibit the gene expression of galectin-1. The prepared siRNA complexes significantly attenuated the expression of methamphetamine potentiates galectin-1 in human monocyte derived macrophages and significantly reduced the effects of metham-

phetamine on human immunodeficiency virus type 1 (HIV-1) infection [81]. These applications are generally demonstrating the potentiality of metal nanoparticles for siRNA delivery. However, determining the cytotoxicity and stability of this class of nanoparticle is a recent challenge and more extensive *in vivo* studies are needed to address several concerns about biocompatibility, biodistribution and tissue accumulation [82, 83].

### 4.3 Carbon-based materials

Allotropic nanostructures of carbon nanotubes and graphene nanosheets are hosting enormous interests as shuttle nanovehicles for drug and siRNA delivery applications. Their extremely large surface area, with every atom exposed on its surface, allows for ultra-high functionalization and loading capacities. In addition to excellent material properties such as ultimate electrical and thermal conductivities and mechanical strength, enlarge and intensify their importance for a wide range of biomedical applications [84-87].

Carbon nanotubes (CNTs) are 1-dimensional hollow carbon nanostructures with a typical diameter of 1-2 nm and length ranges from 50 nm up to 1 cm. This tubular morphology renders them to act as cellular scale nano-needles that are easily and efficiently taken up by cells [88-90]. In the same time, they permit encapsulation of molecules and provide material storage functions as well as protection and controlled release of loaded molecules that are becoming increasingly important in modern delivery applications. Both forms of single-walled (SW) and multi-walled (MW) carbon nanotubes were modified and conjugated with siRNA to be employed against HIV infection and various cancers. Ammonium-functionalization of CNTs and surface modification with cationic polymers such as PEI and poly(diallyldimethylammonium)chloride (PDDA) are commonly applied for simple electrostatic loading of siRNA to their surface (Figure 4). SWCNTs grafted with siRNA showed effective silencing activity by 50-60% to both cluster of differentiation 4 (CD4) receptor and chemokine receptor type 4 (CXCR4) co-receptor of T cells, which are required for HIV entry into human and infection [91]. Zhang *et al.* proposed SWCNTs as an efficient vector for siRNA to suppress murine telomerase reverse transcriptase expression in murine tumor cells on both *in vitro* and *in vivo* levels [92]. This approach also has been successfully used in the control of key signaling regulators in cancer cells, including suppressors of cytokine signaling 1, cyclin A, and extracellular signal-regulated kinase harnessing the potentiality of SWCNTs to deliver siRNA for cancer therapy [93-95].



**Figure 4. Carbon-based nanoparticles for delivery of siRNA.** Applied carbon nanostructures include multiwalled- and singlewalled carbon nanotubes (CNTs) and single-atom-thick sheets of graphene oxide (GO). Ammonium-functionalized CNTs are prepared through non-covalent binding with cetylpyridinium chloride [209], tert-butyl-n-(6-aminoethyl)carbamate [92], and 1, 6 hexanediamine [95]. Polymer coated-CNTs and GO are prepared through the addition of different positively charged polymers to their surfaces, including polyethylenimine (PEI) [106, 209] and poly(diallyldimethylammonium)chloride (PDDA) [94]. Both ammonium-functionalized CNTs and polymer-coated CNTs are conjugated to siRNA by electrostatic interaction. CNTs can also be modified with non-covalent adsorption of phospholipid molecules carrying poly(ethylene glycol) (PL-PEG) chains with terminal amine or maleimide groups. This amine or maleimide terminal on the PL-PEG (red circle) permits the incorporation of thiolated siRNA by disulfide bond formation [91, 93].

In a recent study, 10–30% silencing activity of MWCNTs-siRNA conjugates have been reported in human lung cancer cell line H12991, and efficient suppression and prolonged survival of an animal model were previously reported in another study by Podesta *et al.* [96].

Graphene is a newly discovered two-dimensional carbon nanosheet structure [97-99]. It was described to have additional remarkable properties of facile synthesis, high water dispersibility, good colloidal stability, easily tunable surface functionalization, and good biocompatibility that highlight its promise as a novel nano-carrier for safe and efficient gene transfection [100-106]. Recently, its oxidized form of graphene oxide (GO) were used in a pioneering study by Zhang *et al.* after PEI modification step for sequential delivery of Bcl-2-targeted siRNA and the anticancer drug Dox (Figure 4). The results demonstrated an excellent and effective delivery of siRNA and chemical drugs using GO that significantly enhance the chemotherapy efficacy [106]. The major disadvantage of carbon based nanomaterials to siRNA is their non- biodegradability *in vivo* that can cause a range of adverse health effects. This point was highlighted in several studies and there is need for more future investigations [107, 108].

