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Review article

Strategies for in vivo targeted gene silencing

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Background: Small interfering RNA (siRNA) has attracted extensive attention showing significant promise for the study, diagnosis, and treatment of human disease. However, the specific and efficient delivery of siRNA into cells in vivo remains a great challenge. Targeted modification of siRNA, viral nanoparticle-based vectors, targeted multifunctional/multistage nanosystems, combining ultrasound-targeted microbubble destruction, and tumor targeting in an all-in-one system, provides a useful multimodal approach in targeted delivery.
Objective: We provided an overview of different strategies for siRNA delivery including direct modification of siRNA, nanoparticles and viral vectors.
Methods: We conducted a search of standard database. Relevant primary and summary resources were identified and abstracted. A summary of strategies for in vivo targeted gene silencing was produced.
Results: A list of strategies for gene-targeted delivery in vivo was summarized, including target cells, target genes, target legends, and disease model for each strategy. An overview of strategies for siRNA delivery aimed at in vivo targeted gene silencing was presented.
Conclusions: Integration of the advantages of viral or nonviral vectors into gene silencing could have profound impacts on biomedical research. Recent progress is pointing at answers.

Keywords: Gene delivery, microbubbles, nanoparticles, RNA interference, targeting, ultrasound, vector

RNA interference (RNAi) is a powerful approach for reducing expression of endogenously expressed proteins and a sequence-specific gene silencing mechanism, triggered by the introduction of dsRNA leading to mRNA degradation. It helps in switching on and off the targeted gene, which might have significant impact in therapy. RNAi can be divided into four stages; (1) Double stranded RNA cleavage by the Dicer, (2) silencing complex (RISC) formation, (3) silencing complex activation, and (4) mRNA degradation. It is widely used for biological applications and is being harnessed to silence mRNAs encoding pathogenic proteins for therapy. The major barrier to realizing the full medicinal potential of RNAi is the difficulty of delivering effector molecules, such as siRNA, in vivo [1, 2]. siRNA is known to guide sequence-speci c gene silencing of target mRNAs to which they are perfectly complementary by directing an RNA-induced silencing complex to mediate site-specific cleavage, and therefore, destruction of the targeted mRNA [3-5]. Because of its fast degradation in the physiological milieu, poor cellular uptake, inef cient translocation into the cytoplasm and lack of targeting ability, delivery of synthetic siRNA remains a major obstacle to its therapeutic application [6-10]. Therefore, it is essential to incorporate siRNA into a delivery system to improve siRNA efficacy and specificity, which may also reduce nonspecific RNAi activities of free siRNA. To overcome these problems, siRNA is often complexed with cationic lipids or cationic polymers to form nanoparticles through charge interactions for facilitating intracell delivery to improve its cellular uptake, enhance stability from enzymatic attack, and facilitate siRNA release in the cytoplasm to silence specific genes after endocytosis.

Specificity for the target tissue may be achieved by two main strategies; (1) the therapeutic vector may be preferentially transported towards the target tissue, taken up by the target cells and delivered into the nucleus of target cells (targeted delivery), or (2) the vector may be delivered into several cell types, but the carried nucleic acid is controlled by specific promoter/enhancer elements that allow gene transcription in target cells only (targeted transcription).

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This review introduces the major challenges in achieving tumor-targeted siRNA delivery in vivo and discusses recent advances in overcoming them using chemically modified siRNA, viral siRNA vectors, and physically modified nonviral siRNA carriers. Exerting their superiority may help to look for a noninvasive, specific and efficient gene-targeted strategy in an all-in-one system.

Tumor-targeted siRNA delivery

Tumor-targeted siRNA therapy is a relatively new approach that may be used to silence genes reversibly in vivo by selective targeting. To achieve siRNA in vivo via systemic delivery, it is crucial for siRNA to be efficaciously located in desired tissues or cells. This requires three important processes: prolonging circulation in the body, high accessibility to target tissues, and specific binding to target cells. Targeted siRNA delivery maximizes the local concentration in the desired tissue and prevents nonspecific siRNA distribution. For example, recent studies have reported tumor-targeted siRNA delivery using nanoparticles that specifically bind to tumor-specific or tumor-associated antigens and receptors [11, 12]. Targeting the diseased cell, organ or tissue increases the silencing potency of a given dose of siRNA. Specific cell targeting also prevents side effects by avoiding nondiseased cells [13]. Cell type-specific affinity ligands, such as aptamers, antibodies, and peptides that bind to the signature molecules have been studied extensively for their ability to guide siRNA to the target tissues and cells [14, 15]. Recent advances have led to the identification of various tissue- and cell-specific markers that may be exploited for siRNA delivery [16]. The key consideration here is that the cell receptors should be readily internalized after ligand binding and rapidly re-expressed on the cell surface to allow repeated targeting, as well as to avoid prolonged disruption of their normal ligand-binding functions. Another important consideration of using cell surface receptors for siRNA delivery is to engineer the ligands to enable siRNA 'piggybacking' without disrupting the receptor-binding properties. The carrier should also stabilize the siRNA to enable sufficient half-life circulation. siRNA may be packaged inside nanoparticles made of liposomes or other polymers and the surface of these particles can be modified to incorporate specific targeting ligands. Dickerson et al. [17] designed nanoparticles functionalized with peptides that specially target the erythropoietin-producing hepatocellular (Eph) A2 receptor to deliver siRNA targeting epidermal growth factor receptor (EGFR). The finding showed that the nanoparticles decreased EGFR expression levels and significantly increased the sensitivity of this cell line to docetaxel.

