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REVIEW

Small interfering RNAs (siRNAs) in cancer therapy: a nano-based approach

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Abstract: Cancer is one of the most complex diseases that has resulted in multiple genetic disorders and cellular abnormalities. Globally, cancer is the most common health concern disease that is affecting human beings. Great efforts have been made over the past decades in biology with the aim of searching novel and more efficient tools in therapy. Thus, small interfering RNAs (siRNAs) have been considered one of the most noteworthy developments which are able to regulate gene expression following a process known as RNA interference (RNAi). RNAi is a post-transcriptional mechanism that involves the inhibition of gene expression through promoting cleavage on a specific area of a target messenger RNA (mRNA). This technology has shown promising therapeutic results for a good number of diseases, especially in cancer. However, siRNA therapeutics have to face important drawbacks in therapy including stability and successful siRNA delivery in vivo. In this regard, the development of effective siRNA delivery systems has helped addressing these issues by opening novel therapeutic windows which have allowed to build up important advances in Nanomedicine. In this review, we discuss the progress of siRNA therapy as well as its medical application via nanoparticle-mediated delivery for cancer treatment.

Keywords: delivery strategies, lipoplexes, nanovectors, polymeric nanoparticles, siRNA

Introduction

Cancer has become one of the most complex diseases due to numerous genetic disorders and cellular abnormalities. Cancer disease continues to be a major health concern worldwide being the second leading cause of death in the world. According to the World Health Organization (WHO), nearly 10 million people are estimated to die of cancer by the year 2020. Cancer has been known as the number one cause of deaths in developed countries in the current century.[1–3] While remarkable efforts have been developed during the past few decades in the detection, prevention, and treatment,[4,5] signaling pathway complexities that regulate cancer progression together with its tumor microenvironment heterogeneity and metastasis remain as serious obstacles to find efficient cancer treatments.[6,7] Nowadays, treatments based on single chemotherapeutic drugs generally face lack effectiveness in cancer therapy, whereas two or more combined therapeutic methods involving various mechanisms of action are needed to achieve certain effectiveness in cancer therapy.

The currently used therapies are non-selective leading to side effects responsible for prolonged and expensive recovery, often followed by relapse at later time points. In this context, the selective targeting may provide a platform for the development of novel,

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more effective diagnostic tools and/or less harmful treatments.[8] Targeted therapies using monoclonal antibodies (mAb) against overexpressed receptors (eg, herceptin) have improved clinical outcomes.[9] Moreover, nanomaterials have also become an interesting alternative approach for the administration of drugs reducing the side effects (eg, biodegradable nanoparticles loaded with docetaxel).[10] The impact of these nanomedicines in human health may be enormous whether the right combination of targeting molecules and drugs become available with defined chemical structures. This has been demonstrated with the explosion of the antibodies-drug conjugates field.[11] However, the control of the number and position of the drugs on the antibodies, as well as the design of autoimmolative linkers to help trigger drug release after internalization is still far to be solved although this is an active field of development.[12]

Recently, much attention has been paid to the potential application of RNA interference (RNAi) for cancer treatment.[8,13] RNAi is the term given to the ability of a double-stranded RNA (dsRNA) containing a homologous sequence to a specific gene leading to sequence-specific gene silencing. RNAi is an endogenous post-transcriptional regulation process that consists of small regulatory RNAs including microRNAs (miRNAs) or small interfering RNAs (siRNAs) which are able to silence target messenger RNAs (mRNAs) in a sequence-specific procedure.[14] After the discovery of RNAi in *Caenorhabditis elegans*[15,16] and subsequent demonstration of siRNA activity in mammalian cells, RNAi has received considerable attention as an effective therapy for multiple diseases like cancer and viral infections, particularly for those diseases with "undruggable" molecular targets.[17,18]

Today, RNAi has become a powerful technology for gene function studies and has been recently used in therapeutic applications.[18–20] The main role of the RNAi in cells is the down-regulation of specific proteins (Figure 1). RNAi mechanism is triggered initially by the enzyme Dicer which cleaves double-stranded RNAs (dsRNAs) into short double-stranded siRNAs of 21–25 nt. The siRNA passenger strand is then unwound, and the siRNA guide strand is loaded into the RNA-induced silencing (RISC) complex leading to cleavage of target mRNAs by Argonaute 2 (Ago2) when the guide strand sequence is paired with an mRNA complementary sequence.[21–23] This important mechanism has allowed to open novel therapeutic approaches by designing oligonucleotide molecules through using mRNA transcripts sequences found in the existing human genomic data. Therefore, a careful sequence selection and synthesis of tailored siRNAs may

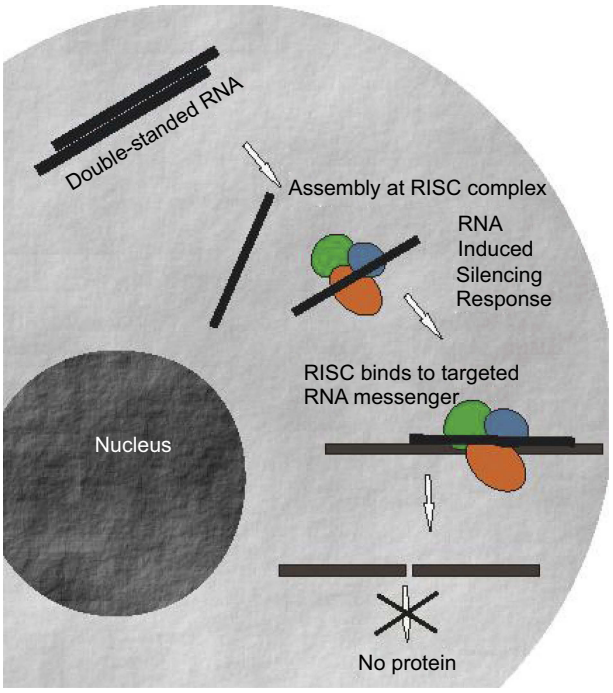

Figure 1 Mechanism of action of siRNA molecules. SiRNA duplexes are incorporated in the RNA-induced silencing complex (RISC). Then, siRNA are unwinded and the strand with lower thermodynamic stability at its 5'end remains in the complex and guides it to the complementary mRNA. The target mRNA is then cleaved and protein expression is abolished or reduced.