#### 4.4 Dendrimers

Dendrimers are spherical and symmetrically branched molecules in which branches radiating from a central core molecule and terminating at functional chemical groups depending on the building blocks, such as charged amine groups in PAMAM dendrimers (Figure 5a,b). Dendrimers have a very precise size and shape defined by the number of generations through controlled chemical synthesis [109, 110]. Dendrimers are water soluble, biocompatible, elicit negligible immune response, and have been successfully used in DNA and oligonucleotides complexation and delivery [111, 112]. Presumably, nucleic acids were stably incorporated into amine-terminated dendrimers *via* the interaction of negative charges on the phosphate on the backbone with the high positive charges of the amino groups, thus protected from nuclease activity. Minko group has previously designed and synthesized a surface-acetylated polyamidoamine generation four dendrimer (QPAMAMNHAc) in which surface amine groups are modified with acetyl group and internal tertiary nitrogen are quaternized (Figure 5c), as a nanocarrier for the targeted intracellular delivery of Bcl-2 siRNA in A2780 human ovarian cancer cells [112-114]. This type of dendrimer has low cytotoxicity and the internal cationic charges for complexation with siRNA to enhance siRNA protection, while the lesser degree of quaternization offers free tertiary amines for potential proton sponge effect [112, 114]. The same group later was able to achieve a targeting siRNA delivery by the addition of LHRH peptide (Figure 5c) that further improved internalization of siRNA by cancer cells and significantly enhanced its intracellular activity leading to a substantial silencing of a targeted Bcl-2 gene [114]. They further improved this design by the addition of PEG and poly-L-lysine (PLL) to form a triblock PAMAM-PEG-PLL nanocarrier, see Figure 5c. In this design, PEG acts as a linker connecting PLL and PAMAM dendrimers and renders nuclease stability for more protection of siRNA, while PLL enhances the electrostatic interaction with siRNA and also acts as penetration enhancer. This

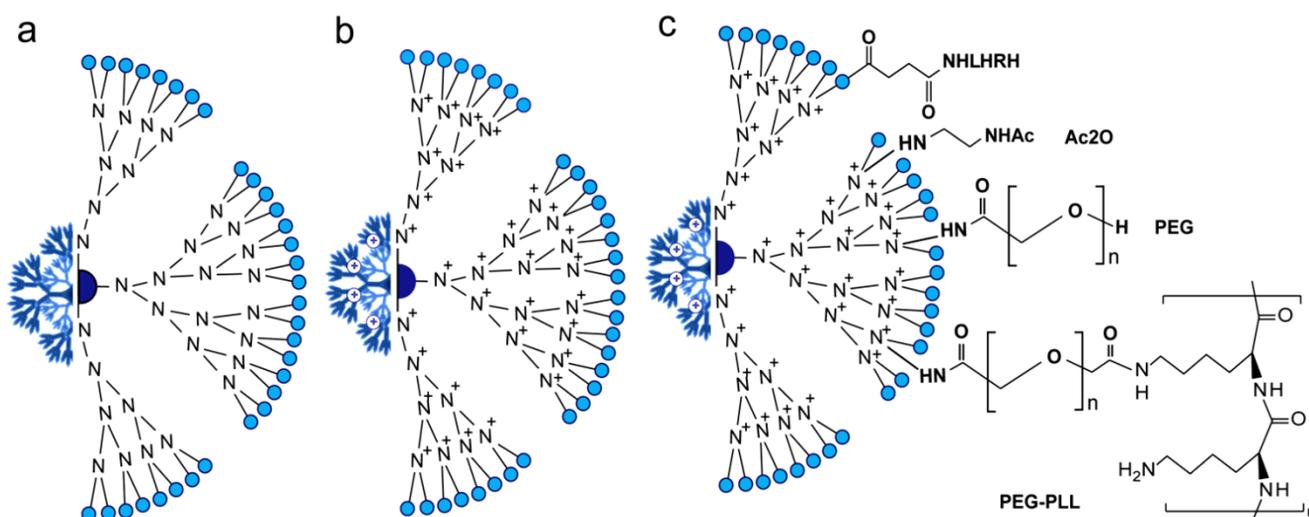
triblock carrier showed excellent stability and efficiency to suppress the target Bcl-2 gene [113]. Despite of these applications that generally introduce PAMAM dendrimers as highly efficient delivery vectors with many possible modifications, further addressing of several limitations, including non-specific cytotoxicity, release kinetics of the associated bio-actives and rapid clearance issues is required to initiate *in vivo* applications [109, 115].

