Review

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Anticancer Use of Nanoparticles as Nucleic Acid Carriers

D. Gozuacik[1,*], H. F. Yagci-Acar[3], Y. Akkoc[1], A. Kosar[2], A. Isin Dogan-Ekici[4], and Sinan Ekici[5]

[1] *Faculty of Engineering and Natural Sciences, Biological Sciences and Bioengineering Program, Sabanci University, Orhanli-Tuzla, 34956 Istanbul, Turkey*
[2] *Faculty of Engineering and Natural Sciences, Mechatronics Engineering Program, Sabanci University, 34956 Istanbul, Turkey*
[3] *Department of Chemistry and Graduate School of Materials Science and Engineering, Koc University, Rumelifeneri Yolu, Sarıyer, 34450 Istanbul, Turkey*
[4] *Department of Pathology, Yeditepe University School of Medicine, Atasehir, 34755 Istanbul, Turkey*
[5] *Department of Urology, Maltepe University School of Medicine, Maltepe, 34843 Istanbul, Turkey*

Advances in nanotechnology opened up new horizons in the field of cancer research. Nanoparticles made of various organic and inorganic materials and with different optical, magnetic and physical characteristics have the potential to revolutionize the way we diagnose, treat and follow-up cancers. Importantly, designs that might allow tumor-specific targeting and lesser side effects may be produced. Nanoparticles may be tailored to carry conventional chemotherapeutics or new generation organic drugs. Currently, most of the drugs that are commonly used, are small chemical molecules targeting disease-related enzymes. Recent progress in RNA interference technologies showed that, even proteins that are considered to be "undruggable" by small chemical molecules, might be targeted by small RNAs for the purpose of curing diseases, including cancer. In fact, small RNAs such as siRNAs, shRNAs and miRNAs can drastically change cellular levels of almost any given disease-associated protein or protein group, resulting in a therapeutic effect. Gene therapy attempts were failing mainly due to delivery viral vector-related side effects. Biocompatible, non-toxic and efficient nanoparticle carriers raise new hopes for the gene therapy of cancer. In this review article, we discuss new advances in nucleic acid and especially RNA carrier nanoparticles, and summarize recent progress about their use in cancer therapy.

KEYWORDS: *Cancer, Drug, Chemotherapy, Nanomedicine, Nanoparticles, RNA Interference, siRNA, shRNA, microRNA, Nanocarriers, Targeted Therapy.*

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*Author to whom correspondence should be addressed.
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INTRODUCTION

In spite of the advances in medical sciences and technologies, cancer is still one of the leading causes of mortality

and morbidity in developed and developing countries.[1] Classical treatment modalities, namely chemotherapy, radiotherapy and surgery, have been used from the beginning of 20th century with limited treatment success rates, especially for advanced stage disease. In the last decades, chemotherapy and radiotherapy approaches evolved to minimize toxicity, invasiveness and side effects while preserving effectiveness. Yet, the basic action mode of both chemotherapy and radiotherapy is perturbation of the division and induction of the death of all rapidly proliferating

D. Gozuacik, M.D. Ph.D., is an Associate Professor in the Biological Sciences and Bioengineering Program at Sabanci University, Istanbul, Turkey. He received his Medical Doctor (MD) degree in 1995 from Hacettepe Medical Faculty, Ankara, Turkey, his D.E.A degree of biochemistry from Ecole Polytechnique, Paris, France, and his Ph.D. degree on Molecular Cell Biology in 2001, from Paris XI University and Pasteur Institute, Paris. Before joining Sabanci in 2006, he worked as a postdoctoral researcher in the Weizmann Institute of Science (2001–2006) on cancer biology. His current research focus is cellular death and stress responses including autophagy in health and disease, RNA interference therapies and nano-drugs. Dr. Gozuacik is a board of directors member of the International Cell Death Society (ICDS), editor of the Autophagy journal (IF: 12) and an *ad hoc* referee of various international journals and granting agencies. He received excellence awards from several institutions including European Molecular Biology Organization (EMBO), Roche Pharmaceuticals and Turkish Academy of Sciences.

H. F. Yagci-Acar, Ph.D. is an Associate Professor in the Chemistry Department at Koç University, Istanbul. She received her B.S and M.S. degrees in Chemistry from Bogazici University, Istanbul Turkey, in 1993 and 1995, respectively. She received her Ph.D. in Polymer Science and Engineering from University of Southern Mississippi, Hattiesburg, in 1999. She worked as a post-doctoral fellow at GE GRC-Niskayuna between 2000 to 2002 and as a lead scientist until 2004, then she joined Koç University in 2004. Her research focuses on the development of novel polymers (living or conventional methods), magnetic nanoparticles, luminescent semiconductor quantum dots and hybrid nanomaterials for biotechnology, medicine and energy applications. She received National L'Oreal Women in Science Reward in Materials Science in 2005.

Y. Akkoc is a Ph.D. student in the Biological Sciences and Bioengineering Program at Sabanci University, Istanbul. He recieved his B.Sc. degree from the Molecular Biology and Genetics Department of Istanbul Kultur University in 2013. His research interests include role of natural polyamines in cancer progression, basic autophagy mechanisms, and gene therapy of autophagy abnormalities.

A. Kosar, Ph.D. is an Associate Professor in the Mechatronics Program at Sabanci University, Istanbul. He received the B.Sc. degree in Mechanical Engineering from Bogazici (Bosphorus) University, Istanbul. He pursued his graduate studies in the Department of Mechanical Engineering at Rensselaer Polytechnic Institute, where he completed his M.S. and Ph.D. degrees. His research interests include micro/nano-scale heat transfer and fluid flow, cooling technologies, magnetic manipulation of nanoparticles and magnetic hyperthermia therapy. Dr. Kosar is a recipient of various national and international awards including Turkish Academy of Sciences Outstanding Young Investigator Award (GEBIP) and TUBITAK (The Scientific and Technological Research Council of Turkey) Incentive Award.

A. Isin Dogan-Ekici, M.D. is an Associate Professor in the Pathology Department at the Yeditepe University Hospital, Istanbul. She graduated from Hacettepe University School of Medicine in 1997. Dr. Dogan-Ekici obtained her pathology specialist degree from Hacettepe University School of Medicine Department of Pathology in 2003. Her research interests include surgical pathology, nephropathology and urogenital system pathology and pathology of gene therapy models.

Sinan Ekici, M.D. is a Professor in the Department of Urology at the Maltepe University School of Medicine. He graduated from Hacettepe University School of Medicine in 1995. He obtained his urology specialist degree from Hacettepe University School of Medicine, Department of Urology in 2000. Dr. Ekici worked as a clinical observer in Memorial Sloan Kettering Cancer Center, New York and worked as research and clinical fellow in the Department of Urooncology, Miami University School of Medicine, USA. His research interests include urooncology, endoscopic urology, cancer biology, gene therapy of urological cancers.

cells in a non-selective way. Consequently, both of these approaches are accompanied with side effects ranging from hair loss and gastrointestinal (GI) problems (hair follicles and GI tract cells are amongst most rapidly proliferating cells in the body) to secondary cancer development (e.g., Leukemias) due to DNA damage. Therefore, development of targeted and cancer cell-selective therapies with minimal short- and long-term side effects is an important challenge.

Recent progress in molecular biology, genetics and biotechnology allowed the development of small molecule drugs targeting cancer-related proteins, antibody-based drugs, immunomodulatory approaches and cellular therapies ranging from improved bone marrow transplantation to stem cell treatments. Some of these novel approaches already entered clinical use, alone or as part of an adjuvant or combinatory therapy with classical treatment modalities.[2] Parallel advances in diagnostic techniques, cancer genetics and pathology led to a better and more detailed classification of cancer subtypes, allowing subtype-specific, even personalized treatment regimens.[3] In spite of all these innovations and efforts in the cancer medicine field, disease-free survival rates are low and prognosis is still bad, especially in advanced and/or metastatic cases. Therefore, a better understanding of cancer biology and development of novel and innovative treatment approaches is still one of the most important challenges of modern science and medicine.

A great majority of small molecule drugs target disease-related enzymes or receptors.[4] Yet, we now understand that complex processes and changes during cancer initiation, progression and evolution leading to drug resistance, result in the mutation and/or dysregulation of enzyme and non-enzyme proteins and even nucleic acids such as microRNAs and long non-coding RNAs.[5] In the post-genomic era, with the advances in genomics and epigenetics of cancer, gene therapy is one of the innovative cancer therapy fields gaining momentum in recent years. Gene therapy approaches offer the possibility of up or downregulation of dysregulated gene products and replacement of modified/mutated transcripts with non-mutated/wild type forms.[6] Experimental treatments using nucleic acids range from DNA/cDNA replacement therapies to RNA-based regimens. Although establishment of general concepts of gene therapy date back to 1960s and 1970s, issues related to the stability and half-life of the nucleic acid molecules, and most importantly lack of suitable delivery and targeting systems limited the success of this promising technology. Until recently, viral systems were the major focus of gene delivery studies and gene therapy protocols. Modified viruses, including adenoviruses, retroviruses, herpes viruses and lentiviruses were examined and tested in detail as gene delivery agents. In addition to severe immune reactions ranging from inflammation to shock, insertional mutagenesis caused by viral integration into the patient genome constituted major side-effects and drawbacks, raising concerns and doubts about the future of gene therapy.[7] With recent advances in nanotechnology, several non-viral gene delivery systems were developed as gene and drug carriers, reviving hopes about the use of nucleic acids as potent drugs against cancer.

SCOPE OF THE REVIEW

In this review, we will summarize and discuss accumulating data about the use of nanoparticles as nucleic acid

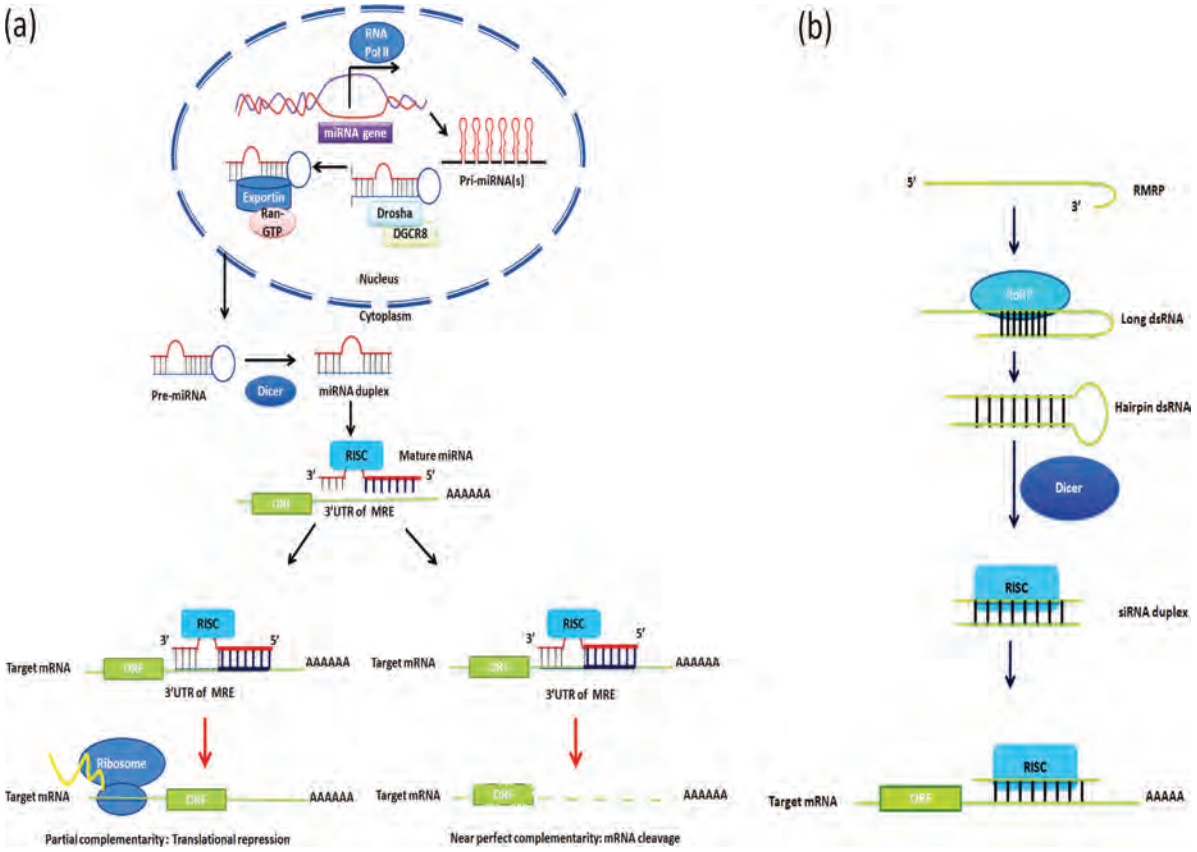
carriers. We will limit ourselves to cancer gene therapy studies exploiting nanocarrier loaded small RNA/DNA molecules, i.e., RNA interference (RNAi) tools such as siRNAs, shRNAs and microRNAs. Merits and contributions of antisense oligonucleotides in gene therapy experience were discussed in detail elsewhere.[8]

RNA INTERFERENCE

Discovery of RNA interference (RNAi) by Andrew Fire and Craig Mello was a breakthrough, that was awarded by the Nobel prize in Physiology or Medicine in 2006. Small RNAs mediating RNA interference control gene expression in organisms ranging from plants and C. elegans to human. Small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs) and microRNAs (miRNAs) are the most studied members of regulatory RNAs.[9] Non-coding small RNAs are not translated; they mainly act at a post-transcriptional level, determining the stability of messenger RNAs (mRNAs) and their translation into proteins.

