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Review

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Interfering Cancer with Polymeric siRNA Nanocarriers

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The ability to specifically silence genes using RNA interference (RNAi) has wide therapeutic applications for the treatment of disease. Numerous studies have demonstrated global gene and protein signatures distinguishing malignant and non-malignant tissues. This worldwide pursuit of optimal cancer targets has so far provided a wide list of potential targets for each cancer type and for each patient, for which RNAi-based therapies can be applied. Nevertheless, due to poor stability of RNAi molecules in physiological conditions and their inability to cross cellular membranes, the delivery of siRNA and microRNA (miRNA) *in vivo* holds a great challenge and remains a crucial issue for their therapeutic success. Supramolecular carriers are often used in order to improve the physicochemical and biopharmaceutical properties of RNAi. Nano-sized delivery systems enable the accumulation of drugs and oligonucleotides (ONTs) in angiogenesis-dependent areas due to the enhanced permeability and retention (EPR) effect, and are able to cross cellular membranes and release the siRNA/miRNA only inside the target cell. In addition, a targeting moiety can increase the selectivity and specific uptake in the target tissue. Several vehicles (dendrimers, nanoparticles, liposomes, polyplex, lipoplex, polymeric nanoconjugates) are being developed for siRNA/miRNA delivery. These vehicles provide an important tool for exploiting the full potential of ONTs as therapeutic agents. In this review we will focus on the polymer-based approaches to deliver siRNA to cancer *in vivo*.

KEYWORDS: *Polymeric Nanoparticles, RNA Interference, siRNA, miRNA, Oligonucleotides, Polyplexes, Polymer Therapeutics, Cancer Therapeutics.*

CONTENTS

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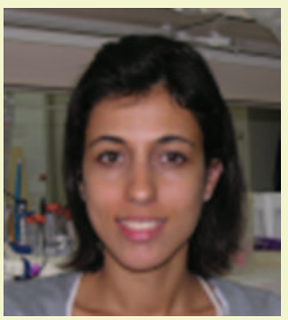
INTRODUCTION

During the last decade remarkable advances have been made in the genome research area that revolutionized the whole field. As a consequence, information may no longer be a bottleneck to understand and tackle complex genetic diseases such as cancer.[1,2] Hundreds of potential new targets are emerging and new technologies are continuously being developed to regulate gene expression. However, all this tremendous amount of knowledge does not seem to translate to novel therapies in the clinic. In this review, we will try to find the reasons for that. We will review the current approaches undertaken in the development of oligonucleotide delivery systems, pointing out the advantages and pitfalls of each one of them.

Three main mechanisms are currently used to regulate the expression of a target gene: antisense oligonucleotides (ASO), ribozymes, and RNA interference (RNAi). ASOs are short pieces of DNA or RNA complementary to messenger RNA sequences, which function by hybridizing with the mRNA inhibiting its transcription. Ribozymes are catalytically active RNA composed of three helices which cleave single-stranded regions of their own or other RNAs by trans-esterification or hydrolysis. Due to

their low stability in serum of only seconds to minutes, ribozymes have not been highly investigated for therapeutic applications. Consequently, the use of small interfering RNA (siRNA) is quickly becoming the new paradigm for gene downregulation, with reports indicating that siRNA is 1000-fold more effective than ASOs in silencing target genes.[3] The knowledge that small RNAs can so exquisitely regulate gene expression has had a tremendous impact on basic and applied research. Small double-stranded RNAs regulate the expression of specific genes at the post transcriptional level by targeting mRNAs for cleavage or translational repression[4,5] (Fig. 1). There are two types of small RNAs central to the RNAi pathway: small interfering RNA (siRNA) and microRNA (miRNA). Cytoplasmic long



Galia Tiram received her bachelor of science in the excellence program for combined life and medical sciences from Tel Aviv University (2009). She is currently undertaking the direct path to Ph.D. studies under the supervision of Professor Satchi-Fainaro. Her research is focused on elucidating the role of microRNAs in tumor dormancy and developing dormancy-promoting nanotherapeutics.

Anna Scomparin is a Postdoctoral fellow in Professor Satchi-Fainaro's laboratory at Tel Aviv University. She graduated a Master of Pharmaceutical Chemistry and Technology (2006) and received her Ph.D. in Molecular Sciences (2010), working in the Drug Delivery laboratory of Professor Caliceti at the University of Padua. She is experienced in bioconjugates for delivery of drugs and proteins. She is currently working on site-specific conjugation of siRNA to polymers for selective delivery to tumors and their vasculature.

Paula Ofek received her B.Sc. (1994) and M.Sc. (1996) from the Hebrew University of Jerusalem and her Ph.D. from Tel Aviv University (2005). Under the supervision of Professor Sara Lavi, she discovered a novel key role of Protein Phosphatase 2C in the regulation of cell cycle as a fine tuner of the p53-Mdm2 pathway. Following a postdoctoral fellowship at the Weizmann Institute of science, studying cellular mechanisms controlling membrane trafficking pathways, she joined Professor Satchi-Fainaro's research laboratory. She is currently working on the selective delivery of siRNAs and miRNAs to tumors and their vasculature.

Ronit Satchi-Fainaro is an Associate Professor and the Head of the Vascular Biology and Nanomedicine Research Laboratory at the Department of Physiology and Pharmacology, the Sackler School of Medicine in Tel Aviv University. She received her Bachelor of Pharmacy from the Hebrew University of Jerusalem, Israel (1995) and her Ph.D. from the University of London, UK (1999). She then completed a post-doctoral fellowship at Harvard University and Children's Hospital Boston, working with Dr. Judah Folkman on novel polymer-conjugated angiogenesis inhibitors to target tumor vasculature. Since 2006 she runs her own research group. Her multi-disciplinary research laboratory focuses on basic research leading to the design of highly-selective targeting molecules integrating biology, chemistry, protein engineering, molecular imaging, computational approaches, material sciences and nanotechnology to selectively guide drugs into pathological sites.