#### 4.5 Polymers (polycations, micelles)

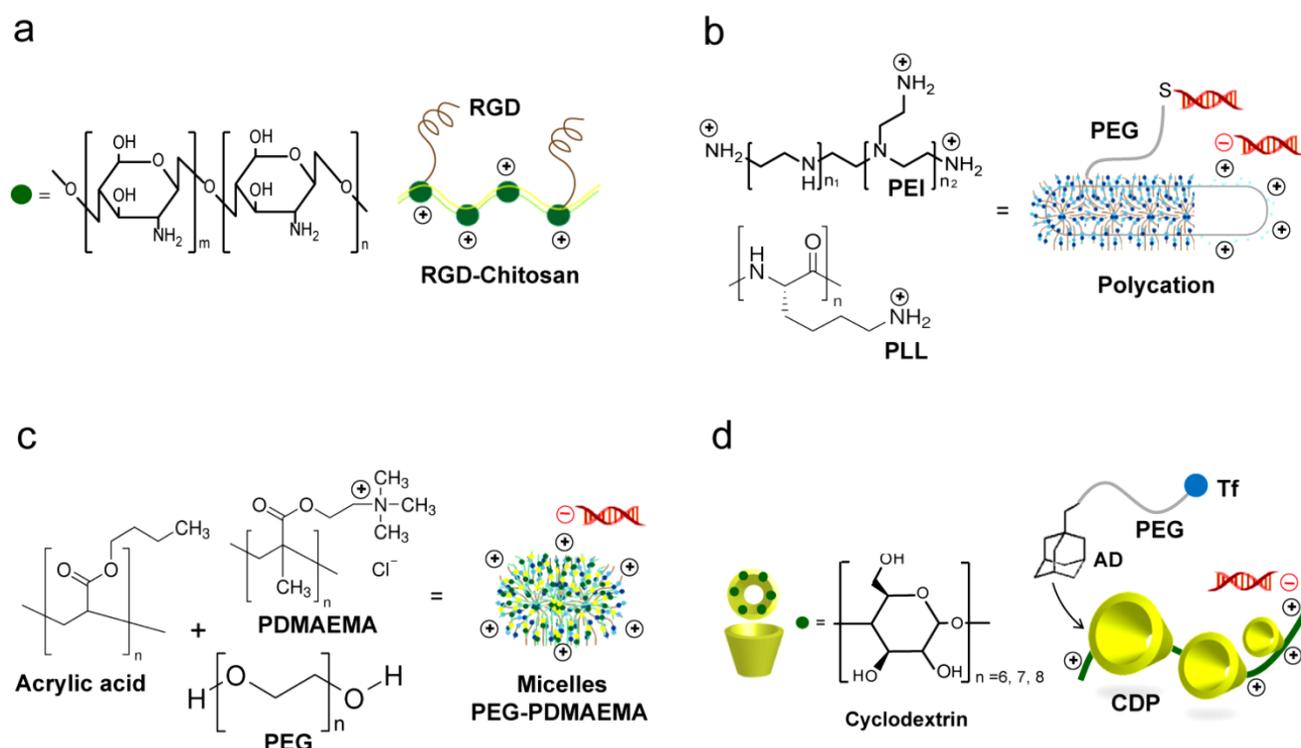
Polymers offer a great promise because their unit-by-unit construction allows for fine-tuning of the properties required for efficient transfection and release of nucleic acids. Their role as carriers for siRNA delivery was extensively reviewed [23, 116, 117], and currently there is a large number of natural and synthetic polymers is already investigated. Natural polymers, such as cationic polypeptides [118-121], chitosan (CH) [122-126], poly(lactic acid-co-glycolic acid) [127-129] and atelocollagen [130-133] have been used due to their nontoxic, biocompatible, and biodegradable properties. Among available natural polymers, chitosan, which is a linear polymer composed of  $\beta$ -(1-4)-2-amino-2-deoxy-D-glucopyranose units (Figure 6a), has been most commonly developed for *in vitro* and *in vivo* delivery of siRNA [122]. siRNA electrostatically incorporated into chitosan nanoparticles (siRNA/CH-NP) successfully delivered siRNAs against POSTN, FAK and Src family in animal model of ovarian cancer [123, 124]. Malhotra *et al.* described a novel method for preparing PEGylated chitosan nanoparticles by chemically modifying C6 position of its

repeating units with PEG and the amine groups at the C2 position of the chitosan were protected using phthalic anhydride. This conjugated nanoparticle delivered siRNA with no toxicity in neural cells. Also, several chitosan-based siRNA formulations were proposed for silencing the expression of human papilloma virus (HPV) E6/E7 oncogenes in human cervical cells. The electrostatic complexation of chitosan with HPV16 E7 siRNA efficiently helped to deliver siRNA into CaSki cervical carcinoma cells and was observed to induce apoptosis of cells [125]. A PEI-introduced chitosan-shell/ poly methyl methacrylate-core nanoparticle was also able to efficiently suppress the expression of HVP oncogene in the SiHa human cervical cancer cells [126].

On the other hand, synthetic polymers applied to siRNA delivery include branched or linear PEI, PLL (two of the most common synthetic polycations), PEG, dimethylaminoethyl methacrylate, polyfluorene and cyclodextrin-based polycations (CDP). Among those synthetic polymers, PEIs, is a class of extensively used cationic polymers, with a wide range of molecular weights and many protonable amino groups, leading to a high cationic charge density at physiological pH, see Figure 6b. However, synthetic polymers like PEI and PLL can trigger cell death through necrosis and apoptosis [134] causing toxicity in living cells. This toxicity is also associated with higher polymer molecular weights and increasing branching. The effect can be reduced by using block copolymers containing PEG and a polycation block [135].



**Figure 5. Composition of the PAMAM delivery systems of siRNA.** (a) Generation 4 PAMAM-OH dendrimer. (b) Internally quaternized PAMAM to form QPAMAM-OH dendrimer with inner cationic charges. PAMAM are frequently quaternized by methyl iodide (ICH<sub>3</sub>) and the terminal surface become very positive allowing the efficient electrostatic binding/loading of negatively charged backbone of siRNA. (c) QPAMAM with different surface modifications, including the addition of acetyl group by direct reaction with acetic anhydride (Ac<sub>2</sub>O) [112], poly(ethylene glycol) (PEG) and poly-L-lysine (PLL) [113], LHRH peptide [114]. This addition of polymer structures such as PEG and PLL is reported to enhance the surface positive charge and circulation of PAMAM nanoparticles. While, the conjugation to LHRH peptides confers targeting ability in PAMAM based delivery applications.