Although recent description of multiple targeted delivery systems heralds future therapeutic applications, there are still a number of concerns and scope for improvement. A suitable targeting ligand is usually added into the carrier to achieve tumor-specific siRNA delivery.

Aptamers

Aptamers are oligonucleic acid or peptide molecules selected for high affinity binding with a protein target [18]. Chimeric RNA molecules that contain an RNA aptamer directly linked to the passenger strand of siRNA may be transcribed in vitro and readily purified in large quantities. Therefore, aptamers can enhance the ability of siRNA to target various cells. McNamara et al. [19] have developed an aptamer-siRNA chimeric RNA capable of specific binding and delivery of therapeutic siRNA into the target cells. The aptamer portion of the chimera had the ability to bind with prostate-specific membrane antigen (PSMA), a cell-surface receptor overexpressed in prostate cancer cells and tumor vascular endothelium, but not the normal cells. siRNA delivered by aptamer-siRNA chimera were internalized and processed by Dicer, resulting in repression of the target protein and cell death. siRNA against a survival gene delivered by an aptamer-siRNA chimera also specifically inhibited tumor growth and mediated tumor regression in a xenograft model of prostate cancer. Studies of Cho and Dassie et al. [20,21] have also shown enhancement of target gene silencing activity and specificity using aptamer-siRNA chimeras.

Peptides

The Arg-Gly-Asp (RGD) peptide has been used to target siRNA to $\alpha_v\beta_3$ integrins overexpressed in the tumor neovasculature. Poly(ethylene glycol)ylated poly(ethylenimine) conjugated with RGD peptides was developed to selectively deliver VEGF siRNA to tumors. In this study, intravenous (i.v.) injected poly-ethylenimine-poly(ethylene glycol)-RGDD siRNA complexes inhibited tumor angiogenesis and the growth of integrin-expressing murine neuroblastoma tumors

in mice[22]. Schiffelers et al. [22] attached siRNA against the VEGF receptor to poly(ethylene glycol) (PEG)-ylated polyethylenimine (PEI) with an RGD peptide as a targeting ligand. They demonstrated the suppression of angiogenesis and the reduction of tumor growth in the murine neuroblastoma N2A xenograft tumor. De Wolf et al. [23] also designed nanoparticles assembled upon complexation of siRNA with cationic liposome (DOTAP/DOPE) and RGD-PEG-PEI, a PEGylated polymer that carries RGD. These authors showed that the circulation kinetics and the overall tumor accumulation of the siRNA complex were similar to noncomplexed siRNA. However, the intratumoral distribution of siRNA was improved by the carriers. The benefits of using the targeted carrier were attributed to the specific transport towards the RGD ligand-mediated tumor. Peptide carriers have been developed that have proven to be effective for siRNA delivery. In their study, Leng et al. [24] demonstrated that the highly branched polymers comprising of histidine and lysine were effective carriers of siRNA. Furthermore, RGD containing peptide carriers showed more siRNA silencing activity in an endothelial cell line (SVR-bag4) compared with the carriers without RGD peptide. Thus, RGD peptide may be used as a targeting ligand for siRNA delivery into the tumor neovasculature and enhance the therapeutic effect. Han et al. [25] developed an RGD peptide-labeled chitosan nanoparticle (RGD-CH-NP) as a novel tumor targeted delivery system for siRNA. This study showed that RGD-CH-NP was a novel and highly selective delivery system for siRNA, with potential for broad applications in human disease.

Transferrin (Tf), has been used as targeting ligands for i.v. siRNA delivery against tumors. In a report of a Phase I study on patients with solid cancers, Davis et al. provided the first clinical evidence of tumor-targeted RNAi using a targeted nanoparticle RNAi delivery system. These nanoparticles were surface-decorated with PEG to increase their circulation time in the body and with human Tf as a targeting ligand evident on the exterior of nanovectors to engage TfR on the surface of cancer cells. This study demonstrated that siRNA-nanoparticles systemically administered to humans may produce a specific target gene inhibition [26]. Bartlett et al. [27] used a Tf-targeting, ^{64}Cu-labeled, cyclodextrin-containing polycation to systemically deliver an anti-luciferase siRNA molecule to Neuro2A-Luc tumor cells. Pirollo et al. reported the development and use of a tumor-specific, nanosized immunoliposome complex for the systemic delivery of siRNA. This complex [TfR single-chain antibody fragment (TfRscFv)/liposome/siRNA] is composed of an anti-HER-2siRNA encapsulated by cationic liposome (DOTAP/DOPE), the surface of which is decorated with a targeting moiety, an anti-TfRscFv [28].