Endogenous siRNAs (endo-siRNAs) are coded not only by transposons and other repeat elements, but they may also originate from regions where both sense and antisense transcripts are transcribed, as well as from sequences giving rise to potential hairpin structures inside pseudogenes and even in protein-coding genes.[10, 11] On the other hand, miRNAs may be intronic or intergenic. Intronic miRNAs are transcribed from intron regions of some protein coding mRNAs, while intergenic miRNAs are transcribed from miRNA genes or gene clusters using their own promoters.

Endogenous siRNAs and miRNAs bear similarities to each other from a structural and functional point of view. Yet, pathways leading to their maturation are rather specific to the small RNA type (Scheme 1(a)). miRNAs are transcribed by RNA polymerase II as primary-miRNAs (pri-miRNAs), and processed by a Drosha-DGCR8 complex in the nucleus to produce hairpin-shaped premature-miRNAs (pre-miRNA).[12] Transport from nucleus is achieved with the help of the exportin-Ran GTPase complexes. Further processing of the pre-miRNA hairpin by cytoplasmic DICER proteins produces ~21–22 nt long miRNA/miRNA* duplexes. One of the mature miRNA strands that originate from the duplex is then loaded onto a complex called the RNA-induced silencing complex (RISC), including the Argonaute (e.g., AGO2) proteins. RISC then guides the mature miRNA strand towards target messenger RNAs (mRNAs). It is believed that target specificity of the miRNA and the fate of the target messenger RNA depends on the degree of complementarity between matching sequences of these two types of



Scheme 1. **RNA interference (RNAi) pathways. (a) miRNA pathways. (b) siRNA pathways. See text for the pathway details. RNA Pol II, RNA polymerase II, Dicer, Dicer enzyme complex into the siRNA. RISC, RNA-induced silencing complex (RISC), RdRP, RNA-dependent RNA polymerase, ORF, open reading frame of the target gene, AAAAA, polyA tail of the mRNA.**

RNAs. While a partial complementarity may lead to the blockage of the translation machinery, protein translation repression, and/or sequestration of miRNA-mRNA complexes in compartments called P-bodies, a perfect match between these two RNAs may result in mRNA degradation (Scheme 1(a)).

Conversely, endo-siRNAs complementary to template mRNAs are synthesized by RNA-dependent RNA polymerases (RdRP), and they are not transcribed from the genomic DNA (Scheme 1(b)). It was long believed that mammals did not have any RdRPs. But this concept is now changing. For example, human telomerase catalytic subunit (hTERT) was shown possess a mammalian RdRP activity required for double stranded RNA synthesis from the mitochondrial RNA processing endoribonuclease noncoding RNA component.[13, 14] In the endogenous siRNA pathway, hairpin containing double-stranded RNA products of RdRP are further processed into functional endogenous siRNAs in either a Dicer-dependent or a Dicer-independent manner (Scheme 1(b)). In general, the sense guide strand of the endo-siRNAs are loaded onto the RISC with AGO2 proteins.

While in general, mammalian miRNAs show partial complementary to their mRNA target sequences and control a wide number of functionally-related transcripts, strict complementarity was thought to be necessary for siRNA function.[9] But, even synthetic siRNAs or shRNAs that are designed to target one and only mRNA were shown to affect so called "off-target genes." Therefore, although a gene with a dominant biological effect will be targeted by siRNA/shRNAs, a spectrum of biological events might be affected by the use of a small RNA. Nevertheless, *in vitro* cell culture and animal studies confirmed that, the biological outcomes of siRNA/shRNAs and even miRNAs were usually determined by their effect on a dominant target gene and/or pathway.[15, 16]

SMALL RNAS AS POTENT DRUGS

Use of potent RNA interference strategies might give us new opportunities for the treatment of diseases such as cancer.[17] Potential advantages of small RNAs over existing small molecule drugs and conventional therapies include, organic composition, natural metabolism, lower drug toxicity, minimal immune reactions when designed optimally and combined with appropriate carriers, possibility of modulating targets that are considered as "undruggable" by conventional drugs, possibility of targeting disease-related tissue-specific isoforms and/or mutant transcripts and standard chemical synthesis protocols. Moreover, RNA platforms are now widely used in high throughput formats for target validation and drug development processes, reducing the product development cycle and cost compared to conventional small molecules.[18]

Modifications to the native RNA structure can create robust drug-like RNA molecules.[19] Synthetic 21-mer RNA duplexes mimicking natural siRNAs and miRNAs are commonly used in RNA-based gene therapy protocols. Alternatively, asymmetric 25/27-mers or 27/29-mers, blunt 25-mers, blunt 27-mers and blunt 19-mers were tested for their stability and potency as RNA drugs with variable merits.[20–23] These different synthetic RNAs might directly be loaded onto the RISC complex or they might first require processing by Dicer. In fact even under *in vitro* conditions, synthetic siRNAs were shown to directly load onto recombinant human AGO2 proteins and form functional complexes.[24] Therefore following delivery, small RNAs may rapidly form functional complexes and alter target protein levels, resulting in therapeutic changes in cells.

Naked RNA molecules are highly susceptible to degradation by nucleases that are abundant in tissues and in the blood circulation. Therefore, to stabilize RNA molecules, increase their half-lives in biological environments and avoid immune reactions, a number of chemical changes might be introduced to the nucleic acid structures.[19] There are several commonly used RNA modifications: 2′-*O*-methyl modifications into the sugar structure of some nucleotides may confer resistance to endonucleases, decrease off-target effects when introduced into the seed region, and minimize Toll-like receptor-mediated immune reactions.[25–27] Introduction of phosphorothioate, boranophosphate or methylphosphonate backbone linkages at the 3′-end of the RNA strands may reduce their susceptibility to exonucleases. Similarly, alternative 2′ sugar modifications such as 2′-fluoro, LNA (Locked nucleic acids), FANA (2′-deoxy-2′-Fluoro-β-d-arabinonucleic acid), and 2′MOE (2′-*O*-methoxyethyl) modifications were reported to increase endonuclease resistance of small RNAs.[28] While all these modifications result in more robust RNA molecules, they might affect the potency of the RNA interference effects obtained during treatment. Therefore, even if the changes were performed according to design criteria or computer tools, experimental testing is required in each separate case to confirm potency of modified small RNA molecules on their mRNA targets.[29, 30]

RNA/DNA NANOCARRIERS FOR CANCER THERAPY

Use of gene therapy and lately small RNAs for cancer treatment was hindered by the lack of a suitable delivery system. Recent developments in nanotechnology allowed the introduction of nanoparticles with very different physico-chemical properties. Novel and sophisticated nanocarriers and their functionalized derivatives are being tested as potent and in many cases targeted RNA drug delivery agents. Some formulations are already in clinical trials and a few of them were even approved by major agencies such as the Food and Drug Agency (FDA) in the USA.[31]

Nanoparticles to be used as nucleic acid delivery agents should meet several criteria. The particles should safely

and effectively deliver RNA molecules to cancer cells and tumors. Therefore, they have to be biocompatible, not immunogenic and they should not get modified or dissolved into toxic substances under biological conditions and before reaching the tumor target. For effective delivery and dosage, nano conjugates have to be stable enough in the blood circulation and resist shear forces, proteases and nucleases that they face with. Complex formation with blood cells, proteins and other blood components might also affect carrier size, charge, availability and efficacy.

Clearance is also an important issue. For nanocarriers to reach therapeutic blood levels, they should not be readily cleared through glomerular filtration in the kidneys or trapped by the reticuloendothelial system. For example, naked siRNA molecules and nanoparticles less than 10 nm may be filtrered and excreted through kidneys following systemic administration. Carriers larger than 100 nm, might be phagocytosed and cleared by monocytes and macrophages residing in the reticuloendothelial system (RES, also called mononuclear phagocytic system) tissues with high blood supply, including pulmonary alveoli, liver sinusoids, skin, spleen etc.[32–36] In addition to the size of the nanoparticles, their geometries, surface charges, hydrophobicities and opsonization by serum proteins might affect clearance by monocytes and macrophages. Additionally, the efficacy of nucleic acid drugs in specific tissues and organs may depend on the ability of nanocarriers to penetrate blood vessels and tissues, and pass through biological barriers such as the tight blood-brain-barrier of the central nervous system.

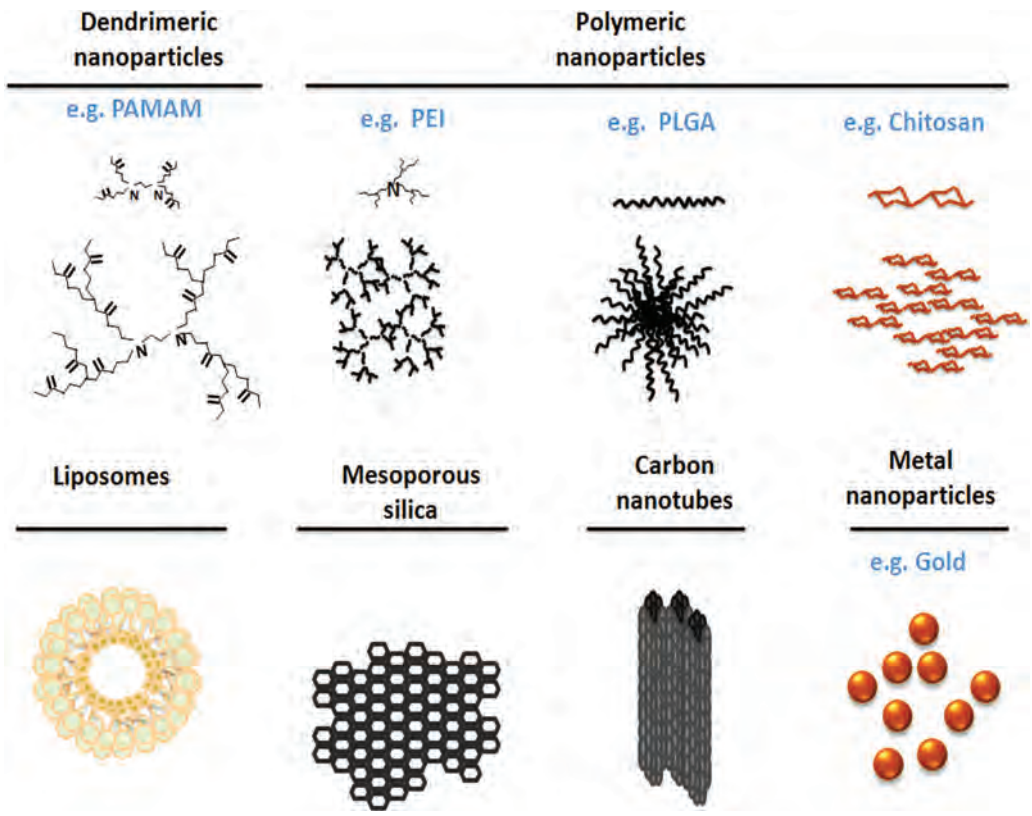
For cancer treatment, small RNA drugs should affect genetic and/or molecular changes specific to cancer cells and that are rate-limiting for tumor growth. The ideal nanocarrier should be able to concentrate within the tumor tissue, and if possible, target individual tumor cells in a selective way (e.g., through receptors or molecules that are tumor-specific or that are enriched in the tumor tissue) and not penetrate normal cells. Enhanced permeability and retention (EPR) effect offers an advantage for solid-tumor targeting. The EPR effect is a result of neovascularisation, new blood vessel formation to feed tumors with sizes beyond passive oxygen and nutrient diffusion limits (beyond 0.1–0.2 mm diameter).[37] Blood vessels feeding tumor tissues are irregular and highly permeable. With the lack of lymphatic drainage, macromolecules are easily retained in and around the tumor area.[38, 39] For example, low molecular weight drugs may diffuse freely in and out of the tumor tissues, but macromolecules (>40 kDa) and nanoparticles of 100–200 nm accumulate in the tumor tissue.[38, 40–43] EPR phenomenon was reported in various human solid tumors as well as in inflammatory tissues.[44]

Different types of nanoparticles were tested as drug carriers and gene therapy tools (Scheme 2). The core structure of the particle may be a vesicle (liposomes), a polymer (e.g., PEI, PLGA), a dendrimer, carbon nanotubes, silica or metal nanoparticles (e.g., Gold). Porous/vesiculate carriers such as liposomes, polymeric micelles or some inorganic particles (e.g., mesoporous silica) may encapsulate or absorb RNA/DNA molecules. Nanocarriers with a cationic nature, including cationic liposomes, cationic dendrimers (e.g., PAMAM) and polymers (e.g., PEI, PDMAEMA) or cationic polymer coated inorganic particles (e.g., PEI covered iron oxide) may form complexes through electrostatic interactions with the negatively charged backbone of nucleic acids. The nature and physico-chemical properties of the nanoparticles may also influence their stability and half-life in blood criculation, efficacy of their delivery into tissues and cells, and their capacity to escape from endosomes in the cell. All these properties are determining criteria for nano drug efficacy.