**Figure 6. Common polymer based siRNA delivery systems.** (a) Chitosan systems. The RGD modified chitosan nanoparticles (RGD-CH-NP) are reported for targeting siRNA delivery. siRNA/RGD-CH-NPs are prepared based on ionic gelation of anionic triphosphate and siRNA [123, 124]. (b) Polycations prepared from polyethylenimine (PEI) or poly-L-lysine (PLL). Their highly cationic nature facilitate strong electrostatic interactions with the negatively of siRNA. PEGylation of polycations with PEG modified with heterobifunctional N-succinimidyl 3-(2-pyridyldithio)propionate allow subsequent coupling of thiolated siRNA via the N-terminal cysteine [136]. (c) Micelle-based nanoparticles. Micelles are prepared from the triblock copolymer poly(ethylene glycol)-poly(n-butyl acrylate)-poly(2-(dimethylamino)ethyl methacrylate) (PEGnBA-PDMAEMA). The presence of amine groups on PDMAEMA allows the electrostatic complexation with siRNA [138]. (d) Cyclodextrin containing polymer (CDP). A commonly reported targeted CDP system is prepared through the addition of adamantane-polyethylene glycol-transferrin (AD-PEG-Tf) [153, 155, 157, 158].

The advantage of polymer-based delivery lies in the unparalleled simplicity for formulation of siRNA and polymer complexes. Cationic polymers with high positive charge usually self-assemble into uniform micellar, polycationic nanoparticle complexes with negatively charged siRNA upon simple mixing (Figure 6a-c), which is driven by electrostatic interactions [116, 136-138]. However, despite this advantage, the electrostatically formed polymer-siRNA complexes are often loose and unstable *in vivo*. This is due to the low charge density of short double stranded siRNA and the subsequent inability to form robust particles with cationic polymer carriers. Recently, several approaches have been proposed to improve the stability of the formed complexes through the use of polymerized siRNA with a high charge density and chemical crosslinking of the complexes [50, 139, 140]. Following these approaches, Mok *et al.* utilized the multimerized siRNA that possesses increased charge densities to produce more stable and compact polyelectrolyte complexes with less cytotoxic cationic carriers than naked siRNA [139]. While, Naito *et al.* utilized phenylboronate with its capability to form reversible covalent esters with 1,2- or 1,3-cis-diols exist on the ribose ring of nucleic acids to assist stabilizing siRNA-polyion complex micelles for intracellular

controlled release of siRNAs [49]. Currently, the disulfide-based crosslinking is particularly interesting approach widely reported to enhance the stability and delivery efficiency of many polymeric carriers. Based on the redox potential gradient existing between extracellular and intracellular environments, this strategy was further tailored to provide a capability for selective and controlled release of siRNA in the cytosolic space of target cell [141, 142]. Several studies have reported the utilization of bioreducible PEI and PEG-based carriers for siRNA. Chung *et al.* indicated that the reducible dimerized siRNAs showed far enhanced complexation behaviors with cationic polymers of PEI or PEG as compared to monomeric siRNA at the same ratio. The reducible siRNA dimeric conjugates showed greatly enhanced cellular uptake and gene silencing effects *in vitro*. These results were mainly explained to be due to the higher charge density and promoted chain flexibility of the dimerized siRNAs that provide more compact and stable siRNA complexes [143]. Also, a bioreducible PEI complexed with VEGF-siRNA was successful to reduce the level of VEGF protein in HepG2 human liver carcinoma cells *in vitro* and to inhibit HepG2 tumor growth in a xenograft mouse model. An important recent approach involved the combined use of bioreducible PEI

with a polymerized siRNA (poly-siRNA) was suggested for more enhanced delivery and gene silencing. The poly-siRNA/PEI complexes exhibited a superior intracellular uptake by murine melanoma cells accompanied with red fluorescent protein (RFP) gene silencing efficiency of about 80%, compared to untreated cells [144]. Furthermore, several other polymers, including poly( $\beta$ -amino ester)s [145], arginine conjugated poly(cystaminebisacrylamide-diaminohexane) [146], hyaluronic acid-graft-poly(dimethylaminoethyl ethacrylate) [147], and dextran [148] were investigated as bioreducible siRNA carrier for efficient gene silencing in various human cancer cell lines, see Table 3. The overall, results suggest their potentiality for the tumor-targeted delivery of siRNA. Most recently, this bioreducible polymer-based strategy for siRNA-carrier complex stabilization was combined with different delivery technologies such as microbubbles, and ultrasound to construct a multimodal delivery system, which successfully synergizes the advantages of each delivery approach to significantly enhance the siRNA uptake and activity *in vitro* and *in vivo* [149].