A tumor-homing peptide (F3) was found to target the cell-surface nucleolin [29]. It binds to the surface of, and is internalized by the tumor cells when administered systemically as a free peptide [30]. Derfus et al. [31] used PEGlyated quantum dots (QDs) core as a scaffold conjugated with siRNA as well as F3 on the particle surface. siRNA attached to the particle by a disulfide crosslinker showed a greater silencing effect compared with a nonreducible thioether linkage. Delivery of the enhanced green fluorescence protein (EGFP) siRNA by an F3/siRNA-QD complex to EGFP-transfected HeLa cells led to significant knockdown of the EGFP signal. By replacing EGFP siRNA with other therapeutic siRNA, the targeted complex may be useful for cancer treatment.

CPPs, short cationic polypeptides with a maximum of 30 amino acids, have been extensively used to obtain enhanced intracellular delivery of a wide range of macromolecules [32, 33]. In their study, Meade and Dowdy reported that HER-2 siRNA complexed with short arginine peptide was localized in the perinuclear regions of the cytoplasm in vitro, further significantly inhibiting tumor-targeted growth of ovarian cancer xenografts [34]. Another type of CPP, MPG-8, was also used to complex cyclin B1 siRNA, and the resulting complexes were further decorated with cholesterol for i.v. injection to the mice bearing human prostate carcinoma and human lung cancer xenografts [35].

Recent findings of Lu et al. [36] suggested that PEI-PEG-APRPG effectively delivers siRNA to tumors overexpressing VEGF, thereby inhibiting tumor-targeted growth. In this study, a targeted delivery system of siRNA/PEI-PEG-APRPG polyelectrolyte complexes was prepared and the efficacy of PEI PEG APRPG as an siRNA-delivering agent was evaluated in vitro and in vivo. This research showed that APRPG peptide as a target substance with PEG-PEI may improve siRNA delivery. It also supports gene therapy as a method to overcome the difficulty of siRNA delivery to the target site.

Antibody

Several studies have suggested that antibodies are good targeting modalities for tumor-targeted siRNA delivery in vivo, when careful selection of target antigen is made. Ideal antigens should be exclusively expressed or substantially overexpressed on target cells. For example, HER-2 siRNA-carrying liposomes decorated with transferrin receptor-specific antibody fragments silenced the HER-2 gene in xenograft tumors in mice, significantly inhibiting tumor growth [28]. A synthetic chimeric peptide consisting of nonamer arginine residues (9R) added to the C-terminus of a rabies virus glycoprotein peptide (29 amino acids) (RVG-9R), was able to specifically deliver siRNA to acetylcholine receptor-expressing neuronal cells after i.v. administration [37]. Pirollo et al. [28] have developed a TfR single-chain antibody fragment-directed nanoimmunoliposome to deliver siRNA to primary tumor as well as metastatic disease. A pH-sensitive histidine–lysine peptide and a modified hybrid (DNA-RNA) anti-HER-2 siRNA molecule were used to enhance the efficacy of this complex. The nanoimmunoliposome anti-HER-2 siRNA complex might silence target gene and its downstream pathway components in vivo, sensitize the tumor cells to chemo-therapeutic agents and inhibit tumor growth in a pancreatic cancer model. Bartlett et al. [27] used positron emission tomography (PET) and bioluminescence imaging (BLI) to quantify the biodistribution and function of siRNA formulated in cyclodextrin-containing polycation nanoparticles in vivo. The nontargeted and transferrin-targeted siRNA nanoparticles showed similar biodistribution and tumor localization through the enhanced permeability and retention effect. However, the transferrin-targeted siRNA nanoparticles decreased luciferase activity in the tumor to a higher extent compared with the nontargeted nanoparticles. The findings by Bartlett et al. demonstrated that the function of the targeting ligand is to enhance the cellular uptake in tumor cells rather than tumor localization.