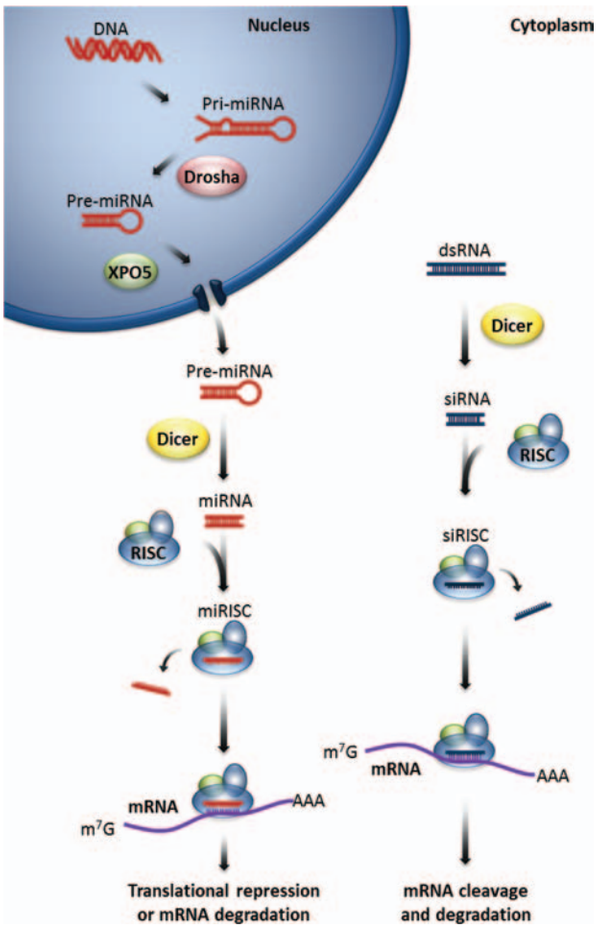


Figure 1. RNA interference mechanisms in mammalian cells. Cytoplasmic long dsRNAs (right side of the figure) are cleaved to siRNA by Dicer and incorporated into the RISC complex, which eliminates the passenger strand (sense strand) of the RNA duplex. The remaining anti-sense strand (guide strand) then directs RISC to a complementary mRNA target, leading to mRNA degradation and gene silencing. Pri-miRNs (left side of the figure) are transcribed in the nucleus, undergo catalysis by Drosha to create pre-miRNAs which are transported to the cytoplasm by exportin 5 (XPO5). Pre-miRNAs are then cleaved by Dicer into mature miRNAs, which are incorporated into the RISC complex. Following elimination of the passenger strand, the guide strand directs RISC to a partially complementary mRNA target, leading to translational repression or mRNA degradation.

double strandded RNA (dsRNA) is cleaved to siRNA by Dicer and is then incorporated into RNA Induced Silencing Complex (RISC). This protein-RNA complex separates the RNA duplex strands and eliminates the passenger (sense) strand. The remaining strand (anti-sense) then guides RISC to the complementary mRNA target, leading to mRNA degradation. miRNAs are first transcribed in the nucleus as primary miRNAs (pri-miRNAs), undergo catalysis by the RNase III drosha to create precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported to the cytoplasm by a RanGTP/exportin 5-dependent mechanism. Once in the cytoplasm, pre-miRNAs are recognized and processed into their mature ~22 nucleotides form by Dicer. From this point, the silencing mechanism is similar to that of siRNA, as it involves generation of miRNA-RISC (miRISC) complex. However, unlike siRNAs, miRNAs can recognize partially complementary target sites, and therefore are able to regulate several mRNA targets.[6,7] The RNA interference pathway can be activated by introducing a synthetic dsRNA into the cell cytoplasm or by exogenous expression of these molecules using plasmids and viral vectors.[8,9] The last two approaches will not be further discussed as they are out of the scope of this review.

Although RNAi has great potential as a therapeutic approach, there are several obstacles that must be overcame for successful and efficient delivery of RNAi-based therapies. This includes intravascular degradation of the naked RNA by RNases, aggregation of RNA nanoparticles, low cellular uptake of RNA attributed to its high molecular weight and hydrophilicity and rapid renal clearance. Local delivery of drugs can solve some of the drawbacks of RNAi delivery, since it bypasses the circulatory system. However, local delivery is not always a feasible option, mostly in cases of inaccessible tumors and their micrometastases. Moreover, local delivery still does not solve the issue of membrane crossing of the negatively charged ONTs. Therefore, most RNAi therapies administered locally in clinical trials are limited to treatment of mucosal tissues.[10] This data emphasizes the need for a proper systemic delivery system. This review will present the different approaches to deliver oligonucleotide *in vivo*, with special emphasis on siRNA-based cancer therapy applications.

DELIVERY SYSTEM CONSIDERATIONS

In order to overcome the *in vivo* limitations of RNAi as anticancer treatment, several non-viral delivery systems have been developed, the majority of them based on a lipidic or polymeric scaffold. Most polymeric carriers exploit the capability of cationic polymers to electrostatically interact with the RNAi molecules, forming polyplexes. The polyelectrolyte complexes are self-assembled through interaction between the cationic polymer and the negatively charged ONTs. In other cases polymers are used as a scaffold for chemical conjugation of RNAi moieties, forming polymeric pro-drugs able to deliver the genetic material to the target site (Fig. 2). The conjugation site of the ONT is of primary importance for maintaining its activity. The ONT duplex has four ends available for chemical modification. Since the antisense strand is the one responsible for the activity, in particular the 5′-terminus, the most preferred conjugation sites are the 3′-terminus of the antisense strand and both the 3′- and 5′-terminus of the sense strand.[11] Several studies combine RNAi conjugation to a polymeric chain with its subsequent assembly into supramolecular structures via electrostatic or hydrophobic interactions.

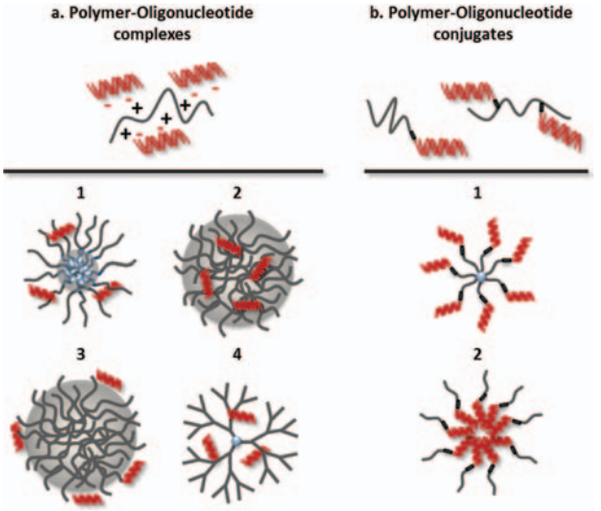


Figure 2. Polymer-oligonucleotide supramolecular structures. Oligonucleotides can be incorporated into delivery systems either via electrostatic interactions (panel a) or via covalent conjugation to the polymer (panel b). The obtained supramolecular structure differs according to the polymeric backbones used. Polymer-oligonucleotide complexes, better known as polyplexes (a), commonly form polymeric micelles (1), polymeric nanoparticles with RNAi entrapped in the core (2) or on the surface (3), and dendritic structures (4). Polymer-oligonucleotide conjugates (b) can form a random coil supramolecular structure, or self-assemble in micelles with RNAi on the external shell (1) or in the core (2) of the micelles. Cleavable linkers commonly used in the literature are disulfide bond, hydrazone bond or β-thiopropionate linkage.

Polymeric nanocarriers used for the delivery of therapeutics are generally made of natural or synthetic biodegradable polymers (e.g., poly(lactic-co-glycolic acid), Poly(ethylenimine), chitosan, etc.), although non-biodegradable scaffolds (e.g., N-(2-hydroxypropyl)methacrylamide, poly(ethyleneglycol), etc.) are also used. However, in order to successfully deliver RNAi, it is further expected from these nanocarriers to be stable in the circulatory system arrive at the target site, facilitate cellular uptake, avoid lysosomal degradation, and bypass rapid renal clearance.[12–16]

The bloodstream poses a great hurdle for RNAi systemic delivery.[17] Naked RNAs have short half-life ($t_{1/2}$) in the circulation due to rapid degradation by RNases and rapid renal clearance attributed to their low molecular weight. In order to overcome the stability problems, several chemical modifications of the RNA molecule itself have been investigated, protecting ONTs from RNase digestion. However, systemic administration of naked RNAs induces an immune response which results in their accumulation in the reticuloendothelial system (RES), i.e., lymph nodes, spleen and liver, where they are digested and cleared by macrophages. In addition, chemically-modified ONTs have been reported to have several drawbacks including off-target effects, formation of toxic metabolites and reduced activity.[18, 19] An adequate delivery system, with a supramolecular structure that exceeds the glomeruli molecular cut-off (~10 nm in diameter, ~60 kDa in Mw), can prevent rapid renal clearance and consequently increase the circulation time of RNAi molecules. In addition, many delivery agents provide steric hindrance from RNase degradation, via physical entrapment of ONTs into supramolecular systems, or via chemical conjugation with polymers. Nevertheless, encapsulation alone cannot prevent the uptake of nanoparticles by the RES. In order to reduce RES recognition, water-soluble polymers like poly(ethylene glycol) are often used as shielding agents.[20, 21]