have enormous repercussion in therapy as almost all genes might be down-regulated as well as splice variants, separate transcripts, or mutations might also be specifically targeted. As a consequence, this powerful approach might help circumvent the limitations exhibited by small molecule drugs in conventional cancer therapy treatments leading to drug development processes based on gene functionality.[21] Therefore, the development of this therapeutic strategy may have a high impact on modern medicine.[20,24,25]

However, siRNA technology faces multiple obstacles regarding efficient delivery and effectiveness. To overcome this issue, siRNA intracellular delivery strategies should be nontoxic and stable into the site of action for succeeding therapeutic applications of RNAi.[26–28] Currently, different methods including mechanical (ultrasound), physical methods,[29] electroporation,[30] hydrodynamic tail vein injections in mice,[31] and the use of a gene gun have been carried out for delivering siRNA in vivo. In addition, local administrations (eg, intraperitoneal, intravenous, subcutaneous injections) and chemical methods based on synthesizing non-viral vehicles (eg, polymers,[32–34] cationic lipids,[35,36] and peptides[37,38]) have also been successfully used among others. To demonstrate

the potential of RNAi-based therapeutics, several proof-of-concept studies including bio-distribution, efficiency of delivery, and toxicity caused by cationic delivery vehicles have been carefully analyzed.

siRNA technology in cancer therapy

siRNA has been investigated as an effective treatment for the viral disease as well as cancer extensively with the aim of blocking multiple disease-causing genes.[39] RNAi was discovered for the first time in the nematode *Caenorhabditis elegans*; however, this process has been also found in many different eukaryotes species including, vertebrates, plants, and insects. In mammalian cells, researchers reported that synthetic siRNAs were also able to promote RNAi and therefore silence the expression of altered proteins.[15–17,40]

Numerous chemical modifications have been proposed in order to increase efficacy and potency in RNAi for in vivo use.[41] In this regard, rational chemical designs have allowed siRNA passenger strands to be more likely to modification than siRNA guide strands. These synthetic strategies have enabled to replace either non-bridging oxygen on the phosphate linkage with a sulfur atom, the 2′-hydroxyl group modification of the sugar ring with a methyl group (2′-OCH_3) and ethyl group (2′-OCH_2CH_3), among others.[42–45] In addition, other strategies have been developed to deliver siRNAs safely in the cytoplasm. While most naked siRNAs have been effective for a good number of tumor cells in vitro, these siRNAs have unfortunately failed when have injected in vivo by systemic administrations.[46]

Immunotherapy has gained increasing attention as a promising strategy for treating cancer by promoting the host immune system activation. This activation may occur by simply introducing cytokines, cancer vaccines, monoclonal antibodies, or using antigen-presenting cells (APC) through Toll-like receptors.[47] Dendritic cells (DCs) are the strongest antigen exhibiting cells that have the capacity to stimulate naïve T cells and stimulate differentiation and growth of B cells. Furthermore, they include a system of leukocytes broadly distributed in all tissues. DC therapy may offer a novel and promising immunotherapeutic method for prevention and treatment of advanced cancer as well as autoimmune disorders.[48,49]

DC immunization has been broadly studied in clinical trials involving several types of malignancies like melanoma, prostate cancer, and renal cell carcinoma (RCC), among others.[50–54] The use of DC therapy has proved to be effective and a safe strategy in inducing antitumor immunity to patients containing advanced-stage cancer diseases.[51,55] Several models have been proposed with the aim of isolating DCs in vitro. For example, Zhang et al developed a method to favor DC differentiation through contact circulating monocytes with natural killer (NK) cell subsets in order to find optimal protocols for DC vaccination.[56] According to in vivo studies, DCs can promote T cell-mediated tumor eradication and controlled tumor-bearing hosts when *ex vivo* isolation have been implemented with tumor antigens. These perceptions have prompted clinical trials to explore both immunologic and clinical impacts of antigen-loaded DCs regulated as therapeutic vaccines to patients containing specific tumors. Some promising outcomes reported from clinical trials in patients with melanoma and malignant prostate tumors showed that immunotherapeutic strategies involving the development of antigen presenting cells in DCs might demonstrate the effectiveness of this process to certain human tumors.[50] However, DC clinical effectiveness has not been as good as researchers expected in some specific cancers probably due to the heterogeneity and different methods to monocytes production as well as DC differentiation.

Interestingly, novel approaches have been implemented involving dendritic cell-based therapies and other therapies like RNAi in order to overcome these drawbacks and find a reliable vaccine for cancer therapy.[57] For example, Qian et al reported that siRNA may be combined to DC-based therapy in order to manipulate CD40 expression levels and therefore reduce the functional capability of DC to stimulate allogeneic T cells. In this regard, silencing of CD40 expression on DC might be used for generating tolerance-promoting DC with the aim of applying in autoimmunity and transplantation processes.[58]

Recently, Liu et al prepared a specific cationic nanoparticle in order to deliver indoleamine 2, 3-dioxygenase (IDO) siRNA and tyrosine-related protein 2 (Trp2) to DCs.[59] The authors were able to inhibit IDO expression, trigger T cell immune reaction and consequently favor the secretion of cytokines like IL-6, IFN-ϒ and TNF-α as well as promote DC differentiation when compared to traditional control DC vaccines. Thus, in vitro and in vivo studies in B16-F10 mouse model proved this approach to be effective in reducing melanoma tumor growth and therefore enhancing immune response in mice.

siRNA-based gene silencing

Great efforts have been made to understand how dsRNAs, siRNAs, and microRNAs (miRNAs) are able to affect

control expression in eukaryotic cells. As described previously, siRNA technology mediates specifically gene silencing at different levels like mRNA degradation, chromatin modification, and translational repression.[60–64] Remarkable findings were also found and confirmed transcriptional gene silencing was conserved in mammalian cells when were mediated by siRNAs.[65] Despite advantages and interest showed by siRNA-based technology as an effective therapeutic approach, many obstacles like efficient cellular uptake, long-term stabilities, and off-target effects have been reported. These important issues have reduced siRNA effectiveness when used in vitro and/or injected in animal models as well.