To improve stability, RNA/DNA loading, target specificity, tracking, cellular internalization, endo-lysosomal escape and intracellular robustness, additional functional units might be introduced to the basic structure of the nanocarriers (Scheme 3).[45] Possible modifications and changes include conjugation to proteins (antibodies, lectins, cytokines, thrombin, fibrinogen, BSA, transferrin), cellular or viral peptides (e.g., RGD, LHRD, TAT, Pep-3, KALA), polysaccharides (e.g., lipopolysaccharides, hyaluronic acid, dextran, chitosan), low molecular weight ligands (e.g., folic acid, anisamide), and polyunsaturated fatty acids (e.g., palmitic acid and phospholipids). These structures can be conjugated onto the nanocarrier surface though hydrolysable or non-hydrolysable chemical bonds and modifications such as amide, ester, silane, hydrazone, or through the use of high avidity molecules such as avidin-biotin. Fluorophores may also be added for particle tracking purposes. To improve the availability of such groups and especially the targeting groups, spacer molecules may be added.

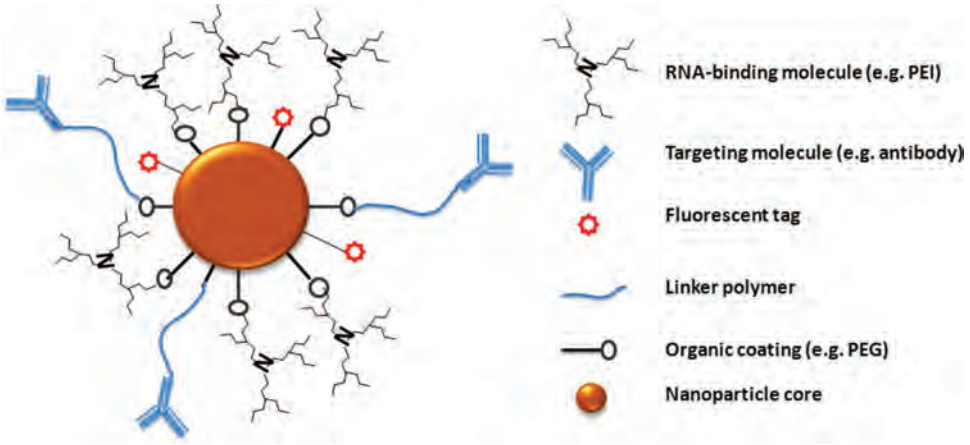
An important modification relevant for biological function is "PEGylation" (Scheme 3).[45] PEG coordinates water molecules and forms an aqueous shell around nanoparticles shielding their charges. PEGylation reduces interaction with serum proteins, decreases opsonization and clearance of nanoparticles by RES monocyte-macrophages, sterically prevent nanocarrier aggregation, and due to increased molecular weight above the threshold for glomerular filtration, reduces elimination of the particles through urinary excretion. Hence PEG increases the stability, improves biocompatibility, prolongs blood circulation time and bioavailability of nanocarriers.[46, 47] Molecular weight and density of PEG chains on the nanocarrier surface may impact the function depending on the nature of the carried molecules, i.e., siRNA.[47]

On the other hand, PEGylation significantly attenuates uptake of nanocarriers by target cells.[48, 49] Moreover, PEG chains were shown to block endosomal escape and cytosolic release of chemical drugs and nucleic acids.[50] Blockage of cellular uptake and endosomal release in the cell may severely attenuate drug efficacy and



Scheme 2. **Major types of nanoparticles used as nucleic acid carriers.**

therapeutic RNA interference effects. Various strategies were adapted to overcome these negative effects of PEG, while exploiting its circulatory advantages. Cleavage of PEG-chains upon delivery into the tumor environment was achieved through addition of tumor-related matrix metalloproteinase sensitive lipids or peptides.[51] pH-sensitive linkers between the PEG moiety and nanocarriers can also be used to release PEG from the particle in the acidic environment of the endosomes, and allow cytosolic release of small RNAs. Modulation of the hydrophobicity of the PEG–nanocarrier conjugate was also tested as a release strategy. In lipid-based nanocarriers, the length of the alkyl chain of the PEG-lipid anchor was shown to determine its affinity for the lipid delivery vehicle, and changing its length modified endosomal escape potential and drug effects.[52] Cholesterol-anchored PEG was also shown to improve endosomal escape capacities.[52]



Scheme 3. **A representative nanoparticle with functional modifications. Various molecules might be added to a nanoparticle to improve its physico-chemical properties and pharmacokinetics (e.g., PEG), to increase RNA/DNA binding (e.g., PEI), to allow better targeting (e.g., antibodies) or tracking (e.g., fluorophores).**

Liposomes

Liposomes have been tested extensively as gene delivery agents. Liposomes are artificial membrane-bound vesicles formed by bilayers of amphipathic lipids. They might be unilamellar or multilamellar. Their biocompatibility, low toxicity and low immunogenicity made them popular agents for both *in vitro* and *in vivo* gene delivery.[53,54] Liposomes are typically made of mixtures of lipids found in biological membranes or their derivatives, including phosphatidylcholine (PC or DOPC, 1,2-Oleoyl-sn-Glycero-3-phosphatidylcholine), phosphatidylethanolamine (PE or DOPE, 1,2-Dioleoyl-sn-Glycero-3-phosphatidylethanolamine), cholesterol and even ceramide.[53] Although liposomes made of neutral lipids are more biocompatible than cationic lipids and have better pharmacokinetics, during preparation of gene delivery vectors, they bind less to negatively charged DNA or RNA molecules, and have lower entrapment efficiencies.[55] Therefore, despite their more toxic nature, cationic lipids are commonly used as liposome-based transfection agents. Cationic lipids posses positively charged headgroups such as amines, quaternary ammonium salts, peptides, aminoacids or guanidiniums, which electrostaticly attract and bind to negatively charged phosphate residues in nucleic acid backbones. The nature of cationic lipid headgroups determines the efficacy of gene delivery. Therefore, various mixtures of lipids with different headgroups and properties were tested to achieve optimal liposome formulations for drug and/or gene delivery.[56–59]

Although liposomes are excellent transfection agents *in vitro*, some side effects and problems were encountered during *in vivo* studies.[56–59] These include, intracellular instability and failure to release small RNA contents, dose-dependent toxicity and pulmonary inflammation that correlated with the production of reactive oxygen species (ROS), opsonization by serum proteins, immunogenicity and uptake by the RES components.[60–63] Additionally, cationic liposome-dependent gene expression changes were observed during *in vitro* experiments with some formulations.[64]

In order to minimize such undesired effects, specialized liposomes were produced through addition of various functional groups. PEGylation and crosslinking within the bilayer of the liposomes was successfully utilized to improve stability and decrease side effects.[65] PEGylation, in general, improved bioavailability and biocompatibility as well. For example, to obtain improved pharmacokinetics, PEGylated cationic liposomes called "solid nucleic acid lipid particles" (SNALPs) were created, and they were successfully used for siRNA delivery. In fact in SNALPs, PEG coating neutralized net charge and increased the blood circulation time of the liposomes significantly.[66–72] Moreover, fusogenic lipids added to liposome formulations improved cellular uptake and endosomal escape.[33] Most importantly, SNALPs were relatively well tolerated *in vivo* even in non-human primates.[32,33] The list of modified/improved liposomes performing well in *in vivo* studies also includes cationic solid–lipid nanoparticles and cardiolipin-based liposomes.[33,73–75] Currently, improved liposomes are one of the most promising tools for gene delivery and patented formulations produced by several companies were successful enough to reach clinical trials.[31]

Lipidoids

Lipidoids are cationic lipids that are created by the conjugation of primary or secondary amines to alkyl-acrylates or alkyl-acrylamides.[76] They were introduced as novel alternatives to liposome formulations used in gene delivery. Changes in the composition lipidoids were shown to improve their therapeutic properties. For example, changes in the alkyl chain length of the PEG lipid was reported to affect PEG deshielding rate and dose kinetics of lipidoids in the blood.[76] Moreover, cholesterol incorporation was shown to improve carrier stability. Small sized lipidoids (50–60 nm) were successfully used for the delivery of siRNA into liver cells avoiding engulfment by Kuppfer cells.[76] Lipidoids may also be used for coating inorganic nanoparticles in order to introduce cationic charges.[77] Lipidoids have lower toxicity and increased efficacy, therefore they present an alternative to classical liposomes for gene delivery studies.

Minicells

Minicells are bacteria-derived, non-living, anucleated nano-sized vesicles that were used as nano drug carriers.[78] Inactivation of *min* genes that control normal division in bacteria such as *Salmonella typhimurium* result in the formation of minicells. *min* gene products ensure that cell division septum is correctly located to the midpoint of the cell. In case of *min* gene product mutations, septation defects result in the formation of minicells devoid of the chromosomal DNA. Therapeutic RNAs may be loaded into minicells by the introduction of shRNA of interest into *min* mutant bacteria, and eventually shRNAs segregate into minicells.[78] Purified minicells prepared by this method are pre-packaged with effective copy numbers of the plasmid. For targeting purposes, tumor-specific antibodies may be decorated onto minicells, by the exploitation of bacterial cell surface components such as *O*-polysaccharide of LPS. Minicell-RNAi combinations proved to be effective in experimental cancer treatment.[79] Other cell derived-vesicles that were tested as nanocarriers include bacterial ghosts, bacterial outer membranes or mammalian cell-derived exosomes.[80–82]

Polyplexes

Materials that self-assemble with nucleic acids into nanocomplexes are called "polyplexes." These complexes generally form though the electrostatic interaction of the

cationic units of the polymers with the anionic phosphate groups of the nucleic acids. Natural and biocompatible (e.g., chitosan, Atelocollagen, cyclodextrin) or more frequently synthetic (e.g., Polyethylenimine, PEI; poly-*L*-lysine, PLL; poly-dl-lactide-co-glycolide, PLGA) polymers are used for polyplex formation.[83–86] Flexibility of the synthetic polymers in terms of chemical nature, molecular weight and architecture (linear , branched, grafted, blocky) provide opportunity to balance their toxicity, improve nucleic acid binding, protection and release efficiencies. Moreover, surface modifications may be introduced for targeting purposes and improved pharmacodynamics. Polymers such as PLGA also benefit from a biodegradable nature which allows clearance of the nanocarriers in time.

PEI

PEI is the most widely and most successfully used polycation for polyplexe formation. It can be synthesized in linear or branched forms, and in variable molecular weights (from 1 kDa to > 1,000 kDa). Higher molecular weight polymers (70 kDa and above) are very effective in nucleic acid binding but they are significantly toxic. Low molecular weight forms (2 kDa or less) are less toxic but they are less effective as transfection agents.[87] Therefore, PEIs at and below 25 kDa with a branched or linear architecture are commonly used as gene delivery reagents.[88–91] The golden standard gene delivery polymer is branched PEI of 25 kDa, which offers a balance between the toxicity, nucleic acid binding/protection and release. Abundance of positive charges due to protonation under physiological conditions allows PEI to spontaneously form complexes with negatively charged small RNAs and DNAs, and protect nucleic acid molecules from nuclease attacks.[84] Moreover, buried inside the polymer, some off-target effects of the small RNAs were shown to be prevented.[92]

The net positive charge of PEI-RNAi complexes also allow interactions with the negatively charged polysaccharides found on the cell surface.[93] These interactions are believed to be an important factor for the endocytosis of the complexes by target cells. A key event for the success of gene delivery is the escape of the nucleic acids from the endosomal pathway in order to avoid accumulation and degradation in the lysosomes of the cell.[93] The escape is thought to be the result of the 'proton sponge' effect where the influx of protons and water lead to endosome swelling, rupture and release of contents, including nucleic acids to the cytosol.

