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RNAi is a powerful gene silencing process that holds great promise in cancer therapy by the use of siRNA. The aim of this review is to give an outline on different approaches to deliver siRNA and to describe the advantages and demerits of these systems. The prospects for siRNA are to be substantially better than other therapies, as they are easily applicable to any therapeutic target. They also promise potent gene inhibition with exquisite selectivity down to the level of single nucleotide polymorphism, and can easily identify offending proteins or variants by screening across gene sequence. The main obstacle of using RNAi technology in cancer treatment is to protect such a fragile and quickly metabolized biological molecule and to efficiently deliver it *in vivo*, to the target cells. Therefore, there is a need of new systems, as nanoparticles for siRNA delivery, to reach and improve their biodistribution in target tissue.

Keywords: cancer therapy • delivery systems • nanoparticles • siRNA

Deregulation of gene expression is a common feature in cancer. The possibility to inhibit the expression of an oncogene at the mRNA level, instead of blocking the function of the gene product, has elicited a great interest for a long time as potential therapeutic application. Therefore, RNAi offers promising new opportunities to very specifically target the genes deregulated in various pathologies including cancer. This strategy has been proposed as a royal road to block *EWS-Fli1* gene expression in Ewing sarcoma [1,2], *RET/PTC* in papillary thyroid carcinoma [3], *HER2* in nasopharyngeal tumor [4], *VEGF* in tumor angiogenesis [5,6], *PD-L1* in ovarian cancers [7] and *FAK* in pancreatic adenocarcinoma [8]. However, *in vivo* delivery of siRNA is a key challenge because the biological efficacy of the siRNAs is hampered by their short plasmatic half-life due to poor stability in biological fluids and low intracellular penetration due to their highly hydrophilic character [9]. So far, a wide variety of approaches including viral vector-based and nonviral delivery systems such as liposomes, nanoparticles (NPs), lipophilic conjugates, polymers and cell-penetrating peptides [10,11] have been investigated to enhance target cells' uptake and silencing potency *in vivo*. However, the safety of viral vectors is questionable due to immunogenicity and possible recombination with oncogenes. Most of the nonviral vectors are designed using polycations, either polymers or lipids whom (cyto) toxicity is now well documented [12]. Therefore, it is an urgent need to introduce new concepts able to impact on the way of siRNA delivery based on the use of more efficient and safe (bio) materials for drug delivery purpose such as squalene, a cholesterol precursor. In this review, the authors describe the major types of NPs that are used to vectorize siRNA, and also their advantages and disadvantages.

Benefits & drawbacks of RNAi in cancer treatment

Antisense oligonucleotides (AS-ONs) and RNAi are the major approaches for therapeutic inhibition of gene expression. Although the activity of both types of molecules is due to their base pairing capacity with complementary cellular nucleic acids, the method by which they inhibit gene expression is different. AS-ONs are single-stranded DNA or RNA sequences of 13–25 bases that can highly selectively inhibit the expression of target genes by hybridizing with their specific 'sense' sequences in mRNA or DNA molecules [13]. RNase H-mediated degradation of complementary mRNA is the major mode of action of AS-ONs. This enzyme

specifically cleaves the RNA strand of RNA–DNA heteroduplexes, leading to the release of the AS-ON that can further bind to a new mRNA strand; the mechanism is therefore catalytic [14]. Whereas, siRNAs are RNA duplexes of 21–23 nucleotides with approximately 2 nucleotides 3 overhangs. One of the siRNA strands, named the guide strand, is recognized by the RNA-induced silencing complex RISC in which the main actor is Ago2 protein (hAGO2 in human). The guide strand serves as a template for the recognition of homologous mRNA, which upon binding to RNA-induced silencing complex, is cleaved by the catalytic activity of Ago2. The other siRNA strand, the passenger strand, is degraded.

It is well known that siRNA and AS-ON can inhibit their specific targets genes, and therefore used for the treatment of many diseases including cancer [15]. Their high specificity, covering a wide range of biomedical applications, allows the inhibition of target proteins that are not easily accessed and modulated by conventional small molecular weight or protein drugs [16]. However, application of gene silencing *via* ONs faces obstacles in gene transfection and tissue targeting [17] because of their poor *in vivo* stability and their inability to transverse intact cell membranes to any significant degree due to their highly hydrophilic and anionic character [18]. Thus, the development of an efficient delivery system is one of the most challenging hurdles to turn ONs into clinically acceptable therapeutic drugs.