Following their route in the circulatory system, nanoparticles are required to accumulate at the target tissue. Most delivery systems exploit the tendency of macromolecules to passively accumulate in inflamed tissues and tumors via the enhanced permeability and retention (EPR) effect (see below).[22] To accomplish successful passive delivery, the size of the nanoparticles should be 50–200 nm, large enough to avoid healthy tissue penetration and rapid renal clearance, and small enough to extravasate through the leaky angiogenic vessels and enter the target tissue. Once arriving at the target tissue, nanoparticles are further required to have the ability to promote cellular uptake. RNAi molecules are not able to cross biological membranes, due to their hydrophilicity and negative charge. To overcome this issue, the negatively-charged genetic material is often complexed with cationic polymers, forming neutral or slightly positively-charged polyplexes, which can be internalized by fluid phase pinocytosis. Improved cellular uptake can also be achieved by addition of a targeting moiety.[23] The targeted nanoparticles are directed into a specific ligand found on the cell's surface and internalized via receptor-mediated endocytosis (active targeting is further elaborated below).

After internalization, either via pinocytosis or endocytosis, the intracellular trafficking of RNAi delivery systems begins in the early endosomes and proceeds in the late endosomes. The endosomal content is then transferred into the lysosomes, where acidic pH (pH ~4.5) and hydrolytic enzymes are responsible for the degradation of macromolecules, including ONTs. Therefore, to maintain the silencing activity of RNAi moieties, nanoparticles must escape endosomal vesicles into the cytoplasm prior to their fusion with the lysosome.

Several mechanisms for endosomal escape have been proposed (Fig. 3):

"Proton sponge" effect-It has been hypothesized that polymeric backbones rich in protonable groups (e.g., poly(amidoamine), dendrimers (repeatedly branched polymers) and poly(ethylenimmine)[24, 25]) may act as "proton sponges." Under acidic conditions, amine groups in the polymeric backbone can adsorb protons, preventing the acidification of endosomal vesicles. This leads to increased

proton and chloride influx, osmotic swelling, endosomal membrane rupture and eventually leakage of the polymer-nucleic acid complex into the cytosol.[26]

Disruption of endosomal membrane-Cell penetrating peptides (CPPs) are short sequences of amino acids, usually cationic and/or amphipathic, which are able to translocate through biological membranes. Under acidic conditions, they fuse into the lipid bilayer of the endosomal membrane with consequentdestabilization of the vesicle and escape of the vesicles content into the cytoplasm.

Pore formation -It has been suggested that in the acidic endosomal environment, certain peptides can undergo a conformational change into an amphipathic alpha-helical structure. In this conformation, the peptides are incorporated into the lipid bilayer, where they are aggregated to form membrane pores responsible for the release of the genetic material into the cytosol.[27, 28]

An essential requirement from an ONT delivery system is its ability to release the genetic material in its active form into the cytosol. In the case of polyplexes, the release occurs via electrostatic dissociation of polymer-ONT complexes in the cytosol, in a process that is still mostly unknown.[29–31] In the case of polymer-ONT conjugates, the release process depends on the nature of the bond between the polymeric backbone and the RNAi strand (Fig. 4). The conjugation strategy often involves cleavable linkers to guarantee the activity of the genetic material. Polymers conjugated to ONTs via a pH-sensitive bond (hydrazone or β-thiopropionate) are cleaved in the acidic endosomal pH, and following endosomal disruption, the free polymer and ONT are released into the cytoplasm (Fig. 4(A)). Polymers conjugated to ONTs via amino-acidic linkers, specifically cleaved by endosomal enzymes,[32] follow a similar pathway of release. Polymer-RNAi conjugates containing reducible disulfide bonds escape from the endosome into the cytosol in the conjugated form, where glutathione promotes the reduction of the disulphide bond and release the RNAi (Fig. 4(B)).

POLYMER-BASED RNAI DELIVERY SYSTEMS

Several polymeric vectors have been employed for the delivery of genetic material, including natural biopolymers (e.g., chitosan, cyclodextrin, etc.) and synthetic polymers (e.g., poly(lysine), poly(ethylenimmine), poly(lactic-co-glycolic acid), etc.) (Table I). A polymeric backbone represents a very versatile platform for delivery of genetic material. Polymers, from natural or synthetic sources, can be tailored in size and charge to maximize the payload of RNAi and to obtain nanoparticles in the nano-sized scale with specific pharmacokinetic and biodistribution profile.[33, 34]

We present here the different polymeric nanocarriers used for ONTs delivery.

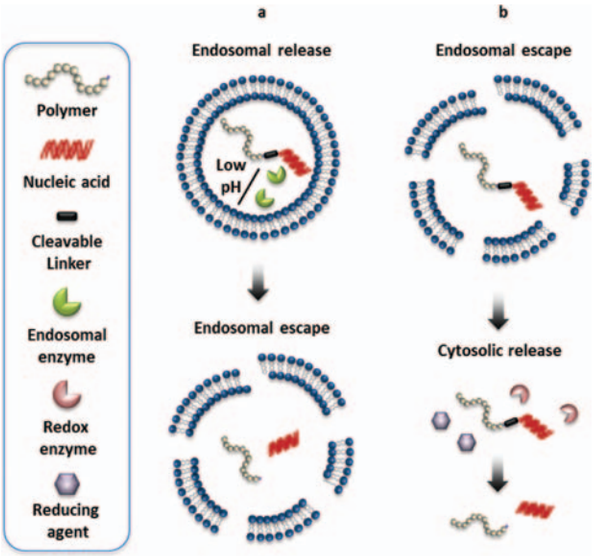


Figure 4. Intracellular release of polymer-oligonucleotide conjugates. (a) Polymers conjugated to ONTs via pH-sensitive bond or by amino-acidic linker are cleaved in the acidic endosomal pH or by endosomal enzymes, respectively, followed by endosomal escape. (b) Polymers conjugated to ONTs via reducible disulfide bonds first escape from the endosome into the cytosol, where they are cleaved by glutathione, by redox enzymes and/or any other reducing agents.