Others

Hyaluronic acid (HA) is a naturally occurring nonsulfated glycosaminoglycan (GAG) polysaccharide composed of *N*-acetyl-D-glucosamine and D-glucuronic acid and is a major constituent of the extracellular matrix. Moreover, HA regulates angiogenesis in many types of tumors, and HA receptors, such as CD44 and RHAMM are abundantly present in tumor cells. Shen et al. demonstrated that a novel target specific siRNA delivery system was successfully developed using hydrophobized hyaluronic acid-spermine conjugates, receptor-mediated tumor targeting behavior of the siRNA/HHSC complex was evaluated in vitro by competitive inhibition with free-HA. The novel conjugate, which may deliver targeting siRNA specifically to the tumor cells containing a HA receptor, should be investigated further for in vivo applications [6]. To improve the efficacy and specificity of gene delivery systems, more recent studies have combined various strategies into the same system and produced a multi-targeting delivery system. York et al. [38] have developed an HPMA copolymer system with multiconjugation of folate ligand, a cancer cell targeting moiety and siRNA. In their study, Murase et al. [39] reported that dual-targeting liposomes modified by APRPG peptide and Gly-Asn-Gly-Arg-Gly peptides containing doxorubicin strongly suppressed tumor growth in colon 26 NL-17 carcinoma-bearing mice. Thomas et al. [40] described the use of low-molecular-weight ligands to target attached siRNA selectively to cancer cells that express the cognate receptor. To monitor the efficacy of siRNA delivery by such small-sized ligands in vivo have used fluorescent conjugates of the targeted siRNA and examined their distributions in live mice, using optical imaging techniques. Their findings showed that by using folate and DUPA (2-[3-(1,3-dicarboxypropyl)-ureido]pentanedioic acid) conjugation as targeting ligands, attached siRNA may be selectively delivered to cancer tissues with high efficacy and speci city. Using these ligands, they demonstrated marked receptor-mediated targeting of siRNA to cancer tissues in vitro and in vivo. In their study, Zhang et al. [41] studies investigated the tumor-targeted efficacy of shRNA vectors harboring chimera hTERT/U6 promoter in telomerase positive cells, which may benefit tumor therapy. Optimized shRNA with hTERT promoter is a potential approach for high potency and sustainable effects of knocking down the purpose genes effectively, leaving tumor therapeutic methods with fewer side effects.

Below is a review of different targeting ligands used for delivery (**Figure 1**) [42]. The questions that remain to be clarified include: (1) delineation of the exact mechanisms of cell entry and the release of siRNA in various targeted delivery approaches; (2) determining whether or not various targeted delivery approaches have sufficient specificity for targeting

cell types with therapeutic relevance in vivo; (iii) determining the economic feasibility of producing and using a drug comprising siRNA and a targeted delivery vehicle.

Tumor-targeted nanoparticles

Targeted delivery of therapeutics to tumor cells and tumor-associated vasculature may enhance the therapeutic targeting by increasing delivery to specific tissues and reduce nonspecific toxicity associated with siRNA. A summary of gene-targeted delivery in vivo is listed in **Table 1**. Tumor-specific or active targeting of such nanoparticles or liposomes to tumor cells may be achieved by coupling ligands to the exterior surface, potentially increasing delivery of siRNA. For this purpose, functional peptides, and folate have been used [43-46]. Tian et al. [43] used a siRNA strategy to silence the Pokemon gene in a cervical cancer model, and applied the RGD peptide ligand and polylysine (K18) fusion peptide to encapsulate a recombinant retrovirus plasmid expressing a siRNA targeting the Pokemon gene and produced the "mimoretrovirus". Findings suggest that the RNAi/RGD-based mimoretrovirus developed in this study is a novel antitumor targeted strategy that may be applicable to most investigations involving cancer therapy. Kim et al. [44] developed hydrophobically modified glycol chitosan (HGC) nanoparticles conjugated with interleukin-4 receptor (IL-4R) binding peptides, termed I4R, and tested them in mice bearing IL-4R-positive tumors. These HGC-I4R nanoparticles exhibited enhanced IL-4R-dependent cell uptake in tumors compared with nonconjugated nanoparticles, leading to better therapeutic and imaging efficacy. Kim et al. observed that the I4R peptide specifically bound to IL-4R, resulting in improved tumor targeting. This study suggests that the intracellular uptake of nanoparticles in tumors is an essential factor to consider in designing nanoparticles for tumor-targeted drug delivery and imaging. Wang et al. [47] reported that folate-functionalized hybrid polymeric nanoparticles (NPs) were prepared as carriers of low water solubility paclitaxel for tumor targeting, which were composed of monomethoxy-poly(ethylene glycol)-b-poly(lactide)-paclitaxel (MPEG-PLA-paclitaxel) and d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS)-folate (TPGS-FOL). Their findings demonstrated that folate-decorated hybrid polymeric NPs are potential and promising carriers for tumor-targeted delivery.