The primary obstacle for translating RNAi technology from an effective research tool into a feasible therapeutic strategy is the efficient delivery of these molecules to the target cells *in vivo*. There is no natural mechanism for the highly hydrophilic polyanionic macromolecules to traverse the cellular membrane due to the reason that the bioavailability of these agents in the intracellular compartment is minuscule. The efficacy of siRNA into cells is offended by the major biological barriers, along with their transient gene silencing effects due to their short-lived nature, and conditioned by the growth potential of dividing cells (between 36 and 48 h for rapidly diving cells and around several weeks for slowly dividing cells) [19].

Another key barrier for the arrival of ONs to the target area in the body is the phagocytes of the reticuloendothelial system. These cells monitor the blood, remove foreign materials such as bacteria and viruses and tend to treat administered macromolecules such as ONs as foreign materials, thus accumulating these materials in hepatic Kupffer cells, splenic macrophages and other phagocyte-rich sites [20]. In addition, naked AS-ONs and siRNAs are relatively unstable in the blood in their native forms, as they are degraded by nucleases due to short half-life of few minutes, and are rapidly cleared from the body by renal excretion or may also aggregate with serum proteins [21]. In parallel, the systemic exposure of siRNA can also induce some nonspecific responses that are caused by CpG DNA oligonucleotides [22]. Passing through the vasculature, ONs must disseminate through the extracellular matrix ECM, which is a dense network of polysaccharides and fibrous proteins that also hinder ONs' diffusion towards the target cells [23]. Thus, the revolutionary potency and selectivity of siRNA can enable improved targeted cancer therapeutics; but the appropriate means of systemic administration and targeted distribution to disseminated metastatic lesions is needed [6].

Strategies to transport gene silencers

A wide variety of approaches including viral and nonviral delivery systems have been investigated to enhance the target cell siRNA-uptake and silencing potency *in vivo*.

Viral vectors

Viral vectors are the most powerful tool for gene transfection. The use of viral vectors is known to be the best way to deliver ON within the cells. Recently, they have been used to deliver siRNA for silencing the hemagglutinating virus of Japan (also called Sendai virus) [24]. However, the safety of viral vectors is questionable due to immunogenicity and possible recombination with oncogenes [25], a general lack of specificity and gene delivery to the target cells of interest, potential toxicity of the viral vectors themselves [26] and even some of them are also able to infect nondividing cells [27].

Nonviral vectors

Nonviral vectors are alternative to viral vectors. Many nonviral vectors have been designed and have become a powerful and popular research tool to elucidate gene structure, regulation and function. Although less efficient than viruses for gene delivery, they offer several advantages: particularly, simple to use, offer trouble-free quality control, biocompatibility and generate little or no specific immune response [28].

Two major nonviral approaches, based on either chemical modification or nanoformulation, were adopted to overcome the above-mentioned limitations concerning ONs' administration. The chemical approach consists of either synthesizing nucleic acids with variations in their natural structure (in order to improve resistance towards degradation) [11] and/or attaching a lipidic, peptidic or polymer moiety associated or nonassociated with a cell-targeting ligand. Attachment of specific targeting ligands can induce binding to protein carriers or uptake by the desired population of cells to be treated. siRNAs can be: coupled with fusogenic peptides, linked to antibodies against cell surface receptor ligands for cell-specific delivery, encapsulated in lipid complexes or cationic liposomes or incorporated in polymers that can be chemically different and able to form NPs [29]. Certain amine-containing cationic polymers such as polyethyleneimine (PEI) can be attached as ligands to the surface of NPs through standard conjugation chemistry. These cationic polymers are capable of inducing membrane permeability, facilitating the entry of NPs into the cells. In particular, PEI has been widely investigated for gene delivery because of its ability to complex with and condenses DNA and transfect a broad range of cell lines with high efficiency [29].