PEI-induced toxicity was proposed to be a result of mitochondrial apoptosis induction by the polymer.[94, 95] Moreover, PEI itself was shown to cause changes in gene expression *in vivo*, which might influence biological outcomes of treatment protocols.[59] PEGylation of the PEI reduces both cytotoxicity of PEI-polyplexes and increase blood circulation half-life. However, a critical balance need to be achieved since PEGylation decreases the surface charge, it impacts the binding capacity and cell internalization of the polyplexes. Alternatively, short PEI chains attached together with hydrolysable links such as disulfide and ester, may provide initially a high molecular weight PEI system for good nucleic acid condensation and protection, but upon dissolution because of intracellular redox conditions, they may act as a low molecular weight PEI polymers with lower toxicity.[87] Additionally, pluronics, that are block copolymers of ethylene glycol-propylene glycol-ethylene glycol, can be used instead of PEG. Similar to PEG, pluronics were shown to improve the biocompatibility and bioavailability of the nanocarriers, but they interact better with the cell membrane due to the propylene glycol units and enhance cellular uptake of the particles.[87]

PLGA

PLGA is a biodegradable copolymer of glycolic acid and lactic acid.[96] It is widely used in biomedical research as an FDA-approved substance. PLGA offers several advantages. The polymer is highly stable, biodegradable and allows sustained release. During nucleic acid delivery, PLGA is easily taken up by cells through endocytosis, and no serious toxicity problems were observed.[97] PLGA binds nucleic acids weakly but it might encapsulate them for drug delivery purposes. Indeed, PLGA nanoparticles were used in several studies for delivery of drugs to tumors through the EPR effects.[98] Yet following endocytosis, PLGA particles do not effectively realease cargo from endosomes.[83, 97] To overcome nucleic acid binding, delivery and endosomal release problems, the surface of PLGA can be decorated with various cationic nanoparticles such as DOTAP, PEI, or polyamine, and may be conjugated to peptides and antibodies.[99]

Dendrimers

Dendrimers are tree-like, highly branched, generally symmetrical and three-dimensional macromolecules. They have uniform size and molecular weight which increases with each new branch (generation). Dendrimers possess a highly functional outer surface, allowing chemical modifications and interactions. Therefore, they may be used as flexible and modifiable gene delivery agents.[100] Besides, the ability of dendrimers to encapsulate cargos add to their potential as drug carriers.[101] Dendrimers, including poly-amidoamine dendrimers (PAMAM), poly-propylene imine (PPI) dendrimers, poly-*L*-lysine dendrimers, triazine dendrimers, carbosilane dendrimers, poly-glycerol-based dendrimers, nanocarbon-based dendrimers, and others, were tested as RNAi delivery agents, PAMAM and PPI dendrimers being the most commonly studied ones. It was shown that introduction of surface-modifications minimized toxicity-related problems, increased RNAi-binding and cellular uptake efficacies of the dendrimers.[102, 103] Of note, *in vivo* gene expression changes were also observed with drug-free dendrimers.[104] Therefore well

controlled experiments are needed before drawing conclusions during RNA interference studies.

Atelocollagen

Atelocollagen is an organic protein-based molecule derived from type I collagen of calf dermis. Since it is obtained following a pepsin treatment, atelocollagen is devoid of telopeptides that are responsible for the immunogenicity of collagen.[105] Moreover, atelocollagen has low toxicity, it was shown to stabilize RNA molecules. Increased cellular uptake and sustained delivery was observed in *in vivo*, making atelocollagen an ideal agent for gene delivery.[106] Indeed, several studies used atelocollagen with success for the systemic or local delivery of RNAi in tumor models.[107–109]

Chitosan

Chitosan is obtained through alkaline deacetylation of the polysaccharide chitin that forms the exoskeleton of crustaceans, some anthropods and insects. Chitosan that is used in biomedical research, is a copolymer of *N*-acetyl-*D*-glucosamine and *D*-glucosamine having a positive charge. In addition to being biodegradable and biocompatible, chitosan has a low production cost. Mucoadhesive properties of chitosan allow the penetration of the substance into epithelial cell layers, including the gastrointestinal barriers. Nucleic acid encapsulation and sustained release was proved to be possible using chitosan.[110–112] Moreover, chitosan prolongs transient time in bowel, improving parenteral drug bioavailability.[113] Nanoparticles of chitosan are taken up into cells through endocytosis to a certain extent. To improve gene delivery efficacies, PEG or deoxycholic acid conjugates, or modifications, including trimethylation, thiolation, galactosylation may be introduced to chitosan.[111] Moreover, chitosan-based carriers possess functional groups that are suitable for conjugation to ligands relevant for targeted tumor delivery. Although inefficient endosomal escape is a problem encountered with chitosan-based carriers, some chemically modified forms showed improved escape properties.[114]

Cyclodextrins

Cyclodextrins are natural polymers. They are cyclic oligosaccharides of a glucopyranose generated during the bacterial digestion of cellulose. Their central cavity is hydrophobic while the outer surface is a hydrophilic, and they can create water-soluble molecular complexes.[115] Native cyclodextrins do not form stable complexes with nucleic acids. But the DNA/RNA complex formation capacities of the molecule can be modulated and improved through molecular modifications, including changes in functional groups, hydrophilic-hydrophobic balance, charge density, spacer length and conjugation to other carrier molecules.[115, 116] Cyclodextrins are not only biocompatible, but they have the capacity to decrease cytotoxicity of other molecules and carriers that are conjugated to them.[117] Moreover, cyclodextrins increase cellular adsorption and intake of molecules.[118] So, in addition to their use as a gene delivery agents, cyclodextrins are used as linking agents or structural modifiers in complex carrier molecules. For example, conjugation of cyclodextrin to PEI resulted in lower toxicity and higher transfection efficiencies.[119] In addition to the advantages cited above, a cyclodextrin polycation delivery system was reported to block immune reactions raised against small RNAs through a masking effect.[120, 121] Importantly, studies in non-human primates revealed that cyclodextrin-based carriers were well tolerated and they do not stimulate significant antibody responses.[120]

Aptamers

Aptamers are nucleic acid-based molecules selected *in vitro* according to their capacity to bind target molecules specifically and with high affinity. The immunogenicity of aptamers is limited due to chemical modifications, minimizing adverse reactions. Moreover, nucleic acid nature and small size of aptamers result in improved transport and tissue penetration. Since they can be specifically designed according to cell or tissue types (e.g., tumor cell components), side effects and off-target effects are minimal. In gene therapy applications, the nucleic acid nature of aptamers allows easy conjugation to RNA/DNA, combining targeting advantages with a therapeutic potential.[122] Alternatively, non-covalent adapter linkages might be created between aptamers and RNA molecules. Functional groups might be added to the 5′- or 3′-termini, allowing covalent or non-covalent conjugation of aptamers to carrier nanoparticles.[123]

Inorganic Particles

Inorganic nanoparticals such as carbon nanotubes, magnetic nanoparticles, quantum dots and silica are the focus of recent efforts in drug and gene delivery. Many of these particles actually offer the opportunity to combine imaging and therapeutic possibilities in the same particle, rendering the nanocarrier a valuable "theranostic" device.[124] Increased surface/volume ratio of inorganic particles provides an opportunity for surface modifications, including conjugations to drugs, oligonucleotides, targeting peptides or other molecules.[125, 126] Yet, inorganic nanoparticles tend to aggregate, and the size of the aggregate might have an impact on its function and biodistribution. Such inorganic nanoparticles are hence coated with organic molecules which provide colloid formation in aqueous and physiological media and stability. Nature of the organic coatings has to be designed for specific purposes, but biocompatibility of the organic molecules themselves and/or their degradation products are critical for drug delivery purposes.[126] Chemistry of the coating might influence the half-life in blood and biodistribution, as well as the toxicity of inorganic nanoparticles and their clearance mechanisms.

Depending on the material they consist of, inorganic nanoparticles may possess a number of different properties such as high electron density and strong optical absorption (e.g., metal particles, in particular Au), photoluminescence or fluorescence (semiconductor quantum dots, e.g., CdSe, CdTe, CdTeSe/ZnS), phosphorescence (doped oxide materials, e.g., Y_2O_3) or magnetic moment (e.g., iron oxide or cobalt nanoparticles). The shape of the nanoparticle is also an important factor influencing its interaction with cells. Many of the above mentioned nanoparticle are spherical in shape except carbon nanotubes that are tubular.

Carbon Nanotubes

Carbon nanotubes (CNTs) easily cross the plasma membrane and translocate directly into the cytoplasm of target cells due to their nanoneedle structure and using an endocytosis-independent mechanism, yet they do not induce cell death.[127–129] CNTs are classified as single-walled CNTs and multiwalled CNTs. Single or multiple graphine layer(s) might have a length ranging from 50 nm to 100 mm, and a diameter of 1 nm to 100 nm. Proper functionalizing of CNTs by covalent or non-covalent strategies (such as coating with PEG or Tween-20) may provide solubility in aqueous solutions and prevent non-specific interactions, thus minimizing toxicity observed with non-functionalized raw particles.[130, 131] Modifications might also increase biocompatibility and blood circulation half-life.[132, 133] CNTs have very strong absorption characteristics, providing an opportunity for photothermal ablation therapy in addition to nanocarrier properties.

Magnetic Nanoparticles

Magnetic nanoparticles are composed of ferromagnetic elements such as Ni, Co, Mn, Fe. Superparamagnetic iron oxide nanoparticles (SPIONS), such as maghemite-γ-Fe_2O_3 and magnetite-Fe_3O_4, are one of the most widely used magnetic particles as nanocarriers due to relatively low cost of production, biocompatability and superparamagnetic nature.[134, 135] SPIONS are approved by FDA for clinical trials. They are commonly used as contrast agents for magnetic resonance imaging (MRI). SPION crystals (less than 10 nm in diameter) are coated for specific purposes with organic molecules such as dextran, amino dextran, BSA, PEI, dendrimers, lipids and trialkoxysilanes, including aminopropyltriethoxy silane (APTES). Coating chemistry and preparation methods influence overall hydrodynamic size, pharmacokinetics and the contrast type (dark-$T2$ or bright-$T1$ agent). Ability to track the fate of nucleic acid carrier magnetic nanoparticles *in vivo* using MRI is highly desirable, since it provides information about the location of the cargo and its biodistribution.[136] Attachment of ligands provides target specificity, and PEGylation may improve biocompatibility and blood half-life. These modifications are commonly introduced to SPIONs that are used for imaging and therapy purposes. Due to their magnetic nature, drug or nucleic acid carrying SPIONs can be concentrated at desired diseased sites such as tumors and they may be dragged magnetically towards the lesion area.[136, 137] Moreover, magnetic nanoparticles offer the possibility of hyperthermia treatment as a result of magnetic heating.[135] Under applied alternating magnetic field, SPIONs cause local temperature increase (41–42 °C) which may be exploited for alternative and efficient cancer therapy. Therefore, SPIONs are one of the most versatile and multifunctional nanoparticles. Consequently, SPIONs were used as popular gene delivery vehicles.[135]

Magnetic nanoparticles may be engineered for oligonucleotide delivery purposes. MRI visible PEI-PEG coated SPIONs, which are tagged with an antibody, have been shown to effectively carry siRNA to cancer cell lines and showed low toxicity.[138] SPIONs coated with both thermo responsive and cationic polymers such as poly[2-(2-methoxyethoxy)ethylmethacrylate]-*b*-poly-[2-(dimethyl-amino)ethyl methacrylate], were reported to have 25–100 times better transfection efficiency than branched PEI 25 kDa, when coupled with magnetic targeting.[139] SPIONs coated with low molecular weight PEI (1.2–2 kDa), which is usually not effective as polyplexes in transfection, were shown succesful in delivering siRNA to mouse macrophages (H. Yagci Acar, WIPO Patent WO2006055447A3). Therefore, magnetic nanparticles are under heavy investigation for the development of multifunctional nanocarriers for cancer therapy and diagnosis.[135]