While the enhancement of siRNA stability has been successfully solved by introducing chemical modifications at the level of sugar and phosphate groups, advances in efficient siRNA delivery systems to transport siRNAs safely into the cytoplasm of targeted cells is a vital element in cancer therapy.[66,67] For this purpose, viral vectors (eg, lentivirus, retrovirus and adenovirus) have become potential vectors for siRNA delivery due to their ability to encapsulate and deliver genetic materials into cells.[68,69] Despite such viral vectors having been shown highly transfection efficacies, their clinical usage is still limited due to possible risks of immune responses, mutation, and inflammation. To minimize such undesirable effects and exploit the potential of RNAi-based technology, a good number of synthetic non-viral vectors have been proposed to impart siRNA delivery safely and effectively.[22,35]

Off-target effects and stimulation of immune response

The specificity of action carried out by siRNA molecules depends upon the uniqueness of the selected sequences; however, many other factors may also have an impact on a lack of specificity.[70] Several types involving siRNA off-target effects have been described and thoroughly reviewed.[71] It is well known that mammalian immune cells tend to express a family of receptors so-called Toll-like receptors (TLRs) which have also the ability to recognize pathogens and molecules derived from microbes. In addition, some synthetic siRNA sequences as well as their non-viral vehicles have proved to promote the activation of immune system cells producing inflammatory cytokines and Type I interferon in vitro and in vivo by activating TLR7 and TLR8 in a sequence-dependent manner.[72]

Other significant off-target effect associated to siRNA delivery falls into the miRNA-like off-target silencing. miRNAs are small non-coding endogenous RNA molecules which have the ability to regulate gene expression of several genes in the same way as siRNAs do. This undesirable sequence-specific activity is mainly produced via siRNA base-pairing with sequences located at the 3′-UTR regions of mRNAs. As a consequence, many transcripts might be affected dealing with multiple-site cleavage translational blocks.[73,74]

Finally, the RNAi machinery saturation mediated by synthetic siRNAs is another source of off-target. Some data have shown that synthetic siRNAs when entered the RNAi pathway tend to compete with endogenous miRNAs for RISC. This process has been observed in certain model studies in which both transcript upregulation and target script downregulation have been observed. Efforts to mitigate or reduce such off-target effects have been effectively studied by designing effective modified siRNAs.[75–79]

Delivery systems

Lipid-based nanovectors for siRNA delivery

Various kinds of systems have been employed for delivering siRNA such as antibody conjugates, micelles, natural polysaccharides, peptides, synthetic cationic polymers, and microparticles among others. Nevertheless, some lipid-based formulations and other lipid-like materials such as liposomes, niosomes, and stable nucleic acid lipid particles (SNALPs) have proved to be effective drug delivery systems as promising strategies for in vivo siRNA delivery[80–85] (Figure 2).

Liposomes and lipoplexes

As non-viral vectors, liposomes have become a powerful platform as a pharmaceutical carrier to facilitate the delivery of small molecule drugs and macromolecules.[86] Liposomes are made up of phospholipids which tend to form closed lipid bilayers in aqueous solvents dealing with particles formation of nanometric size. In addition to loading a good number of substances like active drugs, proteins, peptides, antibodies, and nucleic acids, liposomes have been also used in the transport of photosensitizers needed for promoting photo-dynamic therapy.[87] Second and third generation of liposomes have also been proposed by including certain ligands or loading polymers which have the ability to recognize specific receptors or enhance liposome stabilities, respectively.[88] These advances have allowed liposomes to be used in a good number of clinical

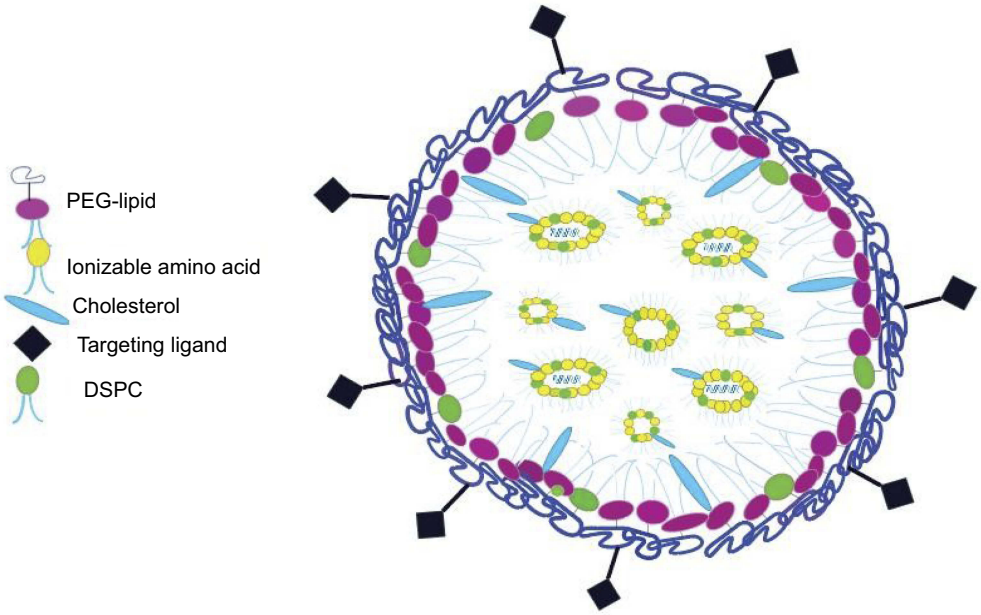

Figure 2 A schematic image of LNPs siRNA showing a nanostructured core.

applications and be also injected according to different administration routes.

Nucleic acids in particular antisense oligonucleotides (ASOs) and siRNAs have been widely incorporated into liposomal nanocarriers (Table 1).[35,62] This electrostatic combination has resulted in obtaining the corresponding lipoplexes which have helped nucleic acids increase both their long-term stabilities and cellular internalization. Positive and negatively charged together with neutral liposomes have been used as vehicles for transporting efficiently siRNA molecules[89] (Figure 3). In this regard, cationic lipids have attracted significant attention as appropriate non-viral vehicles. This interest has allowed to designing convenient synthetic strategies and characterizing a plethora of cationic lipids. Structurally, cationic lipids consist of three general components: i) the lipid tail(s); ii) the cationic head group and iii) the linkers and/or backbones that connect to both parts. Some studies have suggested a relationship between the efficiency and lipid structure; however, this involvement still remains a central goal in siRNA delivery.[82]

Table 1 Summary of siRNA-loaded encapsulated liposomes utilized for siRNA delivery