#### 4.6 Cyclodextrin

Cyclodextrins (CDs) are a group of cyclic ( $\alpha$ ,1,4)-linked oligosaccharides of  $\alpha$ -D-glucopyranose containing a relatively hydrophobic central cavity and a hydrophilic outer surface. CDs form water-soluble inclusion complexes with many poorly soluble drugs. Thus, CDs are widely used in pharmaceutical formulations, and as such, their long-term biocompatibilities are defined in humans [150, 151]. Because of the low toxicity, the lack of immune stimulation, and the absence of enzyme degradation in humans of cyclodextrins, they were chosen as one of the building blocks for the cyclodextrin-containing polymer component. Cyclodextrin-containing polycation nanoparticles can self-assemble with siRNA to form colloidal particles (Figure 6d). The first targeted delivery of siRNA in a human was accomplished with cyclodextrin nanoparticle formulation denoted as CALAA-01 consisting of CDP, which is the main feature of the delivery system, a PEG steric stabilization agent, siRNA against RRM2 gene and human transferring to transferrin receptors for targeted delivery [122, 124, 152-157]. Once approved for use, the four component formulation is self-assembled into 70 nm nanoparticles in the pharmacy and administered intravenously to patients with advanced solid tumors [152-158]. Despite of this wide reputation of cyclodextrins in drug delivery applications, their large-scale commercial is limited by their high cost and concerns regarding their safety [159].