Chitosan

Chitosan is a linear cationic polysaccharide composed of *N*-acetyl-*D*-glucosamine (chitin) and *D*-glucosamine linked by β(1,4)-glycosidic linkage. Chitosan is considered optimal for ONTs delivery since it is biodegradable, biocompatible, has low immunogenicity and it is positively-charged.[35] In the past decade chitosan has been widely used to deliver pDNA (plasmid DNA),[36–38] as well as siRNA[39–43] into cells. Several parameters influence the transfection efficacy and toxicity of chitosan formulations including the molecular weight, the degree of deacetylation (DDA) of primary amines along the chitosan chain, the method of the oligonucleotide association to the chitosan and the *N*/*P* ratio (the mole ratio between the polymer amine groups and the RNAi phosphates groups).[44] Katas and Alpar, one of the first groups to investigate chitosan for siRNA delivery, characterized the *in vitro* behavior of four commercially available chitosan backbones formulated with siRNA via simple complexation or via ionic gelation of tripolyphosphate (TPP) with chitosan. No obvious relation with chitosan molecular weight and silencing activity was reported *in vitro*. However, chitosan-TPP nanoparticles with entrapped siRNA showed greater activity compared to chitosan-TPP with siRNA adsorbed on its surface and chitosan-siRNA complexes. This was attributed to the lack of protection of the genetic material from degradation by the other formulations, either due to exposure of the adsorbed siRNA to nuclease activity or due to dissociation of the

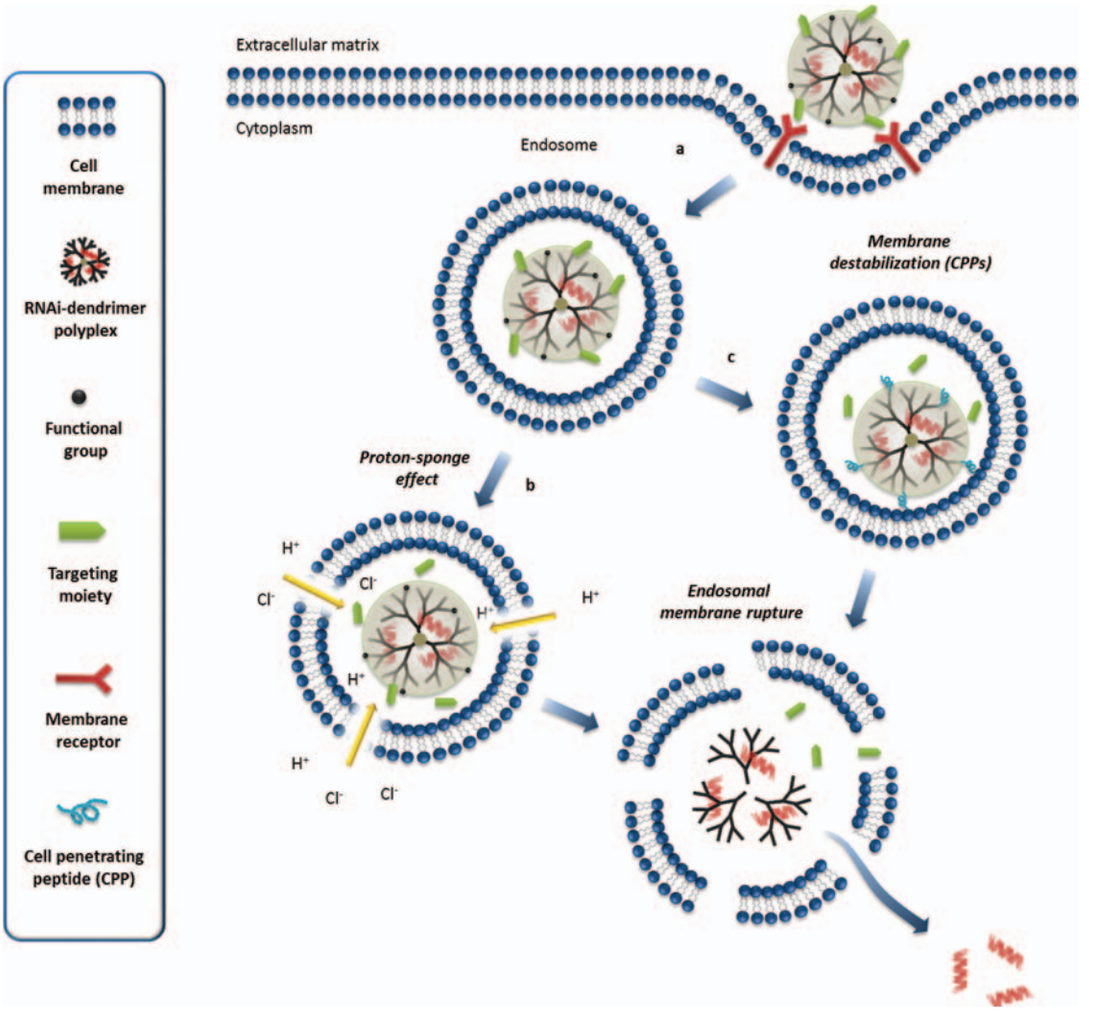


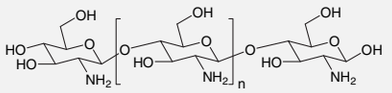
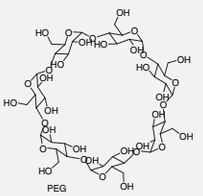
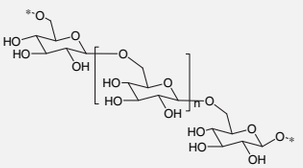
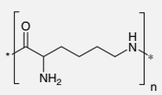
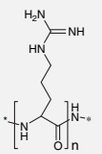
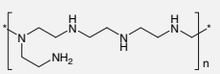
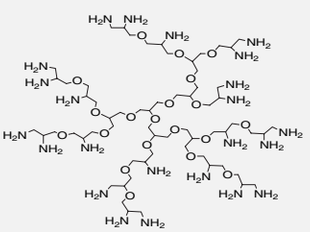
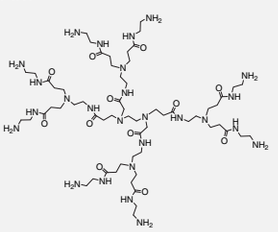
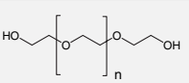
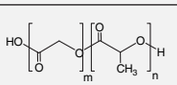
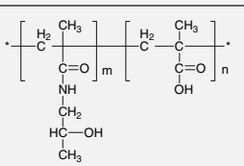
Figure 3. Endosomal escape pathways for polymeric RNAi delivery systems. (a) Polymers containing RNAi molecules and a targeting moiety are internalized via receptor-mediated endocytosis and carried out to the endosome. (b) Polymers with high buffering capacity trigger proton and chloride influx which leads to endosomal membrane rupture and release of its content into the cytosol. (c) Polymeric nanoparticles modified with cell penetrating peptides (CPPs) are able disrupt the endosomal membrane or induce the formation of membrane pores, resulting in the release of endosomal content.

weak electrostatic complexes.[45] Improved siRNA delivery can also be obtained by synthesizing structurally modified chitosan derivatives. Hydrophobically modified chitosans like 5β-cholanic acid modified glycol chitosan (CGC),[46] thiolated chitosan[47] and stearic acid-chitosan[48] were shown to have improved cellular uptake and endosomal escaping capabilities. Combining chitosan with other hydrophilic moieties (e.g., Poly(ethylene glycol) (PEG), glycol, Polyethyleneimine (PEI)), can improve chitosan solubility and transfection efficiency.[41, 46, 49, 50] Huh et al. designed a siRNA nanocarrier composed of glycol-chitosan (GC) and PEI. The addition of PEI to GC formed stable nanoparticles due to the strongly positive-charged surface, which was greater than the surface charge of pure GC alone. GC-PEI NPs were able to form stable and condensed nanoparticles with siRNA targeting Red Fluorescent Protein (RFP), undergo rapid cellular uptake and significantly inhibit RFP gene expression *in vitro* and *in vivo*.[41] Chitosan has also been used as a coating material for other siRNA delivery systems, in an attempt to improve their transfection efficiency. Chitosan-coated polyisohexylcyanoacrylate (PIHCA) encapsulated with anti-RhoA siRNA were shown to significantly inhibit tumor growth in mice bearing MDA-MB-231 tumors.[51] More recently, Jagany et al. synthehsized chitosan coated poly(*D*,L-lactic-co-glycolic acid) (PLGA) NPs complexed with siRNA targeting anti-apoptotic gene Bcl-2. These NPs were able to internalize into cells, achieve effective gene silencing and reduce tumor volume *in vivo* with higher efficiency compared to non-coated NPs.[52]

Cyclodextrin

Cyclodextrins (CD) are natural cyclic oligosaccharides composed of $\alpha(1\rightarrow4)$-linked glucose units, obtained

Table I. Chemical structures of polymeric nanocarriers and their interactions with oligonucleotides.