Liposome components	Target cells	Target genes	References
DOPC	HeyA8, SKOV3ip1	EphA2	13
Egg PC, Chol, PEG-PE, DOTAP, R8	SK-MES-1	HDM2	23
Lipidoid, Chol, PEG-lipids	Hepatocytes	Factor VII, ApoB	23
DOTAP, Chol, PEG-lipids	HeLa	GFP	23
DLinDMA, DSPC, Chol, PEG-C-DMA	HepG2	HBV263, HBV1583	32
DOTAP, DOPE, PEG-PE, Chol, Anti-EGFR	NCI-H322	Luciferase	105

While anionic liposomes are usually cleared from circulation, cationic liposomes (eg, DC-Cholesterol, DOTAP, and AtuFECT01 among others) in combination with helper lipids (eg, Cholesterol, DOPE, and DPhyPE) have proved efficient in siRNA delivery at optimized N/*P* ratios. However, reducing toxicity levels still remains crucial when forming these types of lipoplexes.[90–92] To solve these issues, neutral liposomes [eg, 1,2-dioleoyl-*sn*-glycero -3-phosphatidyl-choline (DOPC)] have been prepared in order to reduce such toxicity and therefore increase in biocompatibility although their entrapment efficiency might be compromised.[93]

The use of neutral lipid-based formulations has enabled the successful siRNA delivery in vivo in mouse models showing tumor growth inhibition and consequently the down-regulation of targeted genes.[67] Several siRNA-based treatments against ovarian cancer were suggested by Sood et al.[94] In this study, DOPC-encapsulated siRNA targeting the oncoprotein Ephrin Type-A receptor

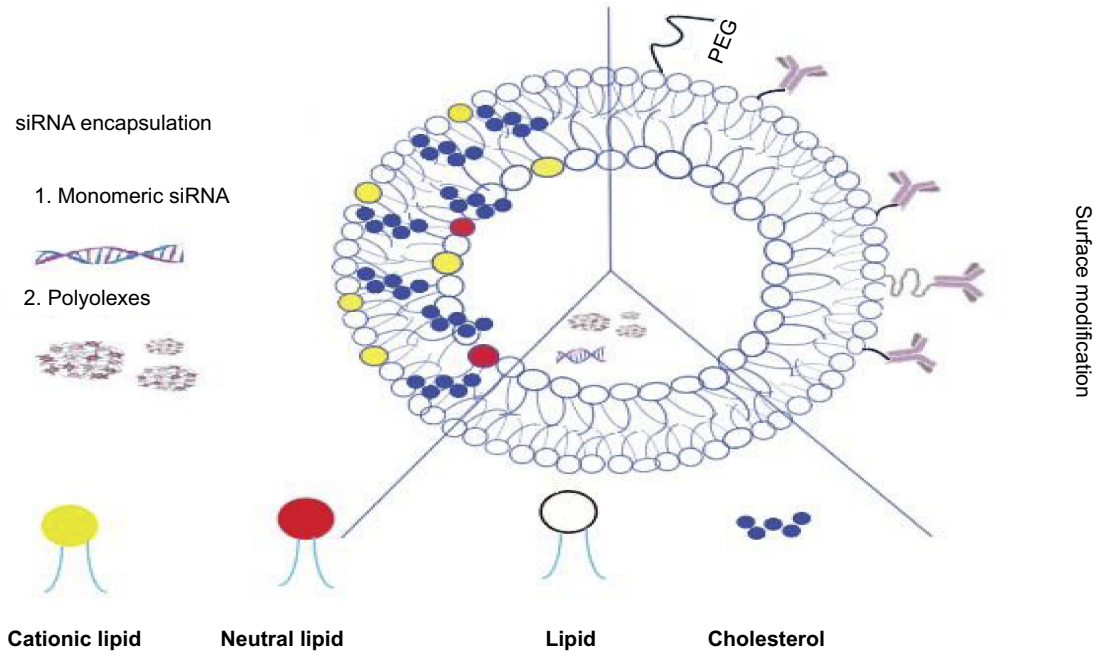

Figure 3 A schematic representation of several strategies for encapsulating siRNA in liposomes.

2 was highly effective (65%) in reducing its expression after 48 hrs of being administered in a single dose in an orthotopic model. Interestingly, the authors found this liposomal formulation when injected both intraperitoneal and intravenous reduced the ovarian tumor size in mice xenograft models with the same efficiency. In addition, treatments with DOPC-based liposomes were also studied in order to target interleukin-8, β-2 adrenergic receptor, and focal adhesion-kinase by mediating intraperitoneal delivery of siRNAs in an ovarian cancer mouse model.[94]

In spite of obtaining powerful and effective nanovehicles based on formulating lipid molecules and siRNAs, liposomes and niosomes[85] have been properly tuned in order to promote targeted delivery in specific organs and tissues. This strategy has enabled the preparation of unique lipid-based platforms bearing the arginine-glycine-aspartic acid (RGD) peptide[95] or decorated with an octa-arginine (R8) cell-penetrating peptide (CPPs).[96] These two strategies have confirmed increased particle stability, biocompatibility, and good siRNA therapeutic response without affecting remarkably cellular viability.

SNALPs

Stable nucleic acid lipid particles (SNALPs) has become a promising delivery platform for siRNA molecules in different animal models. This formulation is made up of a lipid bilayer based on a mixture of fusogenic and cationic lipids which enables endosomal release and therefore facilitates the siRNA cellular uptake. In addition, the SNALP surface is also coated during the formulation process with a PEG-lipid conjugate which provides hydrophilicity and a neutral layer with the aim of stabilizing these particles in the bloodstream when injected in vivo.[35] The first use of SNALPs was reported by Zimmermann et al to silence the apolipoprotein B (Apo B) gene expression in non-human primates with a single dose (2.5 mg/kg) of siRNA.[97] Optimized formulations based on modifying SNALPs chemically increased their siRNA efficacy in vivo by reducing doses until 0.01 $mg \cdot Kg^{-1}$ when targeted a hepatic endogenous gene.[82]

Other important applications of SNALPs have been reported and reviewed in literature.[98] For example, McLachlan et al formulated a siRNA targeting the polymerase gene in vivo in order to fully protect guinea pigs against Ebola virus.[99] More recently, SNALP technology has been successfully employed to favor the silencing of mTTR expression.[100] Clinical results confirmed the suitability and therapeutic effect of this siRNA-SNALP combination in humans giving rise to the first RNAi therapeutic in the market.[68,101]