A polymer combining PEI with the biocompatible polymer polyethylene glycol (PEG; adamantine–PEG) and the polysaccharide chitosan were attached to the surface of iron oxide NPs for *in vivo* gene delivery [30]. The proton sponge effect was used to deliver siRNA-wrapped quantum dots into MDAMB-231 breast cancer cells. By coating quantum dots with a balanced composition of

tertiary amine and carboxylic acid groups, NPs with their siRNA cargo were capable to penetrate cell membrane, released from endosome, carrier unpacking and intracellular transport of cargo [31]. NPs functionalized with a tumor-homing peptide and siRNA cargo have been shown to internalize the cell by ligand-mediated internalization and knockdown of EGFP signal in EGFP-transfected HeLa cells [32]. Aptamers can also be coated onto the surface of NPs for *in vivo* and *in vitro* targeting. Aptamers are short single-stranded DNA or RNA oligonucleotides selected *in vitro* to specific cellular targets with high selectivity and sensitivity [33].

Coupling dual antibodies (the murine 83-14 MAb to the human insulin receptor and the rat 8D3 MAb to the mouse transferrin receptor) to siRNA EGFR at the distal end of PEG on a sterically stabilized liposome by a complex procedure reduced tumor expression of immunoreactive EGFR. Moreover, an increase in survival time of mice with advanced intracranial brain cancer was observed [34]. Several efforts were carried out to evaluate cationic lipids and polymers activities, originally developed from plasmids internalized by nonspecific electrostatic interactions [35]. These plasmids had a good cytoplasmic delivery, but their functional activities remained limited due to the poor entry into the nucleus.

Conjugation of nucleic acids with neutral lipids provides greater metabolic stability and improved bioavailability and offers an interesting alternative [3]. It has been reported that this approach could significantly enhance cellular uptake of AS-ONs and siRNAs, prolong the half-life of these molecules in plasma and increase the efficiency of siRNA-induced gene silencing *in vivo*. Thus, the potential advantages of the chemical strategies are as follows: ON preferential targeting towards specific tissues and cells, expressing specific receptors delivery of ON to appropriate intracellular compartments (i.e., cytoplasm or nucleus), better ON way out from the blood and widespread distribution in tissues due to the small size of the chemical conjugates [36].

A wide variety of nanocarriers, such as liposomes [10], NPs [37], micelles [5] and polyplexes [38], have been investigated so far to enhance target cells' siRNA uptake and silencing potency *in vivo*. Chitosan, a positively charged polysaccharide containing nonacetylated β-linked D-glucosamine residues, has been intensively investigated as a nucleic acid delivery system and has been successfully utilized for *in vivo* delivery of siRNA as chitosan-based [2] or core-shell type (core of poly(isobutylcyanoacrylate) polymer and shell of chitosan) [39] NP. To enhance the circulation half-life, these colloidal systems have been decorated with PEG chains. Moreover, to improve target cell specificity, Dubey *et al.* decided to graft L-arginyl-glycyl-L-aspartic peptide to bind to the integrins on the tumor neovasculature [40]. For example, the intravenous injection of these NPs containing siRNA directed against VEGF receptor-2 led to the selective uptake into subcutaneous tumor xenografts and reduced both tumor growth and angiogenesis [5].

Requirements of nanoformulations

ONs must pass through the tight vascular endothelial junctions to leave the blood circulation, but the molecules larger than 5 nm in diameter can not readily cross the capillary endothelium. In contrast to the normal endothelium, the tumor vasculatures are leakier and characterized by discontinuous structures where pore size varies from 100 to 780 nm, and has a poor lymphatic drainage compared with the pore size of less than 2 nm in normal vascular endothelium in most tissues and postcapillary veins (6 nm) [41]. The tumor endothelium may allow the penetration of high molecular mass macromolecules (>40 kDa) and NPs up to approximately 200 nm in diameter by the translocation process known as the 'enhanced permeation and retention' (EPR) effect [42]. Moreover, on cellular internalization, the ONs must be released from the endosomes (e.g., siRNA loaded on to dicer/Ago-2) to reach the intracellular relevant targets [43]. As the condition in the endosomes/lyosomes is mildly acidic, the acid responsive delivery carriers cause facilitated cytosolic release of ONs, and thus has been a popular strategy to overcome this intracellular hurdle [44]. Several studies showed that naked siRNA are inefficient because of their fast metabolism; therefore, there is a need to protect them against degradation (for review [45]). In a nutshell, an effective delivery strategy must take into account the need to protect the ONs from the enzymatic digestion, improve the pharmacokinetics by avoiding excretion *via* the reticuloendothelial system and rapid renal filtration, allow a better translocation through the endothelium, facilitate the diffusion through the extracellular matrix, enhance cellular uptake and allow endolysosomal escape [29].