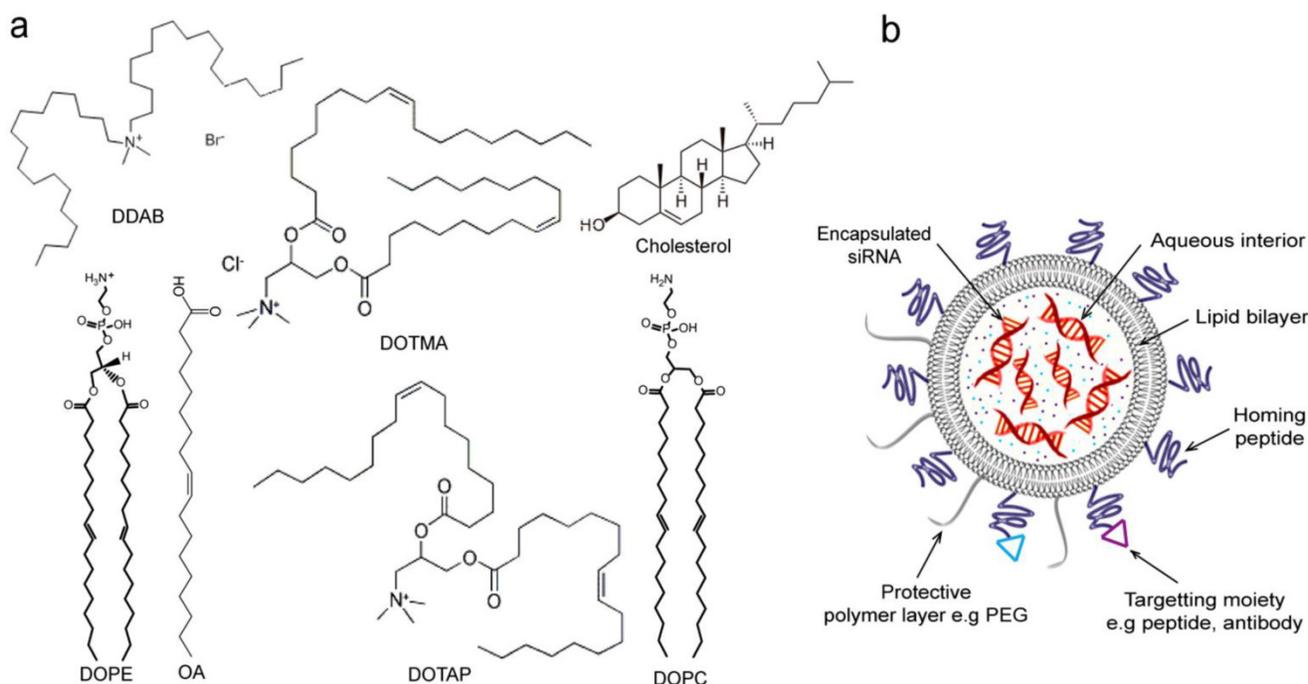
#### 4.7 Lipid-based nanoparticles

Liposomes are among the most advanced nanoparticle drug delivery systems successfully applied in the clinic [160-163]. Several liposomal formulations have been approved by FDA and many more with enhanced features such as stabilized chemotherapeutics encapsulation and tumor-specific targeting versions are currently in development or in clinical trials [164-166]. Liposomes are controlled self-assembly of amphiphilic lipids that form a spherical unilamellar lipid bilayer enclosing an aqueous interior for encapsulation of drug or functional agents (Figure 7). Hydrophobic drugs like paclitaxel can be embedded in the lipid bilayers. Lipid nanoconstructs have also been successfully used in gene delivery. For example, cationic lipids and oligonucleotides complexes have shown good *in vivo* properties as those with stealth liposomes [167]. The flexibility of liposome functionalization allows control over multiple aspects of the formulation. For example, the charge balance can be tuned by the selection of lipid components, as many cationic lipids suitable for nucleotide complexation are available [168]. Figure 7a shows the structure of the most common lipid types reported in siRNA delivery. The composition of lipid bilayers can be chosen to present specific transition temperatures that determines their mechanical rigidity and fluidity in specific temperature range. This has allowed for post-insertion of targeting component, such as human epidermal growth factor receptor 2 (HER2)-targeted single-chain variable fragment (scFv)-PEG-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) to form ad hoc immunoliposomes [169]. Conjugation of functionalized lipids makes liposomes a derivatizable carrier for functional components, such as targeting, contrast agents, probes, and fluorophores, see Figure 7b that represents schematic structure of liposome-based nanoparticle and possible modifications with different functional moieties. PEGylation, *i.e.*, incorporation of PEG chains, is a commonly adapted strategy for prolonging *in vivo* circulation. The PEG chains incorporated in Doxil and most liposomal formulations is around 4-8 mol%. Although the advantages of PEGylation is apparent especially in improving the tumor deposition, the potential downside includes increased difficulty in cell uptake and release of siRNA once in the cellular environment due to inhibition of intracellular trafficking, which is referred to as the PEG dilemma [170]. Hatakeyama *et al.* thus developed a tumor-specific cleavable PEG-lipid to overcome this problem [160]. A PEG-peptide-1,2-dioleoyl-sn-glycero-3-phosphatidylcholine that is cleaved in a matrix metalloproteinase-rich environment was incorporated into a lipid-based multifunctional envelop-type nano device. Similarly, Yingyuad

*et al.* prepared putative enzyme-triggered PEGylated siRNA-nanoparticles through using PEG2000-peptidyl lipids with peptidyl moieties sensitive to tumor-localized elastase or matrix metalloproteinase-2 digestion [171]. Another type of triggered siRNA-nanoparticle system was described by Carmon *et al.* to specifically knockdown hepatitis B virus (HBV) replication in cell culture and in murine hydrodynamic injection models *in vivo*. The described system is generated by the condensation of siRNA with cationic liposomes to form core particles subsequently post-coupled with PEG. In this system, the step of PEG coupling was included to facilitate acidic pH-triggered release of siRNA from endosomes in the target infected cells to suppress HBV [172].

The charged lipids have been initially used widely for siRNA packaging, simulating the success in DNA formulation [173, 174], one of the main challenges has been the concern over toxicity of cationic components and lipids [175]. Therefore, efforts have been made in the development of neutral lipids for the stable association and delivery of siRNA. siRNA encapsulated in neutral liposomes for RNA interference reported by Sood *et al.* is among the more successful examples thus far. They proved conclusively that the neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine-encapsulated siRNA targeting the oncoprotein EphA2 and proangiogenic cytokine

interleukin 8 (IL-8) were highly effective in reducing *in vivo* target gene expression in an orthotopic mouse model of ovarian cancer [176, 177]. In addition, several studies reported the application of neutral lipids for siRNA delivery against viruses. A multicomponent lipid nanoparticles (LNPs) system comprising a mixture of titratable amino lipids (DLin-MP-DMA), PEG lipids (PEG-CDMA), and neutral lipids (cholesterol and dipalmitoylphosphatidylcholine) was described for fully encapsulating short synthetic hairpin RNAs (sshRNAs) that target the internal ribosome entry site of the hepatitis C virus (HCV). This system showed the efficacy of sshRNA against the HCV genome in reducing HCV infection *in vivo* [178]. Stable nucleic acid-lipid particles (SNALPs) were also successfully utilized by Geisbert *et al.* to deliver antiviral siRNA constructs to challenge viral infections of Zaire Ebola virus (ZEBOV) and Marburg virus (MARV). A combination of modified siRNAs targeting the ZEBOV L polymerase (Lpol), viral protein (VP) 24 (VP24), and VP35 formulated in SNALPs were able to protect the tested non-human primate model from lethal ZEBOV infection [179]. For MARV, the treatment with different siRNA targeting MARV VP24, VP35, VP40, NP, and Lpol genes formulated in SNALPs resulted in 60%-100% survival of guinea pigs infected with MARV [180].



**Figure 7. Lipid-based nanoparticles for delivery of siRNA.** (a) Common lipid types and structures reported in siRNA delivery. Cationic lipids include dioleoylphosphatidyl ethanolamine (DOPE), oleic acid (OA) [173], dimethyldioctadecylammonium bromide (DDAB), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) [167]. Neutral lipids include 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) [176, 177] and cholesterol [173]. (b) A schematic showing lipid-based nanoparticle, e.g., liposome. siRNA molecules are encapsulated in the aqueous interior of liposome. Liposome nanoparticles can additionally carry surface protective polymer layer (e.g. PEG) and cellular receptor-specific moieties such as homing peptides and antibodies for enhanced targeting delivery.