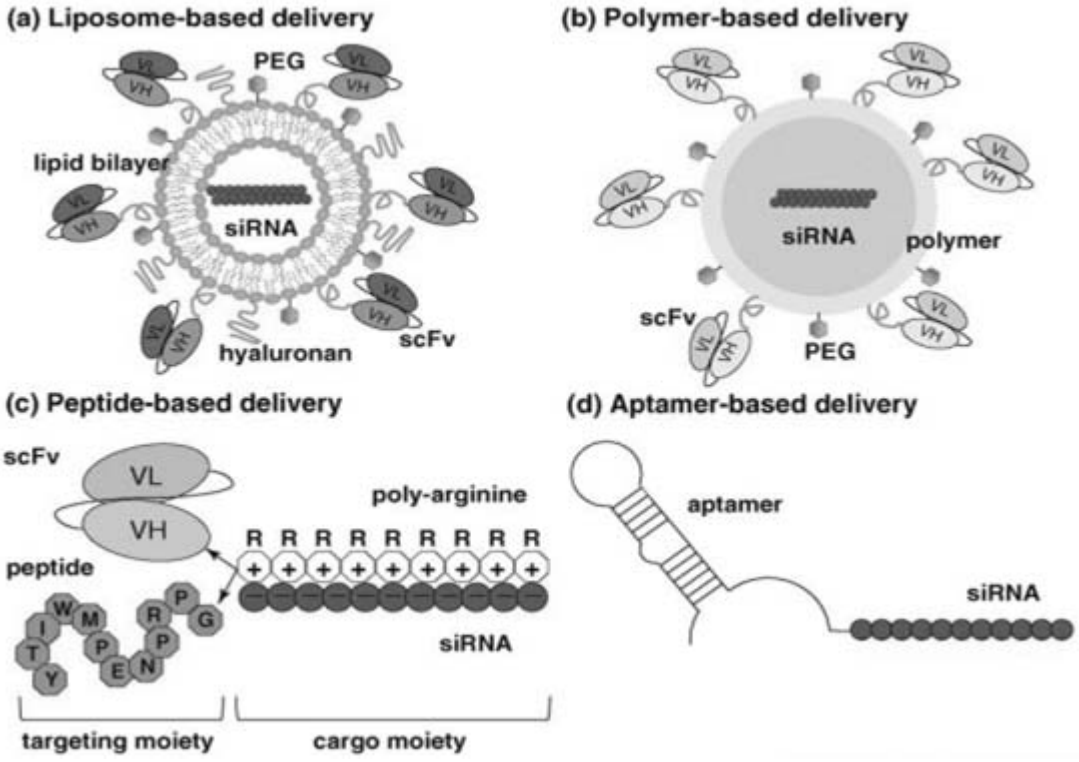

Figure 1. Schematic of strategies for targeted in vivo siRNA delivery

Table 1. List of strategies for gene-targeted delivery in vivo

System	Strategies	Target cell	Target gene	Target ligand	Disease model	Reference
NPs	RGD-NPs	SiHa cells	Pokemon	RGD	Cervical cancer	43
	HGC-I4R NPs	H226/H460 cells	IL-4R	HGC-I4R	Lung cancer	44
	Folate-NPs	Glioma C6 cells		Folate	Cervical cancer	47
	SPIO-PPI G5 NPs			LHRH		50
	MSN-NPs	A549 cells	MRP1	siRNA	Lung cancer	51
	Lipid-based NPs			siRNA	Cancer	52
	Metallic-NPs			HER2	Cancer	54
Viral	ChNPs	DLBCL-Val cells	CD22	Sialic acid		62
	AAV-NPs	HeLa cells		Taxol	Cervical cancer	63, 64
	SPIONs-AAV	HEK293T cells	NGF	Heparin		65
UTMD	UTMD+AAV		TGF-β2	siRNA	PVR	79
	UTMD+ Promoter	MCF-7 cells	CD/TK		Breast cancer	80
	UTMD+ QD-MBs					81
	UTMD+PEI+MBs	HeLa cells	Bax		Cervical cancer	82

Targeting the appropriate tissue or cell is a major limitation for all nanoparticle-based delivery strategies. Targeting has been introduced to siRNA-containing nanoparticles through the modification of the nanoparticles with ligands or antibodies recognizing cell surface receptors or antigen integrins, respectively (**Figure 2**) [48]. Recent examples of ligands used to target siRNA containing nanoparticles in vivo include a monoclonal antibody-protamine fusion protein, which was able to selectively target transferrin, which was in turn able to target tumor xenografts [27].

The targeted nanoparticles are functionalized with an outer layer of receptor-specific ligands, which can be of an antibody, small molecule, antibody peptide, or aptamer. The aqueous core of the liposomal nanoparticle can contain hydrophilic drugs, such as doxorubicin, or nucleic acids, such as DNA or siRNA.

Several promising approaches to targeting have been utilized. Recently, tumor-targeted multifunctional nanosystems have been shown to increase binding and improve therapeutic specificity and efficacy compared with nontargeted nanosystems [49, 50], suggesting that this is a promising approach. Tumor-targeted nanosystems are currently designed based primarily on the intrinsic physicochemical properties of off-the-shelf polymers. Following fabrication, the surfaces of these nanoscale structures are functionalized for passive or active targeted delivery to the tumors.