Chemical structure	Bond/ Interaction	References
Chitosan 	+/–	40–43, 45, 52
Cyclodextrin 	+/–	55–58, 113–115
Dextran 	+/–	61
	Disulphide	116
PLL  PLR 	+/–	66, 67, 117
PEI 	+/–	41, 70, 71, 75, 118, 119
PG-amine  PAMAM 	+/–	58, 83–90, 120, 121
PEG 	+/–	66, 67, 91, 92, 95, 96, 113, 117
	Disulphide	92, 96
	β-Thiopropionate	95
PLGA 	+/–	67, 75, 98, 122
HPMA copolymer 	+/–	104–110, 112
	Disulphide	109, 110

+/– electrostatic interaction

by enzymatic degradation of starch. CDs are mainly used as solubilizing agents for lipophilic drugs due to their ability to encapsulate molecules in their hydrophobic core. However, since CDs are non-toxic, non-immunogenic and able to provide protection from enzymatic degradation, they have also been widely used for drug delivery.[53] CD polycations can be easily complexed with ONTs via electrostatic interactions and therefore are also useful for non-viral delivery of ONTs. Since the first use of CDs for the delivery of genetic materials,[54] numerous studies have utilized them as delivery vectors for ONTs.[55] Due to their unique properties, CDs are often conjugated to other polymers to reduce their toxicity.[56–58]

Dextran

Dextran is a natural, branched, biodegradable polysaccharide formed by linear (1 → 6)- *α-d*-glucose, with branches extending from (1 → 3) position.[59] It is highly soluble in water and can be easily chemically modified via the hydroxyl pendant groups. Thanks to these features, it is widely used as a scaffold for delivery of proteins, low molecular weight drugs and imaging agents.[60] Recently dextran has been proposed as a delivery vehicle for ONTs, alone or in combination with other polymers. Aminated dextran complexed with anti-firefly luciferase siRNA showed efficient silencing *in vitro* on HeLa cells expressing luciferase, with lower toxicity compared with the common controls, PEI and lipofectamine.[61] In a different approach, siRNA oligonucleotide has been conjugated to dextran chains, either via a non-reducible thioester bond or a disulfide reducible linker. Interestingly, this RNAi delivery system did not include any cationic and/or lipid structures, so far considered as responsible for the endosomal escape mechanism.

Poly(aminoacid)

Poly(aminoacidic) polymers are synthetic biopolymers made of repeating units of amino acids. The first generation of cationic poly(aminoacidic) polymers successfully used for nucleic acid delivery into cancer cells were poly(lysine) (PLL) and poly(arginine) (PLR).[62, 63] However, due to their poor endosomal escaping abilities,[64] they are considered as inefficient transfecting agents.[65] Conjugating cationic poly(aminoacidic) polymers to other biodegradable polymers has been shown to improve transfection efficacy, reduce toxicity and facilitate safe and efficient *in vivo* silencing.[66, 67]

Polyethyleneimine

Polyethyleneimine (PEI), synthetic polymer with protonable amino groups at physiological pH, has been widely used for delivery of genetic materials. PEIs are considered a successful scaffold for RNAi delivery due to their positive charge, which promotes the formation of stable complexes with ONTs and facilitates endosomal escape via the proton sponge effect.[25] However, PEI-based delivery systems were shown to cause both cellular and systemic toxicity. PEI's toxicity is mainly attributed to its lack of biodegradability. This can be resolved by introducing reversible linkages to the PEI backbone, to obtain a branched PEI biodegradable scaffold.[68] PEI's transfection efficiency and toxicity correlate with its molecular weight as well as its structure (linear or branched). High molecular weight (HMW) PEI, often used to complex DNA, tends to form large aggregates that interact with erythrocytes and plasma proteins, leading to capillary obstructions and accelerated clearance by the RES.[69] Low molecular weight (LMW) PEI usually forms smaller aggregates compared to HMW-PEI and is therefore less toxic, but also less efficient as a transfecting agent. PEI can be obtained as a linear polymer (LPEI), which contains all secondary amine groups, or a branched polymer (BPEI), which contains primary, secondary and tertiary amino groups. While linear PEI has been proven to be more efficient and less toxic than branched PEI *in vitro*, it's *in vivo* efficacy is still controversial.[70] PEI's toxicity is also attributed to the fact that it is not biodegradable. Gosselin et al. demonstrated that by crosslinking LMW-PEI through degradable linkers to create larger, degradable structures, PEI's transfection efficacy can be improved without augmenting toxicity.[71] However, great efforts are still being made to develop PEI derivatives that will be more suitable and biocompatible for *in vivo* use. Studies have demonstrated that PEI's toxicity can be reduced by shielding its surface with other polymers, including PEG,[72, 73] polyglycerol,[74] chitosan,[41] and PLGA.[75] For example, reduced *in vitro* toxicity was received by introducing glycol chitosan to PEI. Thanks to the biocompatibility of glycol chitosan, siRNA-GC-PEI NPs did not cause any severe cytotoxicity, compared to equivalent concentrations of PEI NPs. Although the *in vivo* toxicity was investigated, GC-PEI NPs showed promising *in vivo* silencing of the target gene.[41] In a comparative *in vivo* study of PEI/siRNA complexes, it was found that PEG-modified PEI was significantly less cytotoxic compared with PEI alone, but increased the immune response to the nanocomplex.[73] Other PEI derivatives with improved biocompatibility profile have been successfully synthesized in the past few years. These include saccharide-grafted PEI,[76, 77] PEI primary amine modified to secondary amines with neutral or anionic substituents[78] and PEI combined with lipid nanoparticles.[79–81]

Dendrimers

Among the emerging polymeric vectors for the delivery of ONTs are cationic dendrimers. Dendrimers are synthetic, repetitively branched, spherical, tree-like macromolecules with three distinct structural features: dendrimer core, symmetrical branch units (each sequential branch unit is termed 'generation') and multiple functional end-groups at the periphery. Multiple dendrimer formulations