## 4.8 Hydrogels

Hydrogels can be defined as three-dimensional networks of responsive polymer materials that are hydrophilic in nature and able to retain large amounts of water or biological fluids. They may exhibit different geometries like macroscopic, microscopic or nano-gels [181, 182]. Both macro- and micro-hydrogel particles were previously prepared and applied for siRNA delivery [182-184]. Nanogels are of current intensive investigations for its nanobased enhanced biomedical application [181, 182]. Due to their characteristic biocompatibility and biodegradability, hydrogels offer excellent opportunities for development of a wide range of controlled siRNA delivery strategies [181, 185-187]. Of particular interest in delivery applications, hydrogels that possess a high degree of porosity, permitting a high loading capacity of siRNA, and can also be selectively surface-functionalized to enable specific targeting applications. Moreover, hydrogel delivery could control and tailor siRNA release time into the target tissues by adjusting the physicochemical properties of gel matrices [182]. This advantage offer great chances for sustained delivery, which has been extensively reported to be necessary for efficient gene silencing by siRNA, and considered as a major disadvantage of the other nanoparticulate siRNA delivery systems that rapidly dispersed in tissues [188].

Naked siRNA have been encapsulated and delivered by multiple hydrogel systems, including alginate [189], collagen [189], dextran [182, 190], polyacrylamide [183, 184], chitosan [191], hyaluronic acid (HA) [192], PEG [193], PEI-catechol [194], and PEI/pluronic [195], see Table 3 and Figure 8a,b. Alginate and collagen were formulated by Krebs *et al.* into hydrogel macro-particle systems that were then successfully applied for localized and sustained delivery of siRNA [196]. Dextran hydrogels loaded with siRNA were adapted to target EGFP in HUH7 human hepatoma cells [182]. Also, Singh *et al.* presented an *in-situ* crosslinkable, injectable formulation containing dendritic cell (DC)-chemo-attractants and dual-mode DNA-siRNA loaded dextran microparticles to attract immature DCs and simultaneously deliver, to the migrated cells, immunomodulatory siRNA and plasmid DNA antigens [190]. Peptide-labeled core/shell hydrogel nanoparticles of poly(N-isopropylmethacrylamide) with a high loading capacity for siRNA have been developed and effectively targeted to ovarian carcinomas by receptor-peptide binding (Figure 8a). The encapsulated siRNA is transported into the cell interior, where it is available for epidermal growth factor receptor (EGFR) gene knockdown [183]. This approach was similarly applied by the

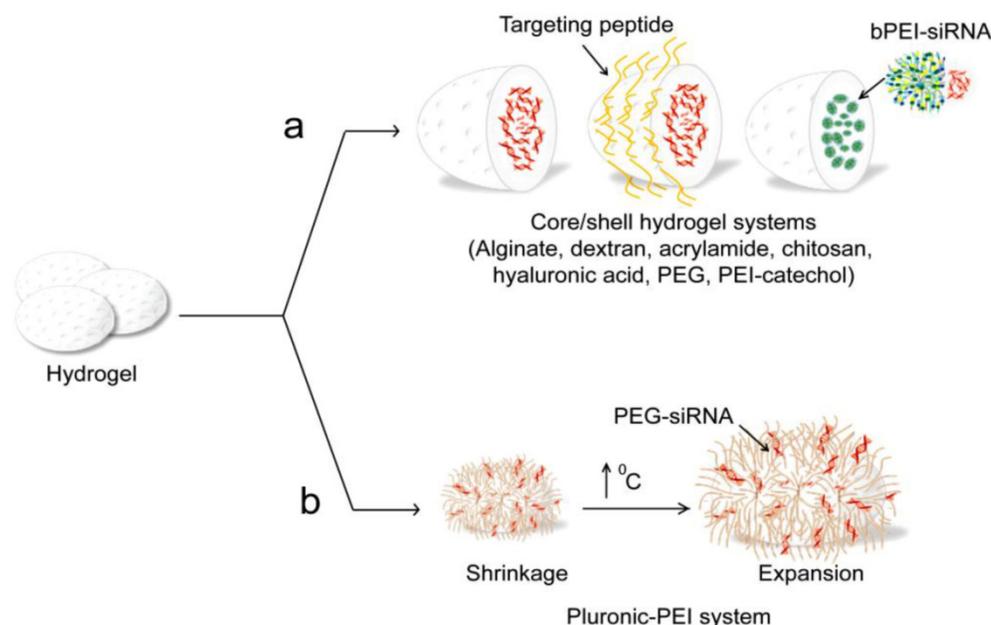
same group as effective strategy for the sensitization of ovarian cancer cells to taxane chemotherapy [184]. Chitosan hydrogel (CH-HG) is another type of particular importance for siRNA applications due to its low toxicity and irritation effects. In another study, CH-HG loaded with either tissue transglutaminase (TG2) siRNA/CH-HG or docetaxel plus TG2 siRNA/CH-HG were effectively delivered in mice bearing A375SM and MDA-MB231 tumors showing enhance localized therapeutic efficacy without risk for systemic side effects [191]. Hyaluronic acid is a naturally occurring nonsulfated glycosaminoglycan polysaccharide, and known to be involved in many biological functions [197]. Hydrogel nanosystems fabricated from HA and physically entrapped GFP siRNA were readily taken up by HA receptor positive HCT-116 cells, and showed a significant extent of GFP gene silencing in both serum and non-serum conditions [192]. In a trial by Takahashi *et al.* to mitigate the soft tissue implant foreign body response by suppressing fibrotic responses around implants; siRNA/branched-PEI complexes released from PEG-hydrogel were applied to inhibit the expression of mammalian target of rapamycin (mTOR) in fibroblasts. This trial resulted in mTOR knock-down and subsequent 70% inhibition in fibroblast proliferation [193]. Similarly, PEI conjugated with catechol groups [194] or crosslinked pluronic acid [195] was adopted by several groups to produce nanogel systems, which were used as a new class of siRNA delivery carriers. The thermally responsive pluronic/PEI system is most interesting. It exhibits a thermally reversible swelling/deswelling volume expansion behavior was synthesized and used for siRNA delivery (Figure 8b). This thermal responsive behavior interestingly allowed for efficient cellular uptake and effective endosome breaking, and a subsequent enhanced delivery for the electrostatically loaded siRNA-PEG on its surface. Recently, Hong *et al.* conducted the first attempt to prepare biologically active siRNA-based microhydrogels using sense/antisense complementary hybridization between single-stranded linear and Y-shaped trimeric siRNAs. These siRNA microhydrogels interestingly exhibited super cellular uptake efficiency and gene silencing activity [198].