Quantum Dots

Semiconductor quantum dots (QDs) are light-emitting nanoparticles of few nanometer in diameter, and they have been increasingly used as biological imaging and labeling probes.[140] Luminescence/fluorescence properties of QDs depend on the crystal size and type. Chemical composition of the crystalline semiconductor core determines the band gap of the material, therefore the emission wavelength range. Within possible spectral window, size of the crystal determines the specific wavelength of luminescence/fluorescence for each and every QD, due to quantum confinement effect. Broad absorption of QDs allow excitation of multiple QDs at a single wavelength, and minimal signal mixing due to the narrow emission band. In addition, they are much more resistant to photobleaching. These two characteristics are the major advantages of QDs compared to organic fluorophores as imaging probes. Utilization of QDs in nucleic acid delivery aims both imaging and therapy, since *in vivo* localization of cargo can be observed using optical imaging systems.[141, 142] For example, cationic CdTe/ZnS QDs conjugated with PEG was demonstrated to form an effective nanoplex with survivin siRNA and successfully transfeced human tongue cancer cells *in vitro*, and provided real time tracking.[143] However, well-established QDs harbour significant toxicity due to constituents such as Cd, Se, Te.[144] Therefore,

toxicity of these QDs is a drawback for their use *in vivo*. As an alternative, silver (Ag) chalcogenites emerged as safer near-infra red-emitting QDs.[145, 146] Ag_2S QDs did not induce cytotoxicity, ROS production, apoptosis, necrosis or DNA damage.[147] For example, Ag_2S QDs (coated with 2-mercaptopropionic acid) emitting between 750–850 nm showed no cytotoxicity in NIH/3T3 cells at even high doses (600 mg/ml) along with good cellular imaging potential.[148]

Gold Nanoparticles

Gold nanoparticles emerged as popular tools for nanocarrier-mediated gene therapy.[149] They are easily synthesized and biocompatible particles that allow addition of functional molecules on their surface due to high surface-to-volume ratio.[114, 150] A variety of surface changes and functional additions were reported, including cationic lipid coating, branched PEI addition or functionalization using cationic quaternary ammonium or cystamine.[114, 151, 152] Indeed, cystamine functionalized gold nanoparticles were shown to effectively bind, deliver and release 35 different miRNA in *in vitro* studies using neuroblastoma and ovarian cancer cell lines.[153]

Silica Nanoparticles

Silica-based nanoparticles are inert, stable, biocompatible and biodegradable particles.[154] They can be rendered hydrophilic, hydrophobic, anionic or cationic using functional surface modifiers, through electrostatic interactions, or by formation of any other type covalent bonds (e.g., ester, amine). Common functionalization strategies to render the molecule cationic and improve nucleic acid binding, include grafting of molecules such as PEI, PEI-PEG and Poly-*L*-arginine.[155] Nucleic acids may also be loaded inside the mesoporous silica particles.[156] siRNA encapsulating mesoporous silica nanoparticles were successfully used for *in vitro* and *in vivo* gene silencing in several studies (For example Ref. [156]).

RECENT *IN VIVO* STUDIES USING RNA INTERFERENCE IN CANCER THERAPY

There is an exponential increase in the number of scientific publications dedicated to gene therapy of diseases using small RNAs and nanoparticles. A literature search using "nanoparticle" and "siRNA, shRNA or miRNA" revealed more than 700 articles published in the last two years. This number is roughly equal to the number of all articles published in this field until two years ago. So the field is expanding, and there seems to form a consensus around the use of nanoparticles as next generation gene therapy tools. We believe that these high expectations are not only a consequence of shifting trends in science and technology. The exponential rise in interest in the field of small RNA carrier nanoparticles is fueled by the advances in the field of nano materials, promising results obtained in small RNA therapeutics by both academic laboratories and industry, and increasing number of successful clinical trials. In this section, we will summarize results of selected recent studies using RNA therapeutics for cancer treatment in preclinical *in vivo* experiments.

Overview of the works dealing with small RNA treatment of experimental cancers and published within the last two years were summarized in Table I. Although several studies were performed using lipid-based nanoparticles (Liposomes, micelles or lipidoids), other particles, such as polymers, organic or inorganic carriers, and compounds mixtures with functional modifications of variable substances were also tested by many research teams with reasonable success. Scheme 4 summarizes the general strategy followed in most of these studies.

Tumor Types and RNAi Therapy

Recent studies in the literature showed that tumors of various tissue origins could be treated using RNA interference strategies (Table I). Fine tuning of nanoparticles through addition of functional moieties or modifications allowed delivery of drugs into almost any kind of tumor tissue with accompanied therapeutic effects. Although several studies used animal tumor models that resulted from the injection of cell lines from most commonly seen human cancers (lung, breast, prostate, cervix or ovarian cancer cell lines), animals with kidney, urinary bladder, head and neck, gastric, pancreatic, melanomas, neuroblastomas, glioblastomas, multiple myelomas or sarcomas were also treated with RNAi/nanocarrier strategy.[81, 156–170] These results give hope about the general use of RNA interference as a strategy to treat cancers of different origins. Although it is difficult to compare the efficacies of different nucleic acid molecules and their interference effects at this point, siRNA or microRNAs as well as shRNA vectors were shown to achieve intratumoral, *in vivo* target gene knockdown and anticancer effects, leading to a decrease in tumor size (For example Refs. [156, 171]). Since in most studies, in addition to RNAi loaded particles, naked nanoparticles or particles loaded with non-specific, control nucleic acids were also used, antitumor effects obtained in these studies are specific and they may be attributable to small RNA molecules rather than the particles themselves, underlining the potential of RNA interference in cancer treatment.

Majority of recent studies with *in vivo* models chose to use human tumor cell line xenografts in immune compromised mice, nude or severe-combined immuno deficient, SCID mice (Table I) (For example Refs. [162, 172]) Syngeneic transplants and established genetically engineered mouse models of cancer were rarely studied in this context.[167, 173–175] Therefore, although antitumor effects and some of the toxic effects (e.g., liver toxicity) might be revealed using immune compromised mice, hematological, immunological and inflammatory side effects of the treatment strategies used in recent studies might need to be revisited using immunocompetent mice.

Table I. **Recent studies with RNAi carrying nanoparticles for the treatment of experimental cancers (Published within the last two years).**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
siRNA	VEGF	Magnetic mesoporous silica nanoparticles	PEI and fusogenic peptide KALA	A549 human lung cancer cells	Nude mice s.c. xenograft	i.t.	N.D.	Knockdown of VEGF. Decreased tumor volume.	[156]
siRNA	Polo-like kinase 1 (PLK1)	pH-sensitive cationic lipid YSK05-MEND (YSK05/POPE/cholesterol = 50/25/25)	PEG	OS-RC2 human renal cancer cells	Nude mice s.c. xenograft	i.t.	N.D.	Knockdown of PLK1.	[51]
siRNA	Kinesin spindle protein (KSP/EG5)	Bioengineered bacterial outer membrane vesicles	Human epidermal growth factor receptor 2 (HER2)-specific affibody targeting	HCC-1954 human breast cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Knockdown of KSP. Decreased tumor volume.	[81]
siRNA	Luciferase	Dioleoylphosphatidic acid-(DOPA-)coated nano-sized calcium phosphate (LCP-II)	DSPE-PEG- and anisamide for targeting, sigma-1 receptor	NCI-H-460 human lung cancer cells	Nude mice s.c. Xenograft	i.t.	N.D.	Knockdown of luciferase.	[211]
siRNA	mTERT	*N*-((2-hydroxy-3-trimethyl-ammonium)propyl) chitosan chloride (HTCC) nanoparticles	Particle loaded with paclitaxel	LCC murine lung cancer cells	syngeneic s.c. graft	oral	No effect	Knockdown of mTERT. Decreased tumor volume. Synergized with paclitaxel	[159]
siRNA	VEGF	Multilayered polyion complexes	Polycation interlayer, and a detachable PEG shell	OS-RC2 human renal cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Knockdown of VEGF.	[212]
siRNA	Multidrug resistance protein 1 (MRP1)	Nanoparticles assembled via layer-by layer deposition of siRNA and poly-*L*-arginine	Hyaluronan (HA) coating for HA-CD44 targeting	MDA-MB-468 human breast cancer cells	Nude mice s.c. xenograft	i.t.	No effect	Knockdown of MRP1. Decreased tumor volume.	[183]
siRNA	STAT3-a	Nanoporous silicon nanoparticles	Arginine and PEI	MDA-MB-231 human breast cancer cells	Nude mice fat pad xenograft	i.v.	No effect	Knockdown of STAT3-a.	[204]
siRNA	Luciferase	Liposomal nanoparticle	Four tandem repeats of human histone H2A peptide intervened by cathepsin D cleavage sites. PEG-Anisamide for targeting	NCI-H-460 human lung cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Knockdown of luciferase.	[199]
siRNA	Polo-like kinase 1 (PLK1)	Hyaluronic acid (HA) based selfassembling HA-PEI/PEG nanosystems	Hyaluronan for CD44 targeting	B16F10 a murine melanoma cells A549-luc human lung cancer cells Hep3B human liver cancer cells MDA-MB-468 human breast cancer cells	Nude mice s.c. graft or metastatic lung lesions by IV injection	i.t.	N.D.	Knockdown of PLK1.	[160]

Table I. **Continued.**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
siRNA	P-glycoprotein drug exporter (P-gp/MDR1)	Mesoporous silica nanoparticles	Polyethyleneimine polyethylene glycol (PEI-PEG) copolymer	MCF-7/MDR human breast cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of P-gp/MDR1. Decreased tumor volume. Synergy with doxorubicin.	[179]
siRNA	Kinesin spindle protein (KSP/EG5)	Multifunctional carrier system with cationic (oligoethanamino)amide core attached to PEG chain	Folic acid for targeting. Inf7 (Influenza peptide) as endosomolytic peptide, coupled to the 5′-end of siRNA	KB human cervix cancer cells	Nude mice s.c. xenograft	i.t. or i.v.	N.D.	Knockdown of EG5. Decreased tumor volume.	[161]
siRNA	Epidermal Growth Factor Receptor (EGFR)	Poly-*L*-arginine and dextran sulfate-based nanocomplex		FaDu human pharynx squamous cancer cells	Nude mice s.c. xenograft	i.t.	N.D.	Knockdown of EGFR. Decreased tumor volume.	[172]
siRNA	Choline kinase (Chk)	Polyethylenimine-polyethylene glycol (PEI-PEG) cografted polymer.	Conversion of nontoxic 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU) by activating bacterial cytosine deaminase (bCD)	PC3-PIP and PC3-Flu human prostate cancer cells	SCID mice s.c. xenograft	i.v.	No effect	Delivery to tumor tissue.	[157]
siRNA	Polo-like kinase 1 (Plk1)	Cationic lipid polymer hybrid nanoparticles (cationic lipid (*N*,*N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-(2-cholesteryloxycarbonyl aminoethyl) ammonium bromide, BHEM-Chol) and amphiphilic polymers (Hydrophobic polylactide core)	PEG shell	BT474 human breast cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of Plk1. Decreased tumor volume.	[213]
siRNA	hTERT	Single-walled carbon nanotubes (SWNTs)	Branched PEI conjugated. DSPE-PEG2000-Maleimide for conjugation with NGR (Cys-Asn-Gly-Arg-Cys-) targeting peptide (recognize tumor neovasculature)	PC3 human prostate cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Knockdown of hTERT. Decreased tumor volume. Synergy with photothermal treatment.	[139]

Table I. **Continued.**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
siRNA	VEGFR2	Targeted polymeric micelles	Poly(ethylene glycol)-block-poly(*L*-lysine) (PEGb-PLL) comprising lysine amines modified with 2-iminothiolane (2IT) and the cyclo-Arg-Gly-Asp (cRGD) peptide on the PEG terminus	HeLa human cervix cancer cells	Nude mice s.c. xenograft	i.v.	Reduced Hepatotoxicty compare to controls	Knockdown of VEGFR2. Decreased tumor volume.	[186]
siRNA	Luciferase	Tetravalent RNA nanoparticles (luciferase siRNA, survivin siRNA, malachite green (MG) binding aptamer and folate labeled RNA)	Aptamer and folate labeling allowed targetin	KB human cervix cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Delivery to tumor tissue.	[158]
siRNA	APRIL and miR-145	Negative lipidoid nanoparticle (98N12-5(1), mPEG2000-C12/C14 lipid and cholesterol)		Not relevant	DMH (dimethyl-hydrazine)-induced colorectal tumors in ICR mice	enema	No effect	Delivery to tumor tissue.	[205]
siRNA	Bcl2L12	Gold nanoparticles	PEGylation	Glioblastoma Multiform	SCID mice s.c. xenograft	i.v.	No effect	Knockdown of *BcL2L12.*	[162]
shRNA	Survivin	Amphiphilic copolymer poly[bis(2-hydroxylethyl)-disulfide-diacrylate-β-tetraethylenepentamine] and polycaprolactone (PBD-PCL)	Hydrophobic chemotherapeutic drugs (Doxorubicin) loaded in core	MCF-7/ADR human breast cancer cells	Nude mice s.c. xenograft	i.v.	Reduced side effects compare to combination therapy	Knockdown P-gp. Decreased tumor volume.	[214]
siRNA	EWS/Fli-1	Biotinylated poly(isobutyl-cyanoacrylate) nanoparticles	Biotinylated with or without anti-glycoprotein cd99 antibody	A673 human Ewing's sarcoma cells	Nude mice s.c. xenograft	i.v.	No effect	Knockdown of EWS/Fli-1. Decreased tumor volume.	[190]
siRNA	Survivin	Peptide-coupled chitosan nanoparticles (TAT-g-CS)	TAT peptide (GCGGGYGRKKRRQRRR) coupled for improved delivery to cells	MCF-7 human breast cancer cells 4T1 murine mammary carcinoma cells	Syngeneic s.c. graft	i.t.	N.D.	Decreased tumor volume.	[174]