have been explored for the delivery of genetic materials into cells including poly (propyleneimine) (PPI),[82] poly(amidoamine) (PAMAM),[83] poly(L-lysine)[84, 85] and polyglycerol (PG)[86, 87] dendrimers. Most of these formulations bear cationic amine groups at the surface, thereby allowing incorporation of the negatively charged ONTs. Polycationic dendrimers offer high transfection efficacy attributed to their high buffering capacity, which facilitates endosomal escape. However, their tendency to interact with negatively charged biological membranes makes them highly toxic.[88] Surface modifications of the terminal amine groups and conjugation of hydrophilic polymers to the dendrimers surface have been reported to reduce their toxicity.[83, 89, 90] Taratula and colleagues formed BCL2- siRNA nanoparticles using PPI-based dendrimers coated with the hydrophilic PEG polymer.[82] The complex was further conjugated with a synthetic analog of luteinizing hormone-releasing hormone (LHRH) peptide, which targets cancer cells. These modifications reduced NP toxicity to A549 cells *in vitro* and improved the dendrimer's systemic delivery by stabilizing it in serum and specifically targeting A549 tumors *in vivo*. Similar modifications were performed by Kim and coworkers, which developed a PAMAM-PEG-PAMAM triblock copolymer. Despite their low solubility and the high cytotoxicity, PAMAM dendrimers are an efficient carrier for gene delivery. The PEG core conferred an enhanced water-solubility compared to the PAMAM dendrimer itself and a little cytotoxicity and high transfection efficiency comparable to that of PEI in 293 cells.[83] Poly(glycerol) (PG) dendrimers have been shown to have low toxicity and high siRNA transfection efficiency *in vitro*.[86] PG with high amine loading (PG-Amine) complexed with luciferase-siRNA demonstrated the best ratio of specific silencing efficiency versus toxicity in U-87 MG human glioblastoma cells infected with the luciferase (Luc) gene, compared with other dendritic formulations. Furthermore, *in vivo* delivery of luciferase–siRNA–PG-Amine to mice bearing luciferase-infected U-87 MG glioblastomas resulted in specific gene silencing with low levels of toxicity.[87]

Poly(ethylene glycol)

PEG is a commercially available polyether compound extensively used in the polymer therapeutics field. It is known for its biocompatibility, water solubility and ability to reduce RES recognition. PEG is usually incorporated onto the surface of polyplexes in order to shield the positive surface charge and reduce non-specific interactions in the circulation.[91] Several studies have utilized PEG itself as a carrier by covalently attaching it to the ONT backbone. Kim et al. synthesized polyelectrolyte complex (PEC) micelles from siRNA conjugated to PEG via a disulfide linker, which was further conjugated to cationic PEI as the core-forming agent.[92] Similarly, EGFP siRNA has been conjugated via a disulfide reducible bond to a poly(PEG methyl ether acrylate) obtained via RAFT polymerization, maintaining *in vitro* activity.[93, 94] In another approach, lactosylated PEG was conjugated to ONTs through an acid-labile linker.[95] Recently, well-defined triblock PEG-siRNA-PEG copolymers were synthesized via a novel method. Both the sense and the antisense strands of siRNA are bound via a reducible disulfide linker to a PEG chain, forming single-strand siRNA-PEG di-block copolymers. The tri-block copolymer was formed by annealing the two complementary strand conjugates, and further complexed with solid lipid nanoparticles as a model carrier. Although PEG was not used as a delivery vector, it was demonstrated that PEG-siRNA-PEG conjugate improved serum stability and maintained *in vitro* gene silencing activity, making it useful for *in vivo* applications.[96]

Poly(lactic-co-glycolic Acid)

Poly(lactic-co-glycolic acid) (PLGA) is a biocompatible and biodegradable FDA-approved polymer, synthesized by co-polymerization of lactic acid and glycolic acid.[66] It is widely used as drug carrier owing to its tunable properties according to polymer composition (lactic acid/glycolic acid ratio), crystallinity and MW.[97] PLGA nanoparticles are negatively charged, a feature which limits the loading capacity of ONTs and their ability to escape from lysosomal degradation. For this reason, they are often being used in combination with other cationic polymers and lipids with high affinity for ONTs, which can facilitate endosomal escape.[98]

N-(2-hydroxypropyl)methacrylamide (HPMA) Copolymer

HPMA copolymer is a non-immunogenic, non-toxic, water soluble polymer widely used for the delivery of low molecular weight anti-cancer drugs.[99–103] Recently, it is also being used for the delivery of ONTs. Unlike the aforementioned formulations which are simply complexed with ONTs via electrostatic interactions, HPMA copolymer is not positively charged and therefore requires modifications and/or addition of cationic moieties to form polyplexes. Indeed, in most reports HPMA copolymer is complexed with ONTs following combination with other cationic polymers like 2-(trimethylammonio)ethyl methacrylate (TMAEM),[104] PLL,[105, 106] N-(3-aminopropyl)methacrylamide (APMA)[107] and N-[3-(dimethylamino)propyl] methacrylamide (DMA PMA).[108] HPMA can also be coupled directly to ONTs through a disulfide bond between HPMA copolymer containing active-sulfhydryl groups and thiolated ONTs.[109, 110] This disulfide bond was shown to be stable in the bloodstream and can be cleaved by intracellular thiols such as glutathione or by redox enzymes.[111] A different approach for conjugation, proposed by Jensen and colleagues, is by covalently attaching ONTs to HPMA copolymer via a lysosomally degradable tetrapeptide spacer.[112] It was demonstrated that this conjugate is able to release the

ONTs from the polymer, escape lysosomal degradation and enter the cytoplasm. It should be taken into consideration that since it is not biodegradable, HPMA copolymer backbone alone should not exceed 50 kDa to allow renal clearance.

RNAI THERAPEUTICS IN CANCER

Until recently, most anticancer therapeutics were mostly based on broad-ranged cytotoxic agents, which often lead to undesirable adverse effects due to their low specificity. In the past few decades, a more personalized approach for cancer therapy has risen, by which each patient's treatment can be tailor-made based on the molecular genetics of the cancer. This approach has brought to the development of several successful targeted therapies, mainly small molecule inhibitors and monoclonal antibodies.[123–126] However, due to the low specificity of small molecule inhibitors and the inaccessibility of specific proteins for targeted antibodies, these strategies are not ideal for many cancer targets.

The realization that RNAi machinery can be harnessed for therapeutic gene silencing in cancer has led to the rapid development of tumor-targeted RNAi-based therapies. Anticancer siRNAs usually inhibit the translation of key players that regulate oncogenic pathways, cell-cycle progression, cellular senescence, tumor angiogenesis (i.e., the formation of new blood vessels from pre-existing ones), invasion and metastasis.[127, 128] Since local administration to tumors is not always feasible, great efforts are being made on the development of vehicles for systemic delivery for RNAi, which are also applicable for the treatment of micrometastases. Most delivery systems rely on the enhanced permeability and retention (EPR) effect to ensure passive accumulation of macromolecules at the tumor site (see below). Ligand targeted delivery to tumors and their stroma can significantly improve the efficiency and specificity of the nanoparticles, reducing non-specific toxicities associated with RNAi. A parallel approach for obtaining improved anti-tumor efficiency is by co-delivery of ONTs with other anti-cancer drugs, to achieve a synergistic inhibitory effect on tumor growth. The following section focuses on some of the recent efforts made to deliver ONTs to tumors *in vivo* using polymeric nanocarriers (Table II).

Local Delivery

Local administration of siRNA enables high bioavailability of the RNAi molecules at the target tissue with low effective dose. Direct delivery also allows for more focused delivery of siRNA, which might circumvent any theoretical, undesired side effects resulting from systemic delivery. Therefore, when the tumor area is limited to one well-defined specific site, reachable from the body surface (e.g., melanoma, lung cancer, breast cancer), local delivery of therapeutics might be the preferred approach. If the tumor site can be easily reached (e.g., intradermally or intranasally), systemic administration has no advantages and many potential hurdles to deal with. However, despite the clear benefits of local delivery derived from the technical ease of the delivery approach, efficiency and selectivity, local administration is applicable for restricted types of tissues, which are accessible for invasive delivery.

Moreover, siRNA is often locally administered to examine the potential feasibility of a novel RNAi-based therapy. This approach is frequently used as a proof of principle for certain therapeutics or delivery systems as exemplified in a study by Ofek et al.[87] A few examples of recently developed local delivery approaches are following.