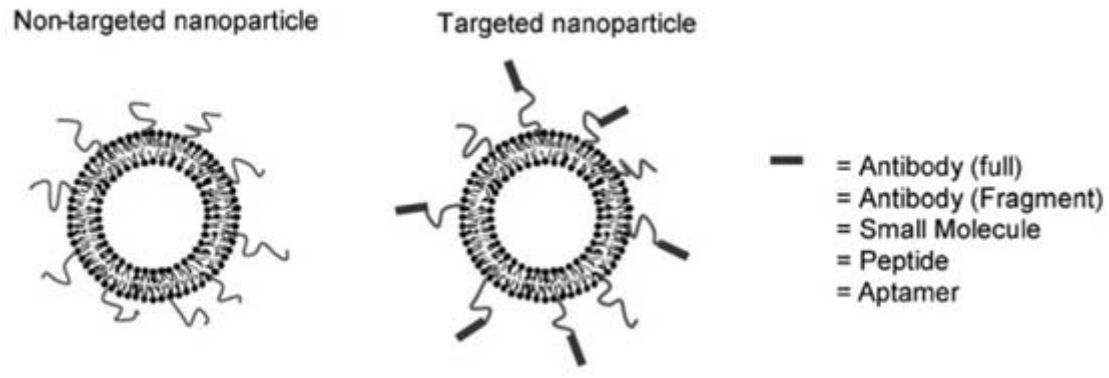

Figure 2. Representation of targeted and nontargeted liposomal nanoparticle systems.

Combinatorial approaches provide several advantages over conventional methods by allowing for the integration of multiple components with varied properties into a nanosystem via self-assembly or chemical conjugation. In their study, Abeylath et al. [49] described a novel approach for the construction of multifunctional polymeric nanosystems based on combinatorial design principles, which further enhance the specificity and efficacy of tumor-targeted therapeutic delivery. Taratula et al. [50] developed a targeted, multifunctional siRNA delivery system for cancer therapy. Poly(ethylene glycol) (PEG) coating and cancer specific targeting moiety (LHRH peptide) have been incorporated into superparamagnetic iron oxide-poly(propyleneimine) generation 5 dendrimers-siRNA (SPIO-PPI G5-siRNA) complexes to enhance serum stability and selective internalization by cancer cells. Such a modification of siRNA nanoparticles enhanced therapeutic siRNA specifically to cancer cells and increased the efficacy of targeted gene suppression in vitro. Taratula et al. [51] also developed a tumor-targeted mesoporous silica nanoparticle-based drug delivery system (DDS) for an inhalation treatment of lung cancer. The system was capable of effectively delivering anticancer drugs (doxorubicin and cisplatin) to cancer cells combined with two types of siRNA targeted to MRP1 and BCL2 mRNA for the suppression of pump and nonpump cell resistance in nonsmall cell lung carcinoma, respectively. A tumor targeting moiety guarantees the uptake of an entire DDS with encapsulated drugs and siRNA specifically by cancer cells that express the corresponding receptor limiting adverse side effects on healthy lung cells. The delivered anticancer drugs and siRNA preserved their specific activity leading to cell death induction and the inhibition of targeted mRNA.

Lipid-based nanoparticle technology has developed from the chemical drug carrier into an efficient multifunctional siRNA tumor targeting delivery system. The tumor-targeted delivery of theranostic agents to the cancer cells is one of the major challenges and an active field of research in the development of cancer chemotherapeutic approaches. Theranostic metallic nanoparticles have gained increasing attention in recent years as a novel tool for theranostic application such as imaging, diagnosis, and the therapeutic delivery of active agents to tumor-specific cells (**Figure 3**) [53]. Akhter et al. [54] reviewed the multidimensional theranostic aspects of multifunctional metallic nanoparticles (MNPs), including passive and active targeting (HER2, folate and angiogenesis).

These strategies have been successfully applied in vitro for specific delivery of peptides, folate, and siRNA, suggesting that they are able to enhance the specificity and efficacy of siRNA by increasing the concentration of siRNA in tumors at relatively lower doses compared with nontargeted nanocarriers [55].

Viral vector targeting

The largest obstacles to the use of viral vectors for human gene therapy have been their potential for infection, insertional mutagenesis, and induction of an innate and/or acquired immune response. Adeno-associated virus (AAV) is a promising mammalian virus vector commonly used for gene therapy applications [56, 57]. It is considered to be one of the safest viral vectors due to its nonpathogenic nature and limited immunogenicity.