## 4.9 Semiconductor nanocrystals

The superior and unique physical and photonic characteristics of semiconductor quantum dots (Qdots), including size and structure tunable emission, high molar extinction coefficient, and high photo and chemical stability propelled them in a plethora of imaging applications [199-202]. Building efficient delivery systems generally involves the synergistic use of diagnostic and therapeutic tools. In this context,

Qdots attracted the interest by being a new generation of nanoparticle carriers that allows real-time fluorescent, multiplexed imaging and efficient delivery of therapeutic agents in living cells [203-207]. Using a PEGylated Qdot core as a scaffold for siRNA and tumor-homing peptides (F3), targeted Qdots conjugates were prepared for siRNA delivery. Delivery of these F3/siRNA-Qdots to EGFP gene-transfected HeLa cells and release from their endosomal entrapment led to significant knockdown of EGFP signal [206]. Also, Qdots modified with different polymers (e.g., amphipols and proton-sponge) were specifically designed for real-time imaging and delivery of siRNA. The polymeric-Qdots systems addressed longstanding barriers in siRNA delivery such as cellular penetration, endosomal release, carrier unpacking, and intracellular transport and showed enhanced

silencing activities [203, 204]. Through a FRET based assay, Lee *et al.* recently utilized cationic Qdots in complex with siRNA for studying intracellular trafficking, unpacking, and gene silencing proving the advances brought by using the photonic properties of Qdots in exploring siRNA delivery processes [205]. Despite of these reports on the apparent applications of Qdots in siRNA delivery, potential problems may arise in their *in vivo* applications. Among these problems, the toxicity of Qdots is a major issue. Their surface coating can be cytotoxic such as mercaptoacetic acid [200, 208]. Also if this coating are compromised, the metallic core made of cadmium and selenium can be highly toxic [208]. Other issues, including particle aggregation, degradation and removal within the body are still largely unknown [200].



**Figure 8. Hydrogel based siRNA delivery systems.** (a) Different core/shell hydrogel systems, including alginate [189], dextran [182], acrylamide [183, 184], chitosan [189, 191], poly(lactide-co-glycolide) (PLGA) [190], hyaluronic acid [192], PEG [193], poly(ethylenimine) (PEI)-catechol [194] are reported for siRNA delivery. These systems are physically encapsulating, where siRNAs are incorporated in the hydrogel network based on electrostatic interaction or non-covalent binding. (b) Thermally responsive pluronic/PEI system [195].

## 5. Concluding remarks

siRNA therapeutics have great potential to impact the future of medicine and, it is recently acclaimed as a revolutionary class of drug molecules. However, there are still many challenges and barriers to achieve their full potential. Systemic delivery is one of the most important challenges. Nanoparticles-mediated delivery is a rapidly emerging and potentially powerful technology. Different nanoparticle systems offer various advantages and disadvantages based on their composition, physical, and chemical characteristics, thus leading to a range of effectiveness when associated with siRNA. Silica nanoparticles, due

to their controllable porosity, allow multi-functional sequential delivery. The metal nanoparticle cores provide multiple functions, including targeted concentration to cells or tissues, multiplexed imaging and real-time tracking. Carbon nanostructures with every atom exposed on its surface hold ultra-high surface modification and loading capacities. The flexibility of liposome functionalization allows control over multiple aspects of the formulation. On other, hydrogels possess a high degree of porosity and biodegradability that permit a high loading capacity and excellent opportunities for development of different controlled delivery strategies. Polymers are rather unique in their unit-by-unit construction that allows for fi-

ne-tuning of the properties required for efficient transfection and release. Here, most of the reported studies were successful in implying these unique advantages of nanoparticles to overcome the known siRNA delivery barriers and to enhance the efficiency of siRNA delivery. However, the overall success has been limited to date by toxicity observations and additional challenges associated with many stages along the delivery process. The development of novel nanoparticles and multifunctional nanostructures that display diverse functionalities to achieve effective multimodal delivery applications and advanced studies to reveal the detailed biological aspects concerning siRNA delivery will greatly help our understanding for this process and subsequently help the design of better delivery systems to reach the full potential of siRNA therapeutics.

## Competing Interests

The authors have declared that no competing interest exists.

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