Choice of nanodelivery system

The interaction of NPs with cellular structures is affected by multiple factors including the intrinsic properties of NPs, target cell type and its physiological state, cellular uptake, toxicity and intracellular localization. During particle–cell interaction, lipid domains, enzymes, carbohydrates and protein receptors of plasma membrane interact differently with the different types of NPs coming in contact with the plasma membrane to ultimately determine the cellular response towards the material. Particle size, morphology and position on the membrane surface decide the various forces of interactions, lead to divergent cellular responses and thus different routes of uptake. For *in vivo* applications, the engineered NPs need to overcome additional biological barriers before being taken up by the targeted cells such as evading undesired immune responses, maintaining colloidal stability in blood circulation, extravasation into the targeted organs and achieving effective tissue penetration [46]. Keeping in view the complexity of NPs' cell interaction and the diverse and sequential presentation of biological barriers on the cellular and the tissue level, NPs need to be designed with dynamically triggered or activated functions or properties [29]. Physicochemical properties of the engineered NPs should be considered while designing a nanomedicine for optimal intracellular uptake and targeting with an aim of being capable to reach its biological target with high efficiency and specificity.

Types & forms of NPs

The nanodelivery system generally uses cationic lipids or polymers that interact with ONs through electrostatic interactions, leading to nanoplexes with size between ten and few hundred nanometers. The expected advantages of the nanoformulation approach are a better stability in biological fluids and an improved cell penetration

and tissue biodistribution. This strategy has already achieved significant success in both cellular and animal studies [47]. However, the toxicity of cationic transfectants either lipidic or polymeric may raise major issues for clinical utilization [25]. In this context, the use of nonionic lipids for ONs' delivery represents an attractive methodology as many lipids are safe, nontoxic and biocompatible, additionally some of them being of natural origin [48].

Four major types of NPs are known to deliver the oligonucleotides: nanoplexes, polyplexes, lipoplexes and micelles (Figure 1). The following paragraphs will describe briefly the characteristics of each type. Few examples are summarised in Table 1.

Nanoplexes: either nanosphere, where nucleic acid is associated with the nanoparticle, or nanocapsule, where nucleic acid is encapsulated into the nanoparticle;

- Polyplexes: core-shell type nanoparticles;
- Lipoplexes: liposome structures characterized by a bilayer lipid membrane and
- Micelles: the results of electrostatic interaction between nucleic acids and copolymers.

Nanoplexes

Two types of nanoplexes can be described: nanospheres where the nucleic acid is dispersed throughout the particle or adsorbed on the surface and nanocapsules where the nucleic acid is encapsulated inside the particle. The authors' laboratory developed two types of nanospheres: type 1 (139 ± 47 nm size and 1.6 mV ζ potential) and type 2 (65 ± 17 nm size and 21 mV ζ potential) were prepared by combining polyalkyl cyanoacrylate (as biodegradable polymer matrix) and chitosan (as a cation). The nanospheres were loaded with oligonucleotides to target EWS-Fli-1 junction oncogene in Ewing sarcoma [2]. In type 1 nanospheres, chitosan was just overcoated; whereas in type 2, it was covalently bound to PACA.

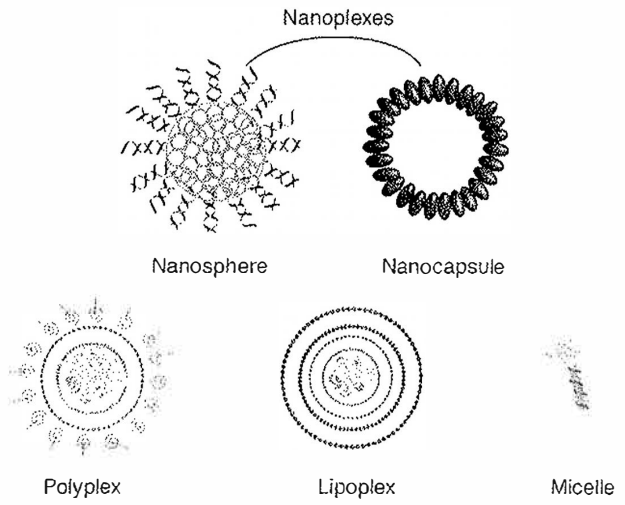

Figure1. Four major types of nanoparticles that are used to deliver oligonucleotides.