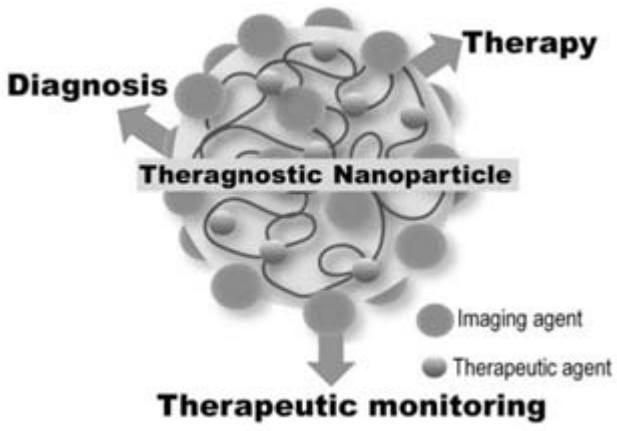

Figure 3. Schematic illustration of a theranostic nanoparticle for simultaneous diagnosis, therapy, and therapeutic monitoring

The merge of viral and nonviral vector study efforts might be an encouraging strategy for the future optimization of the two vector classes. Viral vectors may greatly benefit from chemical modifications to develop chemoviruses, while nonviral vectors may benefit from modifications mimicking viral intracell delivery functions to generate synthetic viruses. In addition, hybrid strategies may also provide interesting solutions.

For the combination of genetic and chemical adenoviral vector particle modifications, a novel cysteine-based vector platform was developed. They genetically introduced cysteines at solvent-exposed positions of the adenovirus bers [58].

The merge of viral and nonviral vector technologies is highlighted as an encouraging strategy for the future. One type of hybrid strategy incorporates a whole viral genome into a nonviral formulation [59]. Another strategy is to generate hybrid vectors combining viral and nonviral elements [60, 61]. For example, an AAV particle was shielded with acid-degradable, siRNA-encapsulating polyketal (PK) shell, resulting in core-shell viral/nonviral chimeric nanoparticles (ChNPs). The AAV core of a ChNP is protected from the immune responses by the PK shell, which also facilitates the intracell trafficking of the AAV core and efficiently releases the encapsulated siRNA into the cytoplasm. ChNPs led to significantly enhanced gene transduction, compared with unmodified free AAVs, and the simultaneous silencing of a target gene, while avoiding inactivation by recognition from the immune system. Furthermore, conjugation of sialic acid on the surface of ChNPs enabled receptor-mediated targeted gene delivery to CD22-expressing cells. The ChNPs developed in this study combine the advantages of viral and nonviral vectors and are a promising platform for targeted co-delivery of DNA and siRNA in inducing synergistic therapeutic effects by simultaneous expression and silencing of multiple genes [62].

Virus nanoparticles have been developed in recent year. Wei et al. [63] tested the covalent conjugation of paclitaxel onto surface-exposed lysine residues present on the virus capsid, "invisible" adeno-associated virus nanoparticles as platforms for the co-delivery targeting of genes and drugs to cancer cells. Musick et al. [64] have reprogrammed the stimulus-responsive conformational change property of a virus nanoparticle (VNP) to enable the surface exposure of metal binding motifs upon heat activation. Their study laid the groundwork for developing stimulus-responsive VNPs that can be used as "smart" building blocks for the creation of higher order structures. Hwang et al. [65] developed a superparamagnetic iron oxide nanoparticle (SPIONs)-guided adeno-associated virus delivery system for enhancing gene targeting delivery to HEK293T and PC12 cell lines. The successful establishment of a magnetically guided AAV delivery system, with the ability to efficiently and rapidly infect target cells, provides a powerful platform for a variety of gene-targeted therapy applications.

Ultrasound-targeted microbubble destruction

Viral vectors have generally been more efficacious, but have safety concerns. Nonviral vectors, however, provide safety, but have often lacked the efficacy of nuclear delivery and gene expression. To this end, ultrasound has been investigated for improving the efficacy of transgene delivery and is a promising noninvasive gene delivery system **(Figure 4)** [66].