Salva et al. demonstrated reduced breast cancer growth and angiogenesis following local delivery of chitosan/Vascular Endothelial Growth Factor (VEGF) siRNA nanoplexes. VEGF is an important angiogenic factor, found to be associated with tumor growth and metastasis., It is widely used as a therapeutic target in several cancer types.[92, 113, 117, 129] These chitosan-VEGF nanoplexes showed a remarkable reduction of 97% in tumor volume, which was correlated with a reduction of VEGF protein levels within the tumors.[43] Tumor targeted mesoporous silica nanoparticles (MSN) coated with PEG were developed for inhalation treatment of lung cancer.[130] The system was capable of effectively delivering anticancer drugs (doxorubicin and cisplatin) combined with two types of siRNA targeted to MRP1 (Multidrug Resistance-associated Protein 1) and BCL2 (apoptosis-regulator protein) in non-small cell lung carcinoma. Suppression of cellular resistance by siRNA effectively delivered inside cancer cells and substantially enhanced the cytotoxicity of anticancer drugs. Local delivery of MSN by inhalation led to the preferential accumulation of nanoparticles in the mouse lungs, prevented the escape of MSN into the systemic circulation, and limited their accumulation in other organs. A chitosan hydrogel (CH-HG) displayed a liquid–solid phase transition in a temperature-dependent manner and formed an endothermic hydrogel in tumor tissue after intra-tumoral injection.[42] Tissue transglutaminase 2 (TG2) is overexpressed in epithelial ovarian cancer (EOC) and promotes intraperitoneal metastasis. For therapeutic proof-of-concept studies, CH-HG including TG2-targeted siRNA significantly inhibited tumor growth in melanoma (A375SM) and breast (MDA-MB-231) tumor models.

Currently in phase I clinical trial is siG12D LODER, a slow release biodegradable polymeric matrix containing small-interfering RNAs for the mutated KRAS oncogene, KRASG12D, (siG12D) for patients with adenocarcinoma of the pancreas (Silenseed Ltd., Israel). Upon intratumoral injection, siG12D is released locally, thereby preventing translation of KRAS proteins and potentially inhibiting growth of tumor cells overexpressing KRAS. KRAS, a member of the small GTPase superfamily, is mutated in

Table II. Selected examples of polymeric nanocarriers used to deliver oligonucleotides to cancer *in vivo*.

Delivery system/ formulation	Route of administration	Active targeting	Target gene	*In vivo* model	Reference
Chitosan	IT	–	VEGF	Breast cancer	[43]
Chitosan hydrogel	IT	–	TG2	Melanoma and breast cancers	[42]
PG-amine	IT/IV	–	Luciferase	Glioblastoma/Breast cancer cells overexpressing luciferase	[87]
PEI	IV	–	hTRT	Liver cancer	[119]
Chitosan-PIBCA	IV	–	ret/PTC1	Papillary thyroid cancer	[40]
Glycol Chitosan-PEI	IV	–	RFP	Melanoma cells overexpressing RFP	[41]
PAMAM-PEG-DOPE	IV	–	GFP	Melanoma cells overexpressing GFP	[121]
PAMAM-hyaluronic acid	IV	–	MVP	Breast cancer	[120]
PAMAM-PEG-α-CD	IV	Folate	Luciferase	Colon cancer cells overexpressing luciferase	[58]
PLGA-PEI	IV	Biotin	PgP	Breast cancer	[75]
PEG-β-CD	IV	Anisamide	VEGF	Prostate cancer	[113]
PEG-b-PLL	IV	RGD	VEGF and VEGFR2	Cervical cancer	[117]
PLGA-PLL-PEG	IV	iRGD	PLK1	Melanoma	[122]

Note: IT, Intratumoral; IV, Intravenous.

over 90% of human pancreatic ductal adenocarcinomas (PDAC) and is associated with tumor cell proliferation and reduced survival.[131, 132]

Passive Systemic Delivery

During the initial stages of tumor development, cancer cells recruit new blood vessels to increase the availability of oxygen and nutrients, in a process termed cancer angiogenesis.[133] The newly-formed vessels are characterized by defective endothelium with wide pores and fenestrations, which lead to tumor vessel leakiness. In addition, tumor tissues usually have impaired lymphatic drainage. These two factors together contribute to a phenomenon by which macromolecules passively accumulate at the tumor tissue, also known as the EPR effect.[22] An efficient passive delivery of polyplexes can therefore be obtained only when the nanoparticle's diameter ranges from 50 to 200 nm. At this size, particles can penetrate tumor leaky vessels but not healthy vessels, and be retained for a long period of time due to poor lymphatic drainage from the tumor site.

Numerous studies have demonstrated a successful systemic delivery of ONTs by polymeric nanocarriers using passive targeting strategies. Recently, a biodegradable PEI formed by disulfide crosslinking of oligoethylenimine (800 Da) and complexed with telomerase reverse transcriptase (hTRT) siRNA, was synthesized. hTRT, a catalytic subunit of the enzyme telomerase, is overexpressed by most tumor types and offers a potential target for cancer therapy, as telomerase inhibition can lead to apoptosis in cancer cells without affecting normal cells. This PEI-siRNA polyplex showed low *in vitro* cytotoxicity, strong siRNA complexation ability, and intracellular siRNA release. However, distribution data showed accumulation in the liver, probably due to opsonization of the complex. The polyplex was also found to have limited activity in inhibiting the growth of HepG2 tumors.[119]

Enhanced antitumor activity was obtained following systemic administration of chitosan-coated poly (isobutylcyanoacrylate) (PIBCA) nanoparticle loaded with siRNA targeting the ret/PTC1 fusion oncogene. Ret/PTC1 mutation is the most common genetic alteration in thyroid papillary carcinomas and represents an interesting target since it is expressed only by tumor cells. Following IV administration of the polyplex to mice bearing RP1 tumors, a significant reduction in tumor growth occurred. RNA extraction from tumoral tissues revealed a correlation between tumor growth inhibition and ret/PTC1 mRNA levels reduction.[40]

Polymeric carriers are often used in combination with other polymeric formulations for improved systemic RNAi delivery. Promising *in vivo* silencing activity has been obtained with self-assembling glycol chitosan (GC)/polyethylenimine (PEI) nanoparticles (GC–PEI NPs) loaded with Red fluorescence protein (RFP)-siRNA. These nanoparticles were able to reduce PFP gene expression in RFP/B16F10 cells *in vitro* and in RFP/B16F10 tumors following systemic administration.[41] Recently, a low molecular weight PEG (mPEG2000) was used as a building block for an amphiphilic triblock copolymer with poly(d,l-lactide) as the hydrophobic component and poly(arginine) as the cationic element. The obtained triblock copolymer was able to self-assemble in a supramolecular structure forming micelleplexes with siRNA. The system was capable to induce cell uptake and endosomal escape, *in vitro*, by silencing the target gene in MCF-7 breast cancer cells. It also showed an impressive effect *in vivo*, inhibiting tumor growth in a MCF-7 xenograft tumor model, without exhibiting significant toxicity and evoking immune response.[66] Similarly, a copolymer formed by conjugating metoxy-PEG with another poly(aminoacidic) polymer, PLL, and with PLGA, has been synthesized to combine the RNAi loading efficiency of PLL, the ability of forming supramolecular NP of PLGA and the shielding properties of PEG. This copolymer was used to form nanoparticles