Chitosan has been chosen as it has the advantage over cetyltrimethylammonium bromide of being less toxic and allowing higher ON loading. When these NPs were intratumorally injected, the authors observed that both types of nanospheres significantly reduce tumor growth and partially decrease the mRNA expression [2].

Polyplexes (core-shell type NPs)

In the core-shell type of NPs, the core consisted of the biodegradable poly isobutylcyanoacrylate polymer and the shell of chitosan (linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and *N*-acetyl-D-glucosamine) for the treatment of papillary thyroid carcinoma. The tumor growth was almost stopped after intravenous injection of the antisense siRNA-loaded NPs in contrast to all control experiments where 10-times increase in tumor size was observed [49].

Lipoplexes

Liposome's structure is characterized by a bilayer lipid membrane mostly composed of phospholipids that can be neutral, positively or negatively charged. Liposomes are being clinically used for chemotherapy and other delivery systems, and are forms of NPs that function as carriers and act as a slow release depot for the drug in the diseased tissue. Usually, a low amount of cationic lipids is added to the formulation to increase charge interactions between the ON and the vehicle rendering the nanoformulation more stable. Moreover, cationic lipids should improve cellular uptake. For example, Sorensen *et al.* showed the reduction of TNF-α expression in the liver and the spleen by delivering siRNA packaged in cationic liposomes [50]. 1, 2-Dioleoylsn-glycero-3-phosphatidylcholine (DOPC) is a neutral lipid, and is successful to deliver AS-ONs *in vivo* without toxicity. DOPC liposomes were used to deliver gene-specific siRNA in orthotopic ovarian cancer models [51]. Specific FAK siRNA in DOPC liposomes was systemically delivered and resulted in efficient FAK downregulation and therapeutic efficacy compared with naked siRNA or cationic liposomes. Treatment with FAK siRNA-DOPC plus either docetaxel or cisplatin chemotherapy was highly effective in inhibiting ovarian cancer growth even in patients with chemotherapy-resistant tumors [10]. This delivery system is not tissue specific, but further liposomes' modifications allow tumor-selective targeting [40].

Micelles

Micelle-forming copolymers such as PEG-polycation di-block polymers with diamine side chains were used as endosome escaping carriers for siRNA [52]. Other types of polymeric micelles designed for nucleic acid delivery include PEG-poly aspartic acid copolymers [53] (exert a 'proton sponge' effect in endosomes), lactosylated PEG (form complexes with poly lysine and AS-ODN thus allow targeting to galactose receptors in liver cells) [54] and PEG-poly methylmethacrylate block copolymers used to produce hybrid organic inorganic NPs [36].

Kim *et al.* spontaneously obtained micelles after conjugation of poly(ethylene glycol) (PEG)-conjugated to VEGF siRNA (siRNA VEGF–PEG) with PEI. These micelles had an inner core of the siRNA VEGF/PEI with a surrounding PEG shell layer.

Table 1. Various nanoparticles being developed for siRNA delivery in anticancer therapy.