Polymeric nanoparticles

The last decades have been witnessed the large development on polymeric nanosized materials as drug carriers becoming some of the top selling drugs.[100,102] Synthetic or naturally-

occurring nanopolymers are colloidal solid materials specially designed to be degraded in vivo without producing toxic components.[103] Several polymeric nanoparticles have been approved as drug carriers for human use.[104] Due to their excellent properties, a variety of polymers including cationic polymers such as polyethylenimine (PEI), polysaccharides such as cyclodextrin (CD) and chitosan have been studied for siRNA delivery.[105,106] As a general rule, polymer nanoparticles exhibit positively charged units to facilitate electrostatic binding of siRNA; however, the use of covalent strategies involving siRNA and polymers have been frequently prepared by using degradable linkers such as disulfide or thiol-maleimide bonds.[107]

Cyclodextrins (CDs) nanoparticles

Cyclodextrins are naturally occurring oligosaccharides that are produced during the bacterial digestion of cellulose. They have been well studied and characterized as pharmaceutical excipients with a favorable toxicological profile[108–110] (Figure 4). Cyclodextrin-containing polycation nanoparticles are exciting polymer-based siRNA delivery systems, as they are able to self-assemble with siRNA dealing to shape colloidal particles of about 50 nm in diameter. In addition, their terminal imidazole groups help in the release and intracellular trafficking of nucleic acids. A complex system consisting of a siRNA, the human transferrin protein to engage transferrin receptors on the surface of the cancer cells, a cyclodextrin-based polymer, and polyethylene glycol (PEG) has been described to increase the stability of nanoparticles in biological fluids.[111,112]

An example of this system is CALAA-01, which is a targeted nanocomplex which includes an anti-R2 siRNA.[113] This targeted therapeutic system has been designed to inhibit tumor growth. The selected siRNA prevents the tumor from growing via RNAi to decrease expression of the M2 subunit of R2 (ribonucleotide reductase). This system was used in clinical studies in patients bearing solid tumors.[114]

Chitosan and inulin nanoparticles

Chitosan and inulin are naturally occurring polysaccharides used for the preparation of siRNA formulations. Chitosan, that contains hydroxyl and amino groups, is widely used as a drug carrier due to its low cost, easy degradability and biocompatibility. Chitosan has positive charges under slightly acidic conditions allowing the formation of nanoparticles or complexes by interaction with siRNA molecules.[115,116] Interestingly, optimized chitosan formulations containing folic acid as a targeting ligand resulted in increasing siRNA cellular uptake to tumor cells producing an enhancement in gene silencing properties in HeLa and OV-3 cell lines.[116] Furthermore, grafting chitosan with polyethylene glycol[117] and polyethylenenimine[118] enable them for in vivo use through intravenous or intraperitoneal administration.[117,118] Chitosan is frequently used in combination with other carriers such as poly(L-lactide) (PLLA) porous microparticle[119,120] or the triblock polymer poly(L-lactide)-poly(ethyleneglycol)-poly(L-lactide) (PLLA-PEG-PLLA) to increase storage stability and decrease immunogenicity.[121] These formulations, prepared by supercritical fluid technology,[122] allowed the addition of multiple polymers as well as other chemotherapeutic drugs such as paclitaxel[121] and doxorubicin.[120]

Another polysaccharide used recently in siRNA delivery is inulin. This polysaccharide made up of fructose and glucose units is usually processed with oligoamines such

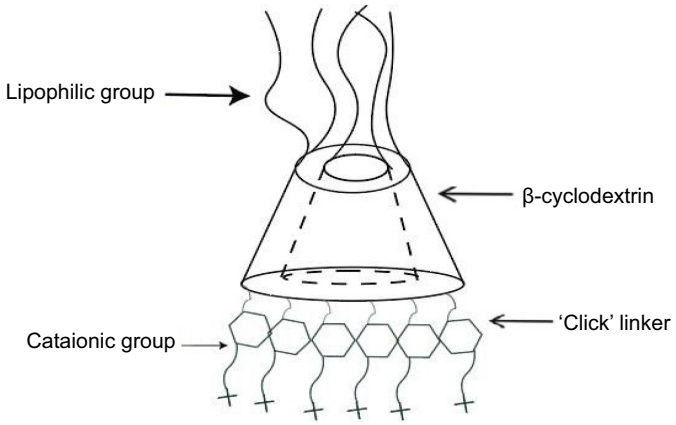

Figure 4 Schematic illustration of a Cyclodextrin structure.

as ethylenediamine[123] or diethylenetriamine and imidazole[124,125] in order to generate cationic groups for siRNA binding and therefore favor endosomal escape.

Polyethylenimine (PEI)

Polyethylenimine (PEI) is one of the most studied cationic polymers for transfection of oligonucleotides, siRNA, and plasmid DNA.[126] It is accessible either in branched or in a linear shape and many molecular weights.[127] The high charge density of PEI is considered a good property for complexation of siRNA and facilitates the endosomal escape by the proton sponge effect.[126] However, high molecular weight PEI has exhibited significant toxicity in many cell lines.[127] The use of PEI as a non-viral vehicle to deliver siRNAs was demonstrated to have an efficient antiviral effect in a guinea pig model of Ebola virus infection[128] and a murine model of influenza infection.[129] The first successful applications of PEI delivery of siRNA in cancer was the inhibition of human EGF receptor 2 (HER2) in mouse models of ovarian cancer.[130] If provided the appropriate molecular weight polymer and structure to avoid toxicities, for systemic use, PEI appears to be a promising siRNA delivery system.[131]

At present, research on PEI is directed towards the development of mixed polymers especially with PEG,[132] and polysaccharides such as chitosan[118] to reduce potential toxic effects and decrease the removal of nanoparticles by the reticuloendothelial system (RES). To direct PEI-siRNA complexes to specific target cells, PEI has been modified with receptor-mediated ligands such as folic acid, mannose, N-acetylgalactosamine, and others.[132]

Anionic polymers

Poly-L-lactic-co-glycolic acid (PLGA) is a biocompatible, well-studied, and biodegradable polymer used for decades in pharmaceutical applications.[133,134] PGLA polymer has the advantage of exhibiting lower toxicity when compared with cationic polymers and cationic lipids. On the other site, PLGA cannot form electrostatic complexes with siRNA as both are negatively charged. One way to overcome this issue is to use PLGA nanocapsules in order to encapsulate oligonucleotides bearing 5′-lipophilic molecules. These resulting polymer microspheres have provided sustained release of the modified siRNAs and antisense oligodeoxynucleotides within 24 hrs when administered subcutaneously.[135] Another interesting approach is to react polyamines or positively charged dendrimers with some of the carboxyl groups of PLGA in order to generate additional positive charges that can be used for electrostatic binding.[134] In addition, siRNAs may also be covalently linked to PLGA via intracellular cleavable disulfide linkers.[136]

Hyaluronic acid (HA) is also an anionic polymer that plays a pivotal role as a ligand against CD44 receptor which is highly overexpressed in tumor cells. The combination of PLGA and hyaluronic acid has also been used to co-deliver paclitaxel together with a siRNA against focal adhesion kinase (FAK) that is overexpressed in breast, colon and ovarian cancers.[137] The resulting PLGA nanoparticles were shown to possess a highly selective delivery of the paclitaxel and siRNA to CD44+ cells.