Table I. **Continued.**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
siRNA	polo-like kinase 1 (PLK1)	PEGylated polycaprolactone (PCL) grafted with disulfide linked poly(2-dimethylaminoethyl methacrylate) (PDMAEMA).	Cleavage of the disulfide linkages facilitates endosomal escape	HeLa human cervix cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of PLK1. Decreased tumor volume.	[169]
siRNA	GFP or Luciferase2	Dimethylaminoethyl methacrylate (DMAEMA) star polymers		MiaPaCa human pancreatic cancer cell H460 nonsmall lung cancer cells	Nude mice s.c. xenograft	i.t.	N.D.	Knockdown of GFP and Luciferase.	[215]
shRNA	p65	Tween 85-s-s-PEI 2K (TSP) bioreducible nanoparticles	Tween 85 improve stability in circulation system and uptake by interacting with low-density lipoprotein receptor. Disulfide bond reduced in tumor environment and release shRNA	MDA-MB-435 human breast cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Decreased tumor volume.	[185]
siRNA	Luciferase and Plk1	Mesoporous silica nanoparticles disulfide bond cross-linked to poly(2-dimethylaminoethyl methacrylate) (PDMAEMA)	Disulfide bonds reduction in cells cleaved PDMAEMA and triggered siRNA release	HeLa human cervix cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Decrease tumor volume.	[216]
siRNA	Anti-apoptosis gene sirtuin 2 (SIRT2)	Ultrasound sensitive nanobubbles with poly(ethylene glycol)-*b*-poly(Benzyloxycarbonyl-*L*-Lysine) diblock copolymer (mPEG-*b*-PCBLLys) siRNA micelles	Low frequency ultrasound enhances cancer cell uptake through cavitation effects	Rat C6 Glioma cells	Nude mice s.c. xenograft	i.v.	N.D.	Decreased tumor volume.	[171]
shRNA	Focal adhesion kinase (FAK) and CD44	Poly *D*,*L*-lactide-co-glycolide acid (PLGA) nanoparticles		Ovarian cancer cells	Nude mice s.c. xenograft	i.p.	No effect	Knockdown of both FAK and CD44. Decreased tumor volume.	[170]
siRNA	EphA2	Nanoparticle-in-microparticle multistage vector (MSV) delivery system: Hemispherical porous silicon particles loaded with DOPC nanoliposome-siRNA	Silicon particles settle at tumor vasculature and liposomal siRNA released when porous silicon degrades	SKOV3ip2 and HeyA8-MDR human ovarian cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Decreased tumor volume.	[188]

Table I. **Continued.**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
siRNA	Bcl-2	Polypeptide micelle nanoparticles: Poly(ethylene glycol)-*b*-poly(*L*-lysine)-*b*-poly(*L*-leucine) (PEG-*b*-PLL-*b*-PLLeu)	Particle combined with Docetaxel	MCF-7 human brest cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of Bcl-2. Synergised with chemotherapy. Decreased tumor volume.	[180]
siRNA	MDM2	pH-responsive diblock copolymer of poly(methacryloyloxy ethyl phosphorylcholine)-block poly(diisopropanolamine ethyl methacrylate) (PMPC-*b*-PDPA)	PDPA hydrphobic at neutral pH but hydrophilic at acidic pH and facilitates PMPC nanoparticle intracellular uptake	p53 mutant H2009 human lung cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of MDM2. Decreased tumor volume.	[200]
dsRNA	p21	Lipidoid encapsulated nanoparticle (LNP)		PC-3 human prostate cancer cells	Nude mice s.c. xenograft	i.t.	N.D.	Decreased tumor volume.	[217]
siRNA	Inflammatory cytokines	Cationic lipid-coated calcium phosphate nanoparticles (LCP)	Cationic lipid-coating and combination with zeledronic acid	B16BL6 mouse melanoma cells	Syngeneic s.c. graft	p.t.	Cytokine induction	Decreased tumor volume.	[175]
miR-34a	E2F3, Bcl-2, c-myc and cyclin D1	Cyclodextrin-PEI conjugate	Conjugate with CC9 (CRGDKGPDC) peptide-penetrating bifunctional peptide	PANC-1 human pancreatic cancer cell 293T human embryonic kidney cells A549 human lung cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Increased miR-34 level. Decreased tumor volume.	[193]
siRNA	EGFP	Dextran conjugates	Complex with cationic polyelectrolytes LPEI and folate for targeting	MDA-MB-435 human breast cancer cells Hela human cervix cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of GFP.	[218]
siRNA	Bcl-2	Chitosan nanoparticles		Hela human cervix carcinoma cells	Swiss Albino mice s.c. xenograft	i.t.	N.D.	Knockdown of Bcl-2. Synergised with chemotherapy.	[176]
shRNA	Twist	Pluronic P85-PEI)/*D*-a-Tocopheryl PEG1000 succinate (TPGS) complex nanoparticles (PTPNs)	Combination with Paclitaxel	4T1 murine mammary carcinoma cells	Nude mice Syngeniec s.c. graft	i.v.	N.D.	Decreased tumor volume.	[178]
siRNA	Luciferase	Cationic liposomal nanoparticles	Conjugation to PEG200 for longer half-life	MDA-MB-435 breast cancer cells	Rag-2m Mice s.c. and i.p. xenograft	i.v.	N.D.	Knockdown of Luciferase.	[219]

Table I. **Continued.**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
siRNA	AIB1	Amorphous calcium carbonate (ACC) particles functionalized with Ca(II)-IP6 compound (CaIP6)		T24 human bladder cancer cells	Nude mice s.c. xenograft	i.t.	N.D.	Knockdown of AIB1 oncogene. Decreased tumor volume.	[164]
miR-34a	E2F3, CD44, and SIRT1	DOTAP:cholesterol liposomes	T-VISA vector system to enhance expression and prolong expression duration	MDA-MB-231 human breast cancer cell	Nude mice s.c. xenograft	i.v.	N.D.	Decreased tumor volume.	[192]
siRNA	Pokemon gene silencing	Biomimetic nanovector with rHDL nanoparticle and cholesterol-conjugated siRNA (Chol-siRNA)	Consist of a polar core containing cholesteryl esters, cholesterol, apoA-I and Chol-siRNA	Hep-G2 human hepatocellular cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of ARF, HDM2, Rb, E2F pathway. Decreased tumor volume.	[220]
siRNA	HIF-1α	Cationic mixed micellar nanoparticle (MNP) (poly(ε-caprolactone)-block-poly(2-aminoethylethylene phosphate) (PCL29-b-PPEEA21) and poly(ε-caprolactone)-block-poly(ethyleneglycol) (PCL40-b-PEG45))		PC3 human prostate cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Knockdown of HIF1-α. Synergy with doxorubicin. Decreased tumor volume.	[184]
shRNA	Survivin	P85-PEI/TPGS complex nanoparticle	Particle combined with Paclitaxel	A549 human lung cancer cells A549/T Paclitaxel resistant human lung cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Synergy with chemoterapeutics. Decrease tumor volume	[221]
siRNA	ID4	Tumor-penetrating nanocomplex (TPN)	Tandem tumor-penetrating and membrane-translocating peptide (R/KXXR/K C-terminal peptide) enabling delivery to tumor parenchyma.	OVCAR-4 human ovarian cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Knockdown of ID4. Decreased tumor volume.	[222]
siRNA	CXCR4	Dextran nanoparticles	Spermine-conjugation to obtain cationic dextran	CT26.WT mouse colorectal cancer cells	Syngeniec s.c. and i.v graft	i.v.	No effect	Knockdown of CXCR4. Decreased tumor volume.	[173]
siRNA	Luciferase	PAMAM dendrimer conjugate with cyclodextrin	Folate-PEG appended for targeting	Colon-26 colon cancer cells	Syngeniec s.c. graft	i.v. and i.t.	N.D.	Delivery totumor tissue.	[163]

Table I. **Continued.**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
shRNA	P21	Lipid nanoparticles (LNPs).		KU-7 cells human bladder cancer cell	Nude mice s.c. xenograft	i.c.	N.D.	Decreased tumor volume.	[223]
siRNA	Mouse VEGF	Cyclodextrin	PEGylated and anisamide added for targeting	TRAMP C1 transgenic mouse prostate cell	Syngeniec s.c. graft	i.v.	N.D.	Knockdown of VEGF. Decreased tumor volume.	[167]
siRNA	Bcl-2	Polyethylene glycol-grafted polyethylenimine (PEG-*g*-PEI)-SPION	Neuroblastoma cell-specific ligand GD2 single chain antibody (scAbGD2) for targeting	SK-N-SH human neuroblastoma cells	Nude mice s.c. xenograft	i.v.	N.D.	Decreased tumor volume.	[168]
siRNA	EGFR	Biodegradable amphiphilic tri-block copolymer of of monomethoxy poly(ethylene glycol), poly(*d*,*l*-lactide) and polyarginine (mPEG(2000)-PLA(3000)-*b*-R(15))	Assembled to cationic polymeric nanomicelles	MCF-7 human brest cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Knockdown of EGFR. Decreased tumor volume.	[203]
shRNA	Survivin	Cyclodextrin-PEI conjugates	Adamantine conjugated paclitaxel encapsulation	SKOV-3 human ovarian cancer cells	Nude mice s.c. xenograft	i.t.	Drug related side effects	Knockdown of Survivin and Bcl-2. Decreased tumor volume.	[181]
shRNA	MDR-1 and Survivin	Bioreducible poly(β-amino esters) (PAEs), poly[bis(2-hydroxylethyl)-disulfide-diacrylate-β-tetraethyl-enepentamine] (PAP)		MCF-7/ADR human breast cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of P-gp and Survivin. Decreased tumor volume.	[224]
siRNA	VEGF	Thiolated glycol chitosan (TGC) nanoparticles		SCC-7 mouse oral squamous cancer cells	Syngeniec s.c. graft	i.v.	N.D.	Knockdown of VEGF. Decreased tumor volume.	[225]
siRNA	MDM2:c-myc:VEGF	Lipid/Calcium/ Phosphate (LCP) nanoparticles	Stabilized with DOPA and coated with cationic lipid and anisamide for targeting	H460 human lung cancer cells A549 human lung cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Delivery to tumor tissue. Decreased tumor volume.	[226]
Anti-miR-155	miR-155/SHIP	PLGA nanoparticles	Modified with cell-penetrating peptide penetratin	NesCre8; mir-155LSLtTA mice	Nude mice s.c. Graft	i.v. and i.t.	N.D.	Increased miR-155 level. Decreased tumor volume.	[196]