Nanoparticle formulation	Size (nm)	Z potential (mV)	Therapeutic agent	Route of administration			Indications	Ref.
				Intratumoral	Intravenous	Intraperitoneal		
Squalene-chitosan (nanosphere)	Type 1 = 139 ± 47 Type 2 = 65 ± 17		siRNA against EWS-Fli1	1 mg/kg siRNA	120 mg/kg siRNA		Ewing's sarcoma	[2]
Squalene (NPs)	165 ± 10		siRNA against RET/PTC1		2.5 mg/kg cumulated dose		Papillary thyroid carcinoma	[3]
Cholesteryl 3b-carboxy amidoethylene-N-hydroxy-ethylamine-DSPE-PEG-FOLATE (NPs)	144.40	−9.40	siRNA against HER2	10 µg/mouse every 2 days			Nasopharyngeal tumor	[4]
PEG-PEI (nanoplexes)	50–80		siRNA against VEGF	500 pmol /mouse once a week	1.5 nmol /mouse 5 injections		Tumor angiogenesis	[5]
RGD-PEG-PEI (nanoplexes)	90	6.00 ± 1.00	siRNAagainst VEGF R2		40 µg/mouse of siRNA 3 times/ week		Tumor angiogenesis	[6]
chitosan-coated poly iso-butylcyanoacrylate (NPs)	60	−11.80	siRNA against RET/PTC1				Papillary thyroid carcinoma	[39]
novel branched PEI-F25-LMW (nanoplexes)			siRNA against VEGF in combination with bevacizumab	siRNA 10 µg/mouse		siRNA 10 µg/mouse + bevacizumab 100 µg/mouse	Colon carcinoma	[57]
Polyisobutyl cyanoacrylate (nanocapsules)			ON against EWS Fli-1				Ewing's sarcoma and PNET	[71]
PEI (nanoplexes)	41.50	19.05 ± 1.46	siRNA against pleiotrophin growth factor			8 µg/mouse of siRNA every 2 days	Orthotopic glioblastoma	[72]
PEI (nanoplexes)	40–60		siRNA against programmed cell death ligand-1			50 µg/mouse	Ovarian carcinoma	[7]
Cholesteryl-3b-carboxy amidoethylene-*N*-hydroxy ethylamine + 5 mol% Tween-80 (NPs)			siRNAagainst RET in combination with Irinotecan	siRNA 10 µg/mouse every 3 days	Irinotecan 30 mg/ kg every 3 days		MTC	[73]

MTC: Medullary thyroid carcinoma; NP: Nanoparticle; PEG: Polyethylene glycol; PEI: Polyethyleneimine; PNET: Primitive neuroectodermal tumor.

After intravenous and intratumoral treatments of xenografted human prostate carcinoma, they observed a significant decrease of tumor growth and an inhibition of *VEGF* expression [5]. The biodistribution on intravenous injection was further studied and compared with the cationic liposome formulation 1,2-dioleyl-3-trimethylammonium-propane/dioleoylphosphatidylethanolamine. Notably, the different carriers changed the intratumoral distribution of siRNA within the tumor [37].

Demerits of delivery services

Dependency on dose & route of administration

One of the most important parameter to be considered for siRNA efficiency is the optimal dose able to inhibit the target gene. Hassani *et al.* demonstrated efficient delivery of nucleic acids to cells by jetPEI, but, when was used to introduce siRNA into the mouse brain *in vivo*, no gene silencing activity was observed in the low-dose ranges tested. This suggests that siRNA dose dependency is fundamental to obtain a therapeutic effect [55].

Several routes of administration were suggested: intranasal administration, intratracheal instillation, aerosol delivery and intravenous injection. It seems that the route must be properly chosen regarding the targeted tissue, for example, for lungs, aerosol keeps long persistence gene expression when compared with instillation [56].

The systemic delivery of siRNA targeting the tumor VEGF resulted marked in antitumor effects in subcutaneous prostate and pancreatic carcinoma xenografts in mice upon intraperitoneal compared with intravenous administration of the PEI F25-LMW/siRNA complexes. Downregulation of *VEGF* expression on mRNA and protein levels were comparable to the treatment with the therapeutic humanized anti-VEGF antibody bevacizumab (Avastin®) [57]. Similarly, the administration through the tail vein, in contrast to intraperitoneal injection, resulted in high siRNA levels in mouse lungs [58].

Charge & size of the delivery system

In the late 1980s, several studies have been performed to assess the physical–chemical properties of different nanosystems' deliveries. It has been shown that charge and size play a key role for passive targeting. For example, positively charged NPs are known to improve the interaction with the bloodstream components reducing the half-life of drugs. As a result, there is rapid clearance of these delivery vehicles from the bloodstream compared with near-neutral particles exhibiting reduced phagocytic uptake [59]. Negatively charged liposomes were believed to be more rapidly removed from circulation than neutral or positively charged liposomes. Kostarelos et al. showed that the negatively charged lipids affect the rate of liposome uptake by the reticuloendothelial system. Moreover, phosphatidylserine, phosphatidic acid and phosphatidyl glycerol containing liposomes are cleared more rapidly than neutral liposomes.