that were loaded with siRNA. *In vivo* systemic administration of the NPs to huh-7 hepatic carcinoma-bearing mice resulted in efficient delivery of siRNA to the tumors. This was attributed mainly to the long circulation achieved by surface PEGylation and the enhanced cellular uptake of the NPs.[67] A novel supramolecular system formed by a triblock copolymer of G(4)-PAMAM-PEG-DOPE has been developed for the co-delivery of hydrophobic drugs (doxorubicin) and siRNA. PAMAM dendrimers provide the cation source for complexation of the siRNA, and the lipid component promotes cell penetration. The obtained mixed micellar system combined the features of dendrimers and polymeric micelles and appears to be a promising carrier for drug/siRNA co-delivery, although the *in vivo* delivery properties have still to be investigated.[121] Similarly, PAMAM dendrimers have been used as scaffold for the conjugation of hyaluronic acid to obtain a supramolecular system for co-delivery of doxorubicin and major vault protein (MVP) targeted small-interfering RNA (MVP-siRNA). The obtained system shows adequate tumor targeting, higher intracellular accumulation, increased blood circulating time and less *in vivo* toxicity compared to free doxorubicin. In addition, delivery of siRNA by PAMAM-HA exhibited efficient gene silencing, enhanced stability and intracellular delivery, and synergistic anticancer activity with doxorubicin.[120]

Active Systemic Delivery

The degree of tumor vascularization can vary within the different tumor types and stages, therefore relying solely on the EPR effect has its limitations. The addition of a targeting moiety can significantly reduce the nanoparticle's off-target effects by specifically directing it to tumor cells or tumor microenvironment cells (e.g., tumor endothelial cells, cancer-associated fibroblasts and immune cells). Once in the tumor site, the targeted nanoparticles are internalized into the target cells via receptor mediated endocytosis. Most polymeric nanocarriers incorporate moieties directed to cell surface receptors or with high affinity to molecules found in the extracellular matrix of the tumor site.[58, 75, 103, 113, 134] Although monoclonal antibodies have been successfully used for targeted delivery of nanoparticles, their use for targeted delivery is limited due to their large size and difficult conjugation.

The most common targeting moieties used to target cancer cells aim for receptors overexpressed on various types of cancers like transferrin and folate receptors,[135, 136] thus offering a broad spectrum of therapeutic activity. The first polyplex formulation to enter phase I clinical trials was CALAA-01 (RONDEL Technology), cyclodextrin-based RNAi nanoparticles bearing transferrin moieties. The nanoparticles were formed by a linear cationic polymer alternated with cyclodextrin units bearing PEG coating on the surface, which were further conjugated with transferrin on the external end of the PEG chains. This complex was loaded with siRNA targeting the M2 subunit of ribonucleotide reductase (RRM2), an enzyme involved in tumor invasion and metastasis. In a phase I clinical trial of CALAA-01 for the treatment of solid tumors (#NCT00689065), i.v. administration resulted in a dose-dependent accumulation of the NPs within the tumors and reduction of RRM2 mRNA and protein levels.[114, 115]

Recently, Arima and colleagues synthesized a generation 3 (G3) PAMAM STARBURST dendrimer conjugate with α-cyclodextrin (α-CD) and decorated with folate-PEG, Fol-PαCs. This structure was able to efficiently load siRNA and internalize into KB cells overexpressing the folate receptor *in vitro*. Fol-PαCs complexed with luciferase-siRNA showed RNAi effects following intratumoral and intravenous administration to mice bearing colon tumors, with negligible toxicity to other organs.[58]

Another ligand widely used for cancer targeting is biotin, as receptors involved in the uptake of biotin were found to be overexpressed in many cancer cells.[137] PLGA-PEI NPs decorated with biotin as a targeting agent were used to deliver paclitaxel and P-glycoprotein (P-gp) targeting siRNA. P-gp is a drug efflux transporter involved in multi-drug resistance (MDR), which is often overexpressed in cancer cells. The combination of co-delivering siRNA with a potent chemotherapeutic agent and a targeting moiety was demonstrated as an efficient method to inhibit tumor growth and overcome drug resistance in tumor bearing BALB/c mice.[75]

Tissue specific targeting, based on markers uniquely expressed on specific tumors, offers an improved, more personalized approach for cancer therapy. Anisamide, a ligand targeting the sigma receptor overexpressed on prostate cancer cells, is widely used to deliver therapeutics to prostate cancer. Recently, PEGylated β-cyclodextrins particles were complexed with siRNA targeting VEGF and conjugated to anisamide to confer *in vivo* active targeting properties. The complex showed significant inhibition of tumor growth and reduction of VEGF mRNA levels following i.v. injection to TRAMP C1 tumor-bearing mice.[113]

Another approach extensively used to deliver therapeutics to tumors is targeting cellular components of the tumor microenvironment. Arg-Gly-Asp (RGD) peptide, which targets the $\alpha v\beta 3$ integrin found on tumor endothelium, is one of the most common ligands used to target tumor microenvironment.[138] Micelles formed from PEG-b-PLL and conjugated with cyclic-RGD (cRGD) were recently used to deliver VEGF and VEGFR2 siRNAs to tumor associated blood vessels. cRGD incorporation improved the cellular uptake *in vitro* and the accumulation in both tumor mass and tumor blood vessels following i.v. administration. Moreover, the micellar NPs were able to effectively reduce VEGF mRNA levels and inhibit tumor growth of HeLa xenografts.[117] Zhou et al. synthesized a similar formulation of PLGA-PLL-PEG, which was introduced with iRGD (CRGDKGPDC) peptide, a modified form of RGD, which is reported to significantly enhance tumor targeting. NPs were further encapsulated

with siRNA targeting the proto-oncogene serine/threonine-protein kinase 1 (PLK1). I.v. injections to mice bearing A549 xenografts resulted in decreased tumor growth with efficient inhibition of PLK1 gene expression.[122] The NGR peptide (Cys-Asn-Gly-Arg) is another ligand used to target angiogenic endothelial cells, as it targets aminopeptidase N found in tumor vasculature. A novel siRNA delivery system was synthesized by Zhang and coworkers, formed by single-walled carbon nanotubes modified with PEI for the complexation of hTERT siRNA, bearing the NGR peptides. The system showed transfection efficiency in PC-3, causing significant tumor cell growth inhibition, and a significant silencing effect towards the target gene both *in vitro* and *in vivo*. In addition, the combined treatment with the SWNT-PEI/siRNA/NGR and near-infrared (NIR) photothermal therapy significantly enhanced the therapeutic ef?cacy in PC-3 tumor-bearing mice.[118]

CONCLUSIONS

Although great progress has already been made in the field of RNAi, the translation of this evolvement to cancer therapy is still limited. The development of an adequate delivery system appears to be fundamental for the application of RNAi in the clinic. Several efforts have been made in order to increase transfection efficacy and reduce toxicity of the delivery systems, and indeed some of the obtained formulations showed good activity and tolerability *in vivo*. However, some obstacles still remain on the way to the clinic. The ultimate goal of achieving RNAi-based therapies cannot be accomplished without improving the safety, effectiveness and reliability of RNAi-delivery systems. Better characterization of the physico-chemical composition of the carrier-RNAi supramolecular structures and a deeper understanding of the intracellular pathways they go through, will lead to rational design of safe and efficient RNAi delivery systems suitable for clinical use. Given the way that RNAi has transformed basic and translational research and the great advances in polymer therapeutics, we will hopefully witness a revolutionary improvement in the RNAi-based nanomedicines field in the coming years.

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