Table I. **Continued.**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
siRNA	RFP (Red Fluorescent Protein)	Capsid nanocarrier complex	RGD peptides for targeting $\alpha v\beta 3$ integrins	B16F10 murine melanoma cells	Nude mice s.c. Graft	i.v.	N.D.	Knockdown of RFP. Decreased tumor volume.	[227]
miR-34a	E2F3, CD44, and SIRT1	Porous silica nanoparticles	Conjugated to a disialoganglioside GD2 antibody	NB1691 and SK-N-AS luc human neuroblastoma cells	SCID mice s.c. xenograft	i.v.	No effect	Delivery to tumor tissue. Decreased tumor volume.	[191]
siRNA	eIF5a	PEI-based SNS01 nanoparticle		KAS-6/1 human multiple myeloma cell	SCID mice s.c. xenograft	i.v.	N.D.	Synergy with chemoterapeu-tics. Decrease tumor volume.	[228]
shRNA	IGF-1R	CombiMAG (commercial magnetic) lipofectamin mixture	Magnetic manipulation	A549 human lung cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Delivery to tumor tissue. Decreased tumor volume.	[229]
siRNA	AR	PLGA-PEG nanoparticles	Conjugation to prostate-specific membrane antigen aptamer A10 for targeting	PC3 human prostate cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of AR. Decreased tumor volume.	[230]
anti-miR-10b	miR10b	Ultrasmall magnetic nanoparticles	Conjugation to RGD peptide for targeting	MDA-MB-231-luc-D3H2LN human breast cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Decreased miR-10b level.	[194]
siRNA	VEGF	Calcium phosphate (CaP)-based nanoparticles		BxPC3 human pancreatic cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of VEGF. Decreased tumor volume.	[231]
siRNA	VEGF	poly(ethylene glycol) and poly(e-caprolactone)/MPEG–PCL-SS-Tat	Tat peptide (GCGGGY-GRKKRRQRRR) coupled for improved delivery to cells	S-180 murine sarcoma cells	Syngeniec s.c. graft	i.v.	No effect	Decreased tumor volume.	[165]
pre-miR-107	miR-107/ Nanog	Cationic lipid nanoparticles		CAL27 human head and neck squamous cell cancer	Nude mice s.c. xenograft	i.v.	N.D.	Increased miR-107 level. Decreased tumor volume.	[198]
siRNA	Negative Control	mPEG-*b*-PLGA-*b*-PLL	Encapsulated with adriamycin	Huh-7 human hepatic cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Delivery to tumor tissue.	[65]
siRNA	RRM2	CDP, AD-PEG, AD-PEG-Tf	Generated with Human transferrin protein (Tf) ligand	Tu212 human head and neck squamous cell cancer	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of RRMP. Decreased tumor volume.	[166]

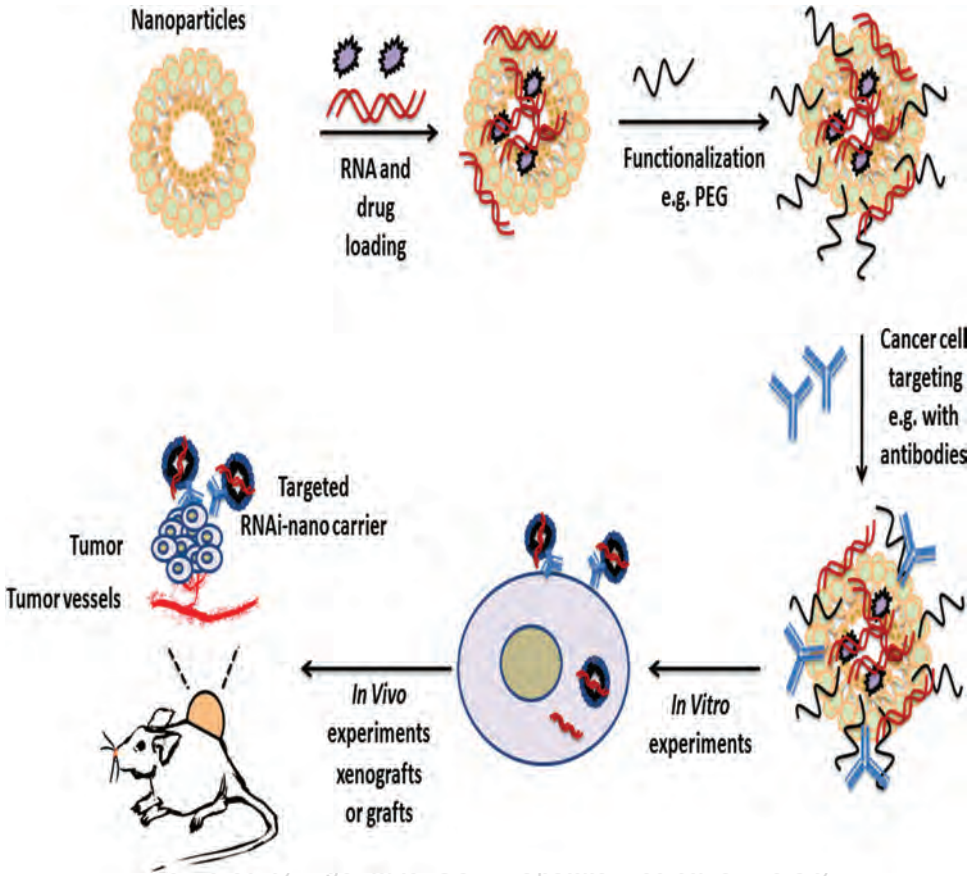
Table I. **Continued.**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
siRNA	Hsp27	Triethanol amine (TEA)-core poly(amidoamine) (PAMAM) dendrimers.		PC3 human prostate cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Delivery to tumor tissue.	[232]
siRNA	VEGF	PEG17-PLL-CA14 and PEG17-PLL-CA32	Modified with cholic acid. Grafted with PEG	TRAMP C1 Murine prostate cancer cells	Syngeniec s.c. graft	i.v.	No effect	Knockdown of VEGF. Decreased tumor volume.	[233]
siRNA	Bcl-2, VEGF and c-Myc	Poly(disulfide amine) polymer (ABP)	Grafted with Arginine	B16-F10 murine melanoma cells	Syngeniec s.c. graft	i.v.	Reduced immune response compare to controls	Knockdown of Bcl-2, c-myc and VEGF. Decreased tumor volume.	[234]
siRNA	MCL-1	Cationic solid lipid nanoparticles (cSLN)	Conjugated with Paclitaxel	KB human cervix cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of MCL-1. Decreased tumor volume.	[182]
siRNA	Ran GTPase	Stable nucleic acid lipid particle (SNALP)		HCT116 human colon DLD-1 human colon HepG2 and HuH7 human liver cancer cells	SCID mice s.c. xenograft	i.v.	N.D.	Knockdown of Ran GTPase. Decreased tumor volume	[235]
pre-miR	miR-155	PEI-based nanoparticles		ID8 murine ovarian cells	Syngeniec s.c. graft	i.v.	No effect	Boosting of anti-tumor immunity. Decreased tumor volume. Synergy with miR-155.	[195]
siRNA	VEGF	2-hydroxypopyl-β-cyclodextrin (HP-β-CD)	Combined with Folic acid and PEI	HeLa human cervix cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of VEGF. Decreased tumor volume.	[236]
siRNA	c-myc	Gold nanoparticles	Conjugated with RGD and PEG	LA-4 mouse lung cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Knockdown of c-myc. Decreased tumor volume	[237]
siRNA	c-myc	Lipid/Calcium/Phosphate (LCP)	Encapsulated with gemcitabine monophosphate (GMP)	H460 large cell lung cancer cells	Nude mice s.c. xenograft	i.v.	Reduced side effects compare to control	Knockdown of c-myc. Synergy with chemother-aupetics. Decreaesed tumor volume	[238]

Table I. **Continued.**

Nucleic acid	Target	Nanoparticle	Modification	Cell line	Tumor model	Delivery	Side effect	Outcome	Ref.
siRNA	Kras	Poly(ethylene glycol)-*block*-poly(*l*-lysine) and poly(ethylene glycol)-block-poly(dl-lactide)	Combined with arsenic	PANC-1 human pancreatic cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Decreased tumor volume. Synergy with chemother-aupeutics.	[239]
siRNA	RFP	Gelatin (tGel)	Modified with sulfhydryl groups/Thiolation	RFP/B16F10 murine melanoma cells	Syngeniec s.c. graft	i.v.	No effect	Knockdown of RFP.	[240]
siRNA	Survivin	Hyaluronic acid (HA) nanoparticles	Combination with PEI and PEG	A549 non-small lung cancer cells H69 small cell lung cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Decreased tumor growth.	[187]
siRNA	PLK1	Chitosan, polyethylene glycol	Combined with peptide CP15	SW480 human colon cancer cells	Nude mice s.c. xenograft	i.p.	No effect	Delivery to tumor tissue. Knockdown of PLK1. Decreased tumor volume.	[201]
siRNA	XIAP	Triple-shell calcium phosphate	Combined with doxorubicin and unbound caveolin-1	H292 human lung carcinoma cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of XIAP. Decreased tumor volume.	[177]
siRNA	PLK1	mPEG45-*b*-PAEP75-Cya	Coated with PEGylated anionic polymer PPC-DA	MDA-MB-231 human breast cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Delivery to tumor tissue. Decreased tumor volume.	[241]
siRNA	AR	Cationic lipid nanoparticles		LNcaP human prostate cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of AR.	[242]
siRNA	PLK1	Poly(lactic-co-glycolic acid) and poly(ε-carbobenzoxyl-l-lysine)	Conjugated with avidin palmitic acid and PEG	A549 human lung cancer cells	Nude mice s.c. xenograft	i.v.	N.D.	Knockdown of PLK1. Decreased tumor volume.	[243]
shRNA	Survivin	*D*-α-tocopheryl polyethylene glycol 1000 succinate (TPGS)	Conjugated with RGD peptide and encapsulated with paclitaxel	A549/T human Paclitaxel resistant lung cancer cells	Nude mice s.c. xenograft	i.v.	No effect	Decreased tumor volume.	[202]
microRNA	miR-29b	Liposomal nanoparticle	Conjugated with Transferrin	MV4-11 B leukemia cells	NO/SCID-gamma	i.v.	No effect	Delivery to tumor tissue.	[244]

Notes: N.D: Not Determined; i.v., intravenous; i.p., intraperitoneal, i.t., intratumoral; i.c., intracavitary; p.t., peritumoral.



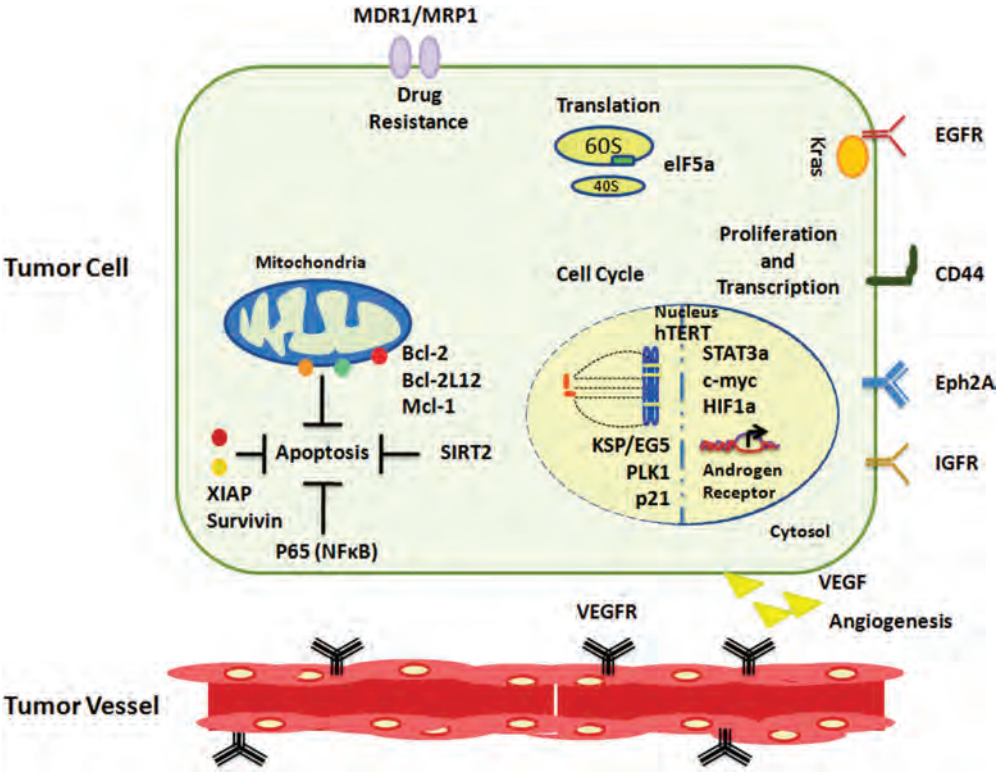
Scheme 4. **A typical study testing the antitumor potential of nanocarriers and RNA interference.**

Combination Treatments

Combination therapies of small RNA molecules and chemotherapy agents such as paclitaxel, doxorubicin or gemcitabine were tested to obtain synergistic antitumor effects.[139, 159, 176–180] Several studies chose to load the chemotherapy agent to RNA carrier nanoparticles rather than systemic chemotherapy administration.