The size of NPs should be between 40 and 200 nm depending on the targeting tissues. The characteristic solid tumors have higher interstitial fluid pressure in the center of the tumor compared with the periphery. An outward convective flow reduces drug diffusion to the center of the tumor, and the particles and drugs that gain interstitial access have higher retention times than in normal tissues. This aberrant vasculature and higher interstitial fluid pressure create an EPR) effect on NPs. Particles that are smaller than the fenestration can gain access and be retained in the tumor [60,61]. Thus, the macromolecule complexes preferentially accumulate in tumors through the EPR effect. However, these large macromolecules exhibit limited diffusion into the extracellular spaces such as tumor interstitium and in the complex intracellular environment [62]. In both situations, restricted movement will severely limit efficacy by preventing uptake by a sufficient number of cells or hindering the ability of the delivered particles to localize in to intracellular compartments such as the nucleus [63]. In short, too big NPs cannot efficiently enter the cell, whereas too small particles cannot remain and accumulate inside the tumor after translocation through the wide fenestration of the tumor endothelium [64].

Transfection & target delivery

An intracellular conformational change in the NPs is another problem in efficient targeting. Internalization of quantum dots into cells by cationic liposomes such as lipofectamine complexes formed aggregates in the cytoplasm [65]. Fluorescent semiconductor quantum dots (alloy nanocrystalline colloids such as CdSe/ZnS and CdTe1-xSex), delivered by electroporation into live cells, formed similar aggregates of up to 500 nm in diameter rather than being delivered individually. Aggregates of quantum dots were observed both inside and outside of the cell even when crosslinked with bovine serum albumin coat and delivered via electroporation. This suggests that the stabilization of surface ligand is not sufficient to prevent aggregation and that the applied electric potential induced quantum dot aggregation [32]. A major limitation in most of the NP delivery strategies is the difficulty of achieving active targeting of specific subcellular organelles after entry into the cell. The surface of the NPNPs should be modified to improve cellular uptake and to allow specific subcellular delivery [29].

Disparity of the results between in vitro *&* in vivo

Various studies demonstrated a high discrepancy in results between *in vitro* and *in vivo* experiments. In the authors' group, two examples reflected these discrepancies. In 2005, Maksimenko *et al.* used two types of nanospheres carrying AS-ONs targeting EWS/Fli-1 oncogene and found that both were inefficient *in vitro* (no inhibition of mRNA and protein expression). However, the lack of the type 2 nanosphere efficiency was not due to their inability to cross the cell membrane, as their intra cellular presence was demonstrated by confoncal analysis; but NPs might be entrapped in an inefficient cellular compartment within the cell. Nevertheless, both types once injected *in vivo* by intratumoral route showed a significant, but incomplete, inhibition and only partial mRNA inhibition was observed. Once injected intravenously, only type 2 formulation was found to be efficient [2].

In 2011, Raouane *et al.* conjugated siRNA against *RET/PTC1* oncogene with squalene and found that the NPs are efficient *in vivo* but not *in vitro*. The authors postulated that in cell culture conditions, the limited enzymatic content did not allow the cleavage of the squalene needed for the disaggregation of NPs before further recognition of mRNA sequence [3].

Advantages & uses of squalene NPs

Many different cationic lipids have been synthesized and tested for transfection of genes into cells. Cationic complexes have advantages of nonimmunogenic compared with viral vectors but the problems of low efficiency and cytotoxicity of cationic lipids could not be ignored [66]. Cationic cholesterol derivatives are composed of three distinct parts: a cholesteryl skeleton, a cationic amino group and a linker arm between the cholesteryl skeleton and cationic amino group. Derivatives with different combinations of these parts were reported, and some have high transfection efficiency [67]. Among them, cholesteryl-3b-carboxyamidoethylene-*N*-hydroxyethylamine, a cationic lipid with a hydroxyethyl group at the amino terminal showed the most efficient transfection efficiency for plasmid DNA (pDNA) and antisense oligodeoxynucleotide delivery [68].