Cationic dendrimers

Cationic dendrimers with extremely branched peripheral chain ends are well-defined artificial macromolecules. These can be synthesized by adding several layer branches. Each branched layer represents a superior generation molecule.[133] The precise core-shell nanostructures of dendrimers enables drug loading by surface adsorption, interior encapsulation, or chemical conjugation (Figure 5). The dendrimer surface can be functionalized to build a variety of functions for a good number of applications. Dendrimers have a well-defined chemical structure and size in comparison with alternative linear polymers with a spherical form. To deliver negatively charged plasmid DNA, siRNAs, and antisense oligonucleotides, dendrimers

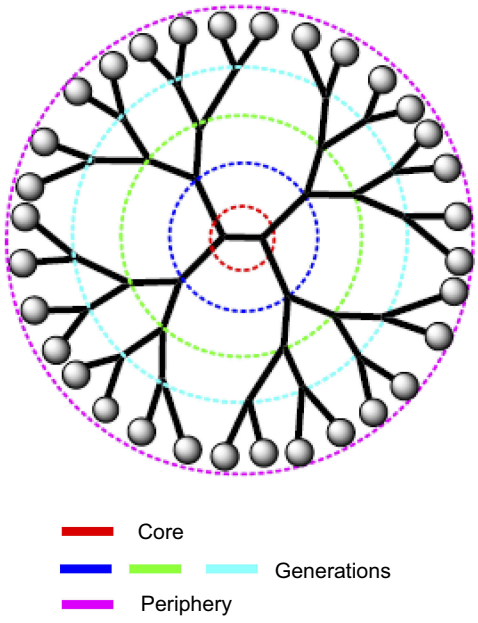

Figure 5 A schematic representation of a dendrimer showing the central core, the peripherical sites, and the consecutive generations.

containing high density of positive charges on the surface have been used.[138,139] Polycationic dendrimers including poly-propylenimine (PPI) and poly-(amidoamine) (PAMAM) dendrimers have been investigated for siRNA delivery in past years. PAMAM-mediated siRNA delivery is usually found at nucleolus and even perinuclear locations. In a study designed for increasing the siRNA loading capacity, dendrimers were additionally improved with magnetofluorescent nanoworms to create "dendriworms."[140] After adding the dendriworms carrying siRNAs to human glioblastoma cells, the siRNA-dendrimers quickly internalized into the cells and escaped into the cytosol. The delivered siRNAs were demonstrated to silence expression of the targeted gene in vivo.[141]

Carbon nanotubes (CNTs)

Carbon nanotubes (CNTs) have been considered as potential nucleic acid and drug delivery vehicles within the nanomedicine field.[142,143] They can be either single-walled CNTs (a single layer of graphene sheets) or multi-walled CNTs which can be prepared using multiple layers. In addition, CNTs can be modified with additional functional groups and also offer a structural advantage due to their very large surface. This can be used for loading therapeutic drugs like proteins and nucleic acids, and they. In order to form complexes with siRNA, positively charged functionalized single-walled CNTs (SWCNTs+) have been prepared. In a model study, siRNA designed to inhibit telomerase reverse transcriptase (TERT) was complexed with SWCNTs+ and incubated with tumor cells. The complexes TERT siRNA:SWCNTs+ were effective in delivering siRNA and knockdown the expression of TERT in a variety of tumor cells.[144]

Inorganic nanoparticles (INPs)

In the last decades, inorganic nanoparticles (INPs) have emerged as alternative nanomaterials to traditional lipid formulations for siRNA delivery.[145] Specifically, magnetic nanoparticles and gold nanoparticles have been extensively studied for siRNA delivery and in vivo imaging because of their biocompatibility and reduced toxicity.[145,146] For example, mesoporous silica nanoparticles (MSNPs) have been used as interesting vehicles for delivering siRNAs in cancer cells. Recently, Ngamcherdtrakul et al successfully prepared 50 nm INPs decorated with PEI, PEG, and an antibody.[147] These authors were able to establish a lyophilization protocol for these modified INPs and therefore promote the formation of complexes with siRNAs. Interestingly, these siRNA nanoconstructs were stable, showed luciferase silencing inhibition, and were able to display anti-proliferative effect in vitro. Other INPs like carbonate apatite have also studied by Tiash et al. In this work, the authors used these NPs as vehicles for delivering siRNA targeting GFR genes simultaneously (egfr1 and erbb2) in mouse models showing the ability of these siRNA complexes to reduce tumor growth and reduce cell viability.[148]

Magnetic nanoparticles (MNPs)

Magnetic nanoparticles (MNPs) have been extensively employed as magnetic resonance imaging (MRI) contrast agents for tumor imaging and drug delivery as well. Iron oxide nanoparticles can be synthesized to obtain particles of small nanometric size with narrow and high magnetization values. In addition, MNPs have shown to induce cancer cell apoptosis by magnetic heating because of their excellent contrast signal in MRI.[149] In addition, MNPs can be coated with siRNAs as well as other chemotherapeutic agents in order to promote delivery to the tumor site by applying external magnetic fields. Some representative examples inducing cellular death by magnetic heating have been reported.[150,151]

Gold nanoparticles (AuNPs)