In fact in the literature, small molecule drug delivery using nanoparticles was studied extensively with the goal of achieving higher local doses in tumors following low dose systemic drug administrations. Very promising experimental results were obtained, and nanocarrier loaded drugs even entered clinical use.[31] Nanoparticles loaded with chemotherapy agents, including liposomal formulations of doxorubicin, daunorubicin, irinotecan, vincristine, paclitaxel/docetaxel, lurtotecan and oxaliplatin, and polymers carrying camptothecin and docetaxel reached clinical trials, and even some of them are already in the market.[31]

Combinations of small RNAs and chemotherapy agents obtained by loading both components into/onto same nanocarriers may offer several advantages. Experimental results obtained with these combinations were encouraging. For example, combination of paclitaxel with mTERT siRNA or survivin shRNA in experimental lung cancers,[159, 178] with survivin and Bcl-2 shRNA in ovarian cancer[181] and Mcl-1 siRNA in cervix ca models[182] resulted in synergistic antitumor effects. Similar results were obtained with survivin shRNA combined with doxorubicin in breast cancer.[171] Small RNA combinations were used to counteract multiple drug resistance during chemotherapy. siRNA targeting major multiple drug resistance proteins P-glycoprotein drug exporter/MDR1/ABCB1 or MRP1/ABCC1 in nano combinations with doxorubicin enhanced antitumor effects of the chemotherapy in experimental breast cancer models.[179, 183] HIF-1α knockdown using siRNAs sensitized prostate cancer tumors to doxorubicin and MDR1 downregulation was also observed in this model.[184] In an alternative strategy, a drug-activating enzyme (bacterial cytosine deaminase, bCD) was delivered together with the siRNA against choline kinase (Chk).[157] In this context, while bCD activated local conversion of nontoxic prodrug 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU) inside prostate tumors, siRNA Chk targeted choline metabolism offering the possibility of combined treatment.[157] Therefore, packaging of small RNAs together with chemotherapy agents in multifunctional targeted nanoparticles, might further sensitize cancer cells to the toxic effects of chemotherapy agents, fight multi drug resistance and allow the use of local activation strategies.



Scheme 5. **Schematic representation of proteins targeted by RNA interference in recent cancer studies. Targeted proteins fall into 6 major groups based on their cellular functions. 1-Cell cycle 2-Apoptosis, 3-Proliferation and transcription, 4-Angiogenesis, 5-Translation, 6-Drug resistance.**

RNAi-Targeted Genes

Genes that were frequently targeted by siRNA/shRNAs or miRNAs in recent studies fall into a few categories (Scheme 5). In recent studies, cell cycle regulators such as Polo-like kinase 1 (PLK1), kinesin spindle protein (KSP/EG5), p21, antiapoptotic proteins Bcl-2, BCL2L12, MCL-1, survivin, XIAP, or p65/RELA, and proteins-related to tumor angiogenesis namely VEGF and VEGF receptors were targeted by several groups to block growth of tumors of different origins.[162, 165, 176, 177, 182, 185–187] Additionally, growth factor receptors, such as epidermal growth factor receptor (EGFR), were targeted in head and neck and breast cancers, while epithelial cell receptor protein-tyrosine kinase (EphA2) downregulation showed antitumor effects in ovarian tumors.[172, 188]

EWS/Fli-1 is an abnormal protein produced by the 11; 22 chromosomal translocation and the fusion of the *N*-terminal of part of the EWS protein to the DNA-binding domain of the Fli-1 protein. Resulting EWS/Fli-1 chimeric transcription factor was shown to drive the development of the pediatric bone cancer called Ewing sarcoma.[189] The work of Ramon et al. showed that siRNA-mediated knock-down of the EWS/Fli-1 chimeric oncogene using targeted nanoparticles led to tumor regression *in vivo*, confirming the use of this strategy for cancer-specific proteins produced by chromosomal abnormalities.[190] Moreover, oncogenes, such as MYC, STAT-3, hTERT were targeted by nanoparticle-coupled small RNAs leading to tumor treatment.

miRNAs and Antagomirs

A number of microRNAs or antagomirs (anti-miRNA oligonucleotides) were used as anticancer molecules as well. The list includes miR-34a, miR-10b, miR-107 and miR-155.[191–196] miR-34 expression is lost in a very broad range of cancer types, pointing out to its key role in the regulation of tumor suppression.[191–193] This miRNA was shown to have various anticancer effects, including blockage of cell proliferation and metastasis, and induction of apoptosis.[197] Recent works showed that miR-34 coupled to nanoparticles could block the growth of pancreas, breast and neuroblastoma experimental tumors.[191–193] Nano-targeted delivery of the tumor suppressor miR-107 into head and neck cancers also led to the treatment of experimental tumors.[198] On the other hand, overexpression of miR-155 in mice led to lymphoma development, and transplanted tumors of miR-155 overexpressing lymphomas responded miR-155 antagomirs (anti-miR-155) delivered on nanocarriers.[196] Similarily, antagomirs against miR-10a (anti-miR-10a) delivered in nanoparticles could prevent lymph node metastasis of xenografted breast tumors and decreased primary tumor volume.[194]

Targeted Delivery Strategies

A number of targeting strategies were used to concentrate nanoparticles in and around the tumor mass. A number of studies relied on the enhanced permeability and retention (EPR) effect and/or cleavage-mediated release of PEG components in the tumor area as a result of low pH environment and metalloproteases.[180, 184] Others used ultrasonic cavitation or magnetic manipulation as means of tumoral delivery.[171] Different targeting strategies, including attachment of antibodies (e.g., anti-GD2 antibodies for targeting neuroblastomas, anti-CD99 antibody for Ewing's sarcomas, anti-Human epidermal growth factor receptor 2 (HER2) antibodies for breast cancers and anti-CD44 antibodies for gastric cancers and melanomas), aptamers (prostate-specific membrane antigen (PSMA) aptamers for prostate cancers), peptides (e.g., RGD or NGR peptides targeting tumor vasculature), proteins (e.g., Hyaluronan to target CD44 receptors on breast tumors) or molecules such as anisamide (targeting sigma-1 receptors) or urea-based PSMA-targeting moiety were exploited to selectively deliver nanoparticles and RNA drugs to tumor tissues and cells.[81, 157, 183, 186, 190]

Another problem encountered during nanoparticle-based therapies is the efficacy with which the particles enter cells. Here, in addition to relying on single or multilayered lipids for cell membrane fusion, the delivery of many particles into cells was achieved by the addition of peptides or proteins onto the particles, including TAT, CC9, KALA peptides or hyaluronan, that facilitate endosomal uptake by target cells.[156, 174, 183, 193]

A bottleneck in the effectiveness of RNA-based therapeutics is encountered following endocytosis. In fact, endosomes mature into lysosomes through acidification of their interior and acquirement of lytic enzymes, including proteases, nucleases and lipases. Delivery to the tumor tissue and endocytosis *per se* do not guarantee anticancer effects, and small RNAs carrier particles might well end up in lysosomes and degraded before having the chance to show any therapeutic effect. To circumvent lysis in lysosomes, several strategies were applied in the reviewed works. As mentioned in previous sections, an essential dilemma stems from the use of PEG. While PEGylation changes surface charges allow addition of functional molecules and prolong half-life in blood circulation, PEG prevents endosomal escape. In order to benefit from the positive effects but still allow the release of the particle to the cytosol where small RNA action occurs, several strategies were followed. The strategies included addition of pH-sensitive and/or cleavable linkers to PEG itself or to the linkers of the RNA molecules.[51, 199, 200] Inclusion of PEI to the particles was another strategy to destabilize endosomes and release the contents to the cytosol through its proton sponge effects.[157, 178, 179, 201, 202] Finally, in some studies, authors preferred to add peptides such as Influenza Inf7 peptide or Arginine-rich polypeptides that facilitated endosomal escape of the particles.[161, 172, 203, 204] Indeed, in almost all cases, manipulations favoring the release of RNA and/or particles from endosomes increased transfection efficacies and antitumor effects of RNA carrier nanoparticles (For example Ref. [161]).

Delivery Methods

Most commonly studied nanoparticle delivery mode in experimental cancers appears to be intravenous or intratumoral injections of RNA-nanocarrier complexes. As expected, intravenous systemic injections were more effective, if targeting of the nano drugs to tumorous tissues using antibodies, pepetides etc were achieved (For example Ref. [81]) Strikingly, in a few studies, oral administration and enema were tested.[159, 205] Ideally, oral administration would be the most practical administration method for any drug, be it a small molecule or an RNA-based drug. Although intravenous or intratumoral administrations better meet consistency and reproducibility concerns during animal studies, and they are viable alternatives for cancer treatment, drug development efforts need to include per os (oral) and other alternative delivery methods as well and deal with problems related to gastrointestinal environments and absorption.

CONCLUSIONS

Studies cited above and others are the proof that there is an increasing interest in nanoparticle-based drugs for cancer treatment. The ideal cancer drug should have several properties, including high efficiency, high selectivity for cancer cells, and minimal side effects in normal organs and tissues. In addition, drugs should have limited effects on the life standard of patients during the treatment period and after. Lower metastasis rates and higher rates of complete remission and cure are expected in the ideal treatment of cancer. Moreover, some nanoparticles, such as quantum dots and SPIONs, might offer advantages for more accurate and sensitive diagnosis and in the follow-up of relapses and metastasis. Nanoparticles might fit the description of such "magic bullet drugs," making close to ideal medical approaches possible. Progress in the fields of nanotechnology and biomedicine, and experiences obtained during both preclinical and clinical studies about the use of nanoparticles as anticancer molecule carriers will surely pay off in the coming years.

Nanoparticle carried and targeted drugs are studied extensively. In addition to nanocarrier-delivered conventional chemotherapeutics that are currently in advanced clinical phases or already in the market, several RNAi-based drugs entered or are entering clinical trials.[31, 206, 207] Today, nanoparticles that reached clinical phases are liposomal or lipid-based RNAi formulations, and they are mainly tested in patients with solid tumors (References herein [31] and for example clinical trial code NCT01505153). Yet, clinical studies for the treatment of non-solid cancers, including non-Hodgkin's lymphomas

and multiple myelomas are ongoing (e.g., clinical trial codes NCT01733238 and NCT01435720). Publications of the results of a clinical study using lipid nanoparticles carrying siRNAs against VEGF and kinesin spindle showed that trials using the right strategies and combinations may be well tolerated, less toxic and effective against advanced stage cancers (here, liver metastases in endometrial cancer).[208] Additionally, studies with some of the siRNA carrier non-liposomal particles were reported to be relatively safe and feasible both in non-human primates and human.[120, 209]

Overall, data provided here point out to the fact that nucleic acid nanocarriers have a great potential as optimal cancer drugs. Effective carriers that may be synthesized using feasible chemistry allowing large scale production and having reasonably long shelf-lives will surely be available for routine use in clinics in the near future. As a consequence, global market size of nano-based pharmaceuticals is expected to increase exponentially in the coming years.[210]

ABBREVIATIONS

siRNA, Small interfering RNA
shRNA, Small hairpin RNA
miRNA, Micro RNA
GI, Gastrointestinal
cDNA, Complementary DNA
RNAi, RNA interference
AGO, argonaute
RISC, RNA-Induced Silencing Complex
RdRP, RNA-dependent RNA polymerases
hTERT, Human telomerase catalytic subunit
RMRP, RNA processing endoribonuclease
LNA, Locked nucleic acids
FANA, 2′-deoxy-2′-Fluoro-β-d-arabinonucleic acid
RES, Reticuloendothelial system
NPs, Nanoparticles
EPR, Enhanced permeability and retention
PAMAM, Poly(amido amine)
PEI, Polyethyleneimine
PDMAEMA, Poly(dimethylaminoethyl methacrylate)
RGD, Arginylglycylaspartic acid
PEG, Polyethylene glycol
PC, Phosphatidylcholine
PE, Phosphatidylserine
DOPC, 1,2-Oleoyl-sn-Glycero-3-phosphatidyl-choline
DOPE, 1,2-Dioleoyl-sn-Glycero-3-phosphatidyl-ethanolamine
SNALPs, Solid nucleic acid lipid particles
LPS, Lipopolysaccharides
PLGA, Poly-dl-lactide-co-glycolide
PLL, Poly-*L*-lysine
DOTAP, *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate
PPI, Poly-propyleneimine
CNTs, Carbon nanotubes
SPIONs, Superparamagnetic iron oxide nanoparticles
APTES, Aminopropyltriethoxy silane
QDs, Quantum Dots
SCID, Severe-combined immuno deficient
mTERT, Mouse telomerase catalytic subunit
MDR1, Multidrug resistance protein 1
Chk, Choline kinase
bCD, Bacterial cytosine deaminase
5-FC, 5-fluorocytosine
5-FU, 5-fluorouracil
PLK1, Polo like kinase 1
KSP, Kinesin spindle protein
XIAP, X-linked inhibitor of apoptosis protein
VEGF, Vascular endothelial growth factor
VEGFR, Vascular endothelial growth factor receptor
EGFR, Epithelial growth factor receptor
EphA2, Tyrosine kinase stimulates
HER2, Human epidermal growth factor receptor 2
PSMA, Prostate-specific membrane antigen
NGR, Asn-Gly-Arg peptide.

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