The use of neutral cholesterol derivatives such as squalene was used as a safe alternate as drug carrier biomaterials for several anticancer molecules as gemcitabine [69] and more recently by the authors for siRNA [3]. Squalene have several advantages such as being naturally present in the body, precursor of cholesterol, used as excipient in several pharmacological preparations and a nonionic nontoxic biocompatible vector [69]. A squalenoyl conjugate of siRNA RET/PTC1, the junction oncogene frequently found in papillary thyroid carcinoma, was synthesized. The acyclic isoprenoid chain of squalene has been covalently coupled with siRNA RET/PTC1 at the 3 -terminus of the sense strand *via* maleimide–sulfhydryl chemistry. Squalene NPs (size = 165 ± 10 nm) hiring siRNA RET/PTC1 display a significant regression of tumor growth (i.e., 70%) *in vivo* paralleled with inhibition of *RET/PTC1* oncogene and oncoprotein expressions in papillary thyroid carcinoma [3]. However, mechanism of action should be investigated, as the same NPs show relatively low cellular uptake *in vitro*. Improvements in squalene-based nanoformulations can be done by grafting on their surface-specific ligands recognized by tumor cells, in order to improve their efficiency both *in vitro* and *in vivo*.

Expert commentary & five-year view

Despite the recent progress in cancer treatment, a considerable number of patients relapses and develops recurrent resistance. Therefore, siRNAs could constitute an area of important research in emerging therapies alone or as combined treatment. The first siRNAs discovered in mammalian cells and the enormous potential of these biological molecules to be used as drugs have been described for the first time in 2001 [70]. Nowadays, if we look at the ongoing clinical trials, we can be surprised to find how many therapeutic siRNAs are already in Phase II and III [101]. Naked siRNAs are used for local treatments in a large number of different diseases such as age-related macular degeneration, melanoma, intraocular pressure, however, for cancer treatment, naked siRNAs do not spontaneously enter either into the cells in culture or tumor tissue. To efficiently deliver siRNAs, several strategies were developed using nanocarriers, although only two NPs encapsulating siRNA are actually on early Phase I for patients with neoplastic pathology. This is probably due to the toxicity related to the polymers or compounds used to generate the nanovectors. Much more effort should be done exploring new biological materials (such as lipids, cholesterol and squalene) in order to substitute toxic polymers and improve the number of therapeutic siRNAs that could be delivered. The high specificity of siRNAs is the gold standard characteristic of these potential drugs; hence, an individual therapy related to genomic difference from one patient to another can be now much more realistic within the next 5 years. Furthermore, the synthesis of covalently bound siRNAs with natural lipids or related compounds able to rearrange and form NPs (i.e., squalene) could be a new strategy to improve the encapsulation yield, and consequently to reduce frequency and administration doses, fundamental for patients' wellbeing and comfort.

Our point of view is that therapeutic potential of siRNA is not yet enough exploited and this because of not yet well optimized delivery systems. In the next 5 years, we can expect that new nanoformulations will be conceived and can scale up to clinical trials very quickly.

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Key issues

- RNAi offers promising new opportunities to very specifically target the genes deregulated in various pathologies including cancer.
- Naked antisense oligonucleotides and siRNA are relatively unstable in the blood in their native forms, as are degraded by nucleases due to short half-life of few minutes and are rapidly cleared from the body by renal excretion or may also aggregate with serum proteins.
- A wide variety of approaches including viral as well as nonviral delivery systems have been investigated to enhance the target cell siRNA uptake and silencing potency *in vivo*.
- Physicochemical properties of the engineered nanoparticles should be considered while designing a nanomedicine for optimal intracellular uptake and targeting with the aim of being capable to reach its biological target with high efficiency and specificity.
- This review helps to understand the parameters that are important to obtain an efficient siRNA delivery system in terms of charge, size, dose and administration route.
- Four major types of nanoparticles are known to deliver the oligonucleotides: nanoplexes, polyplexes, lipoplexes and micelles. This review describes briefly the characteristics of each type.
- In our opinion, much more effort should be done exploring new biological materials (such as lipids, cholesterol and squalene) in order to substitute toxic polymers and improve the number of therapeutic siRNA that could be delivered.

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