Gold nanoparticles (AuNPs) have been used as effective nanomaterials for siRNA delivery applications for several reasons.[146,152,153] First, functional diversity can be easily obtained with the creation of multifunctional monolayers and second, AuNPs can be prepared in a scalable fashion with low size disparity. AuNPs have been widely utilized for gene therapy targets in preclinical animal model and in vitro studies because of their low toxicity, rapid endosomal escape, high payload, efficient uptake, increased half-life; specific, efficient, extensive transcriptional activation of the innate immune response, and selective gene silencing and transfection.[154] AuNPs were effectively employed as a platform for the delivery of Bcl-2 (B-cell lymphoma 2) or VEGF (vascular endothelial growth factor) siRNAs to a human cervical carcinoma cell line or into a human glioma cell line, for silencing of EGFP (enhanced green fluorescent protein) and LUC (luciferase) reporter genes.[155,156]

Limitations to the siRNA therapeutic approach

Table 2 summarizes some of the limitations described for siRNA delivery.[157] Degradation in serum of siRNA therapeutic molecules is one of the major limitations for the

Table 2 Intracellular and extracellular limitations of RNAi

Limitation	Solution(s)	References
A. Intracellular		
mRNA targeting	Chemical modification of siRNA	42
Endosomal escape	Acid-responsive polymers/lipid complexation	126
	Conjugation or complexation with fusogenic peptides	160
B. Extracellular		
Targeting to specific cells	Vector modification with targeting ligands	104
Degradation in serum	Peptide/polymer/lipid complexation	162
	Chemical modification with PEG, etc.	112, 113
	Nanoparticle encapsulation	172
Internalization	Peptide/polymer/lipid complexation for charge neutralization	158
	Conjugation or complexation with CPPs	161
	Ligand modification for receptor-mediated endocytosis	170, 171

therapeutic use of these molecules. Double-stranded siRNA molecules are more stable than single-stranded antisense oligonucleotides. For this reason, unmodified siRNA in saline buffer has been described to be successful for human treatments. However, these cases have been mostly focused for the treatment of ocular diseases by local administration.[158]

Most of the siRNA molecules used for therapeutic uses are partially modified since a large number of modifications may block RISC binding to siRNA and therefore prevent RNA interference mechanism.[159] Frequently these modifications are located near the 3′ and 5′-ends of the passenger and guide strands where it has been demonstrated that have a strong impact in the following properties: i) avoid exonuclease degradation action;[74,159] ii) increase affinity to RISC;[76] iii) prevent the passenger strand loading;[76] iv) lower innate immunostimulation response;[79] and v) avoid miRNA-like effects.[75] Another interesting siRNA chemical modification is the preparation of siRNA conjugates covalently,[160] especially lipid conjugates such as cholesterol. Such lipid siRNA conjugates have exhibited exonuclease resistance have promoted cellular uptake and also increased serum circulation time by binding to serum proteins.[161,162]

Carbohydrate-siRNA conjugates and especially N-acetyl-galactosamine (GalNAc)-siRNA conjugates have also being used for in vivo targeting to hepatocytes. A large impact on cellular uptake was observed with siRNAs that were conjugated with triantennary GalNAc residues. Transfection experiments corroborated high specificity and gene silencing levels in liver.[163] Peptide-siRNA conjugates especially cell-penetrating peptides (CPPs) and fusogenic peptides[160] have been also described to potentiate cellular uptake and facilitate endosomal escape being a good alternative for anti-cancer siRNA delivery.

Most of the clinical trials involving systemic delivery of siRNA molecules have used lipid formulations including liposomes, SNALPs, and other types of lipid formulations that contain cationic lipids to form complexes with siRNA molecules. One of the first human clinical trials for cancer treatment used two siRNA molecules formulated in lipid nanoparticles (LNP) to treat liver metastasis of colon cancer.[164] These formulations were actively taken up by the liver but treatment of cancer to other tissues was not demonstrated.

As shown in this review, the development of novel approaches based on nanomaterials is one of the most active areas of development for future medicines for cancer treatment. Polymeric nanoparticles are one of the most promising alternatives for the near future. The knowledge generated in the last decades for the delivery of chemotherapeutic agents using biocompatible nanopolymers will trigger the development of novel formulations that may include combinations of chemotherapeutic agents together with one or several siRNA molecules targeting overexpressed proteins for a more efficient and less toxic medicines. These nanomaterials as well as gold and SPION nanoparticles also allow the introduction of specific targeting molecules that will direct the accumulation of the therapeutic molecules near the tumors increasing the passive accumulation of nanoparticles by the enhanced permeability and retention (EPR) effect. The addition of acid responsive polymers such as PEI and peptides may also increase the efficacy by facilitating endosomal escape. A limitation on the use of nanomaterials is the potential in vivo aggregation with serum proteins and subsequent removal by the RES. This undesired effect may be prevented by the incorporation of PEG.[136]

Finally, a novel approach with high potential is the use of DNA scaffolds for the delivery of siRNA.[165] For example, DNA dumbbell,[166] DNA nanoribbons,[167] and spherical

nucleic acids (SNA)[168] have been described as alternative systems which are biodegradable, non-toxic, and also non-immunogenic. These DNA scaffolds have proved to be effective when have incorporated targeting ligands, siRNA drugs as well as chemotherapeutic drugs with high precision.[169,170]

siRNA in clinical trials for cancer therapy

Recently, RNAi-based treatment has been rapidly developed into clinical trials. In addition, it has been studied for treating diverse diseases, such as cancer, respiratory infection, AMD (age-related macular degeneration), glaucoma, and hypercholesterolemia, among others.[171] So far, at least 20 clinical trials have been initiated using siRNA- and miRNA-based therapeutics.[172] Although there are many ways to engage RNAi pathways, currently the majority of the clinical trials involve siRNA technology.[114,173–175]

In a recent report from a phase I study on patients with refractory or relapsed solid cancer, Davis et al provided the first clinical evidence that RNAi could be achieved by administering siRNA against the M2 subunit of ribonucleotide reductase (RRM2).[109]

Recently, the therapeutic potential of RNAi as a revolutionary modern class of medicine was demonstrated in cancer therapy for the treatment of inaccessible solid tumors via systemic administration by delivering Atu027[176] and CALAA-01[114] developed by Silence Therapeutics and Calando Pharmaceuticals Inc, respectively (Table 3). In 2008, CALAA-01 was employed for treating the first patient in a phase I clinical trial. In these studies, a siRNA nanoparticle targeting protein kinase N3 (PKN3) or RRM2 was administered intravenously for the treatment of solid tumors.[114] Recently Atu027, a siRNA targeting protein kinase N3 (PKN3), is being used in a clinical trial together with gemcitabine for the treatment of advanced or metastatic pancreatic cancer (clinicaltrials.gov Identifier: NCT01808638).

An interesting therapeutic approach has been shown by Tabernero et al.[177] In this study, two different siRNAs targeting two genes (vascular endothelial growth factor, VEGF and kinesin spindle protein, KSP) involved in angiogenesis were formulated in the same lipid formulation. The authors showed that siRNAs were able to promote mRNA cleavage and antitumor activity in humans. This lipid formulation (ALN-RSV) (Alnylam Pharmaceuticals) demonstrated complete regression of liver metastases and endometrial cancer in some cases.

A similar lipid formulation is being used for silencing Myc oncoprotein which is deregulated in over half of human malignancies. In a recent dose-escalation work, the clinical activity of DCR-MYC was evaluated in patients with advanced solid tumors, multiple myeloma, or lymphoma showing good clinical and metabolic responses over various dose levels.[178]

Recently, the use of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine liposomes for systemic siRNA delivery was evaluated in phase 1 clinical trial to inhibit Ephrin type-A receptor2 (EphA2) gene.[179] The liposomal formulation was well tolerated, and clinical trials are ongoing.

Table 3 Selection of clinical trials against cancer using siRNA drugs

siRNA drug	Formulation	Target	Disease	Phase	Sponsor	References
CALAA-01	Rondel® Nanoparticles (CD)	M2 subunit of ribonucleotide reductase (RRM2)	Solid tumors	I	Calando Pharmaceuticals	114
Atu027	siRNA with 2′-O-Me and Cationic lipid	Protein Kinase N3 (PKN3)	Advanced solid tumors (metastatic pancreatic cancer)	I	Silence Therapeutics GmbH	177
ALN-RSV	Lipid nanoparticles	VEGF gene and kinesin spindle protein (KSP) gene	Solid tumors (Liver metastasis from colon cancer)	I	Alnylam pharmaceuticals	178
DCR-MYC	Lipid nanoparticles	Myc	Hepatocellular carcinoma	I	Dicerna pharmaceuticals	179
siRNAEphA2-DOPC	DOPC liposomes	Ephrin type-A receptor2 (EphA2) gene	Advanced cancers	I	M.D. Anderson Cancer center	180
siG12D-LODER	LODER® (Polymer)	KRAS (mutation G12D in KRAS oncogene)	Solid tumors (advanced pancreatic cancer)	II	Silenseed Ltd	181

Abbreviations: 2′-O-Me, 2′-O-methyl-RNA units; CD, cyclodextrins; DOPC, 1,2-dioleoyl-snglycero-3-phosphatidylcholine neutral liposomes; LODER®, LOcal Drug EluteR; VEGF, vascular endothelial growth factor.

In another approach, Silenseed has used the insertion of a specialized bio-polymeric "scaffold" containing the siRNA drug into the solid tumor core. The LODER® polymer matrix has been used in a clinical trial for silencing a mutated K-RAS oncogene relevant for pancreatic ductal adenocarcinoma.[180]

Although there are not a large number of siRNAs in advanced clinical trials, RNAi is a noteworthy mechanism for advanced novel therapeutics because in cancer cells, the functionality and expression of overexpressed damaging genes are significantly reduced by siRNAs. Nonetheless, once applying RNAi techniques in vivo, the most obstacle to achieving gene silencing is the delivery of therapeutic siRNA molecules.[178] Thus, the clinical application of siRNA therapy faces several challenges to achieve effective dosages in target cells at safe and maintaining oligonucleotide stability in circulation.

The strategies for the observation of the distribution and therapeutic effects as well as techniques for enhancing cellular uptake also are essential.[18,155] First clinical trials have shown encouraging results. An interesting direction is the use of combinations of chemotherapeutic agents and siRNA as well as the combination of several siRNA targeting overexpressed proteins involved in different metabolic routes such as oncogenes, control of the nucleotide pool, angiogenesis, telomeres, antiapoptotic genes, and so on. Finally, the use of exosomes derived from mesenchymal stromal cells will be evaluated in a clinical trial for the delivery KrasG12D siRNA in patients of pancreatic cancer with KrasG12D mutation (clinicaltrials.gov Identifier: NCT03608631). Exosomes are natural lipid-particles secreted by cells that play a crucial role in protecting and transporting macromolecules such as endogenous microRNA and mRNA. These interesting properties have triggered the interest in the use of such exosomes as potential nanovehicles for internalization of therapeutic molecules.

Conclusions

Cancer is accounted for one of the most prevalent diseases with high mortality rates in the world. It is considered one of the major challenges of medical treatment and health.[181] For instance, colorectal cancer with more than 1.2 million new cases causes 600 thousand deaths per year and is the fourth cause of deaths worldwide.[182] Nearly 50,000 people will die in the United States per year and nearly 135,000 new cases will be diagnosed.[183]

In addition to surgery and classical chemotherapeutic and radiation methods, there is a need for novel and less aggressive treatments. RNA interference can be considered a promising alternative for cancer therapy as it is less toxic than classical chemotherapy. In addition, siRNAs have been studied for the treatment of various human diseases such as genetic disorders, ocular conditions, cardiovascular diseases, viral infections, and cancers. The ability to target virtually any gene(s) is one of the most attractive aspects of siRNA therapeutics when treatments involving protein-based drugs or small molecules cannot be properly used.

However, a major restriction within the therapeutic applications of siRNA is the low cellular uptake of unmodified siRNAs as they cannot penetrate the cells with high efficiency. For this reason, siRNA molecules need to be complexed or conjugated with an appropriate carrier system. In addition, their fast degradation in cellular cytoplasm and plasma lead to short half-lives. For this reason, numerous strategies involving the combination of biocompatible and versatile non-viral carriers with siRNAs containing modifications and optimized sequences have resulted in potential RNAi-based drugs as efficient medicines into the clinic.

Although a large number of excellent works have been described, future studies are required to concentrate on the in vivo safety profiles of the nanoparticle-based delivery systems like polymers, cationic lipids, dendrimers, and inorganic nanoparticles, including undesirable cytotoxicity and immune stimulation. For the clinical usage of siRNA-based cancer therapeutics, the development of biocompatible, biodegradable, and safe biodegradable nanoparticle delivery systems is still necessary together with the development of simple and reproducible protocols for the production of batches for regulatory assessment and clinical trials.

Disclosure

The authors report no conflicts of interest in this work.

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