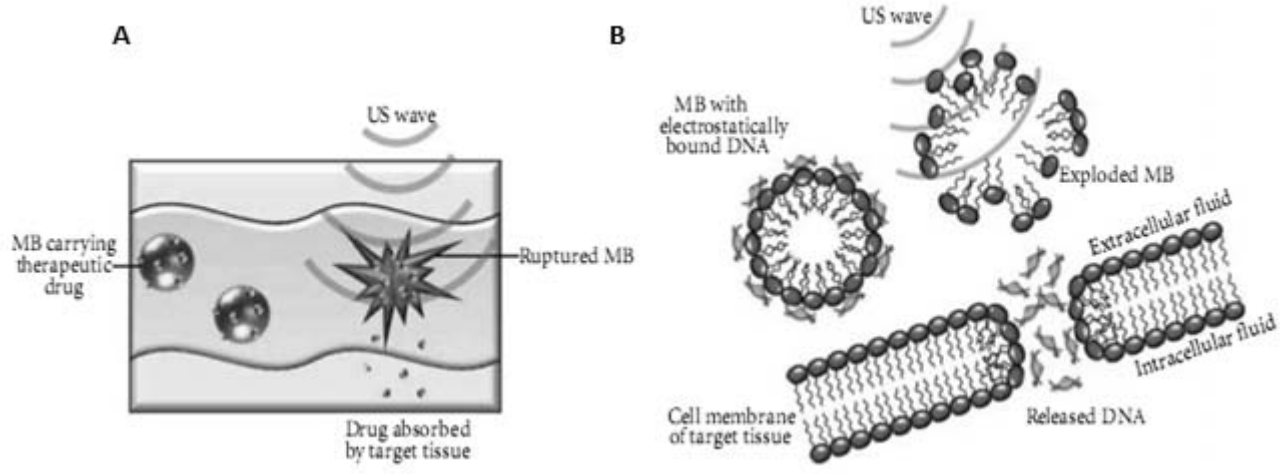

Figure 4. Sonoporation mechanisms for therapeutic delivery. **A:** Sonoporation for drug delivery, **B:** Sonoporation for gene delivery

Ultrasound-targeted microbubble destruction (UTMD) is a new, safe, targeted and noninvasive gene transfection technology. Ultrasound contrast agent is a good carrier for genes and drugs. Ultrasound contrast agent may be destroyed by ultrasound irradiation in a specific space (focus area) and at a specific time, generating cavitation and an acoustic chemical reaction resulting in the cell gap among the target cells being widened, an increase in membrane permeability, and the formation of a transient hole on the cell surface. Shock waves and microacoustic streaming generated by the rupture of the microbubbles (MBs) may lead the genes or drugs released from the microbubbles into the target cells. Ultrasound-targeted microbubble destruction has been used in various organ systems and in tumors to successfully deliver drugs, proteins, gene therapy vectors and gene silencing constructs. Several proof-of-principle studies have demonstrated its potential as a noninvasive delivery tool [67-74]. The safety and efficacy of delivering naked siRNA into cells in vivo by UTMD had been demonstrated [75].

The basic strategy to target MBs is to couple covalent or noncovalent targeting ligands to the shell [76]. Specific ligands, such as monoclonal antibodies, receptors, glycoproteins, carbohydrates, peptides or peptidomimetics have been used. Coupling of a targeting ligand to the MBs surface may be achieved by a direct covalent bond or through a biotin-avidin linkage. Coupling strategies include binding of an amino group of the ligand to a carboxyl group on the MBs shell. To account for immunogenic effects of ligand-labelled MBs, Borden et al. presented an extremely sophisticated targeting surface model concealing the ligand by a polymeric overbrush of, for example, PEG. By applying ultrasound radiation force, local revelation of the targeting ligand may be achieved [77].

Zheng et al. [78] demonstrated that the combinatorial use of UTMD and recombinant adeno-associated virus-mediated RNA interference-targeting transforming growth factor-β2 and platelet-derived growth factor-B might serve as a novel approach to enhance the targeting of gene delivery.

The tumor-specific promoter technology has been proven to be effective in protecting normal cells and has achieved the targeted killing of tumor cells [79]. The VEGF receptor (KDR) promoter is highly expressed in tumor vascular endothelial cells as well as most tumor cells, but not significantly in normal tissue cells. Using the KDR promoter sequence to regulate suicide gene expression may allow suicide genes to be expressed in tumor vascular endothelial cells and tumor cells specifically, achieving the dual role of targeted gene therapy. In their study, Li et al. [80] transfected MCF-7 cells with the KDR promoter and LSl74T cells without the KDR promoter with the recombinant plasmid pEGFP-KDRP-CD/TK using UTMD. These findings demonstrated that UTMD is a safe, effective and targeted gene delivery system. Additionally, the KDR promoter may drive the expression of the CD/TK double suicide gene target in MCF-7 cells, and the targeted killing effect of the KDRP-CD/TK gene on MCF-7 cells in vitro has good synergy with expression of the CD/TK fusion gene. Therefore, UTMD combined with tumor tissue-specific promoter-modified genes has shed new light on the targeted gene therapy eld.

The QDs-modified MBs maintained the ability of ultrasound imaging, as well as having the potential to be used as a targeted system to deliver the QDs for cell and tissue fluorescent imaging by UTMD [81]. Chen et al. [82] developed a noninvasive, novel combination of UTMD with PEI that might effectively enhance targeted gene delivery and gene expression in tumor xenografts at intravenous administration without causing any apparently adverse effect, thus being a promising candidate for gene therapy. At present, echogenic PLGA nanoparticles may be further modified to enhance their potential for a longer half-life circulation and for enabling spatial targeting. The use of ultrasound is an efficacious tool to further enhance gene delivery by PLGA or alternative echogenic particles *in vivo*. Echogenic PLGA nanoparticles are an attractive strategy for ultrasound-mediated gene delivery. Surface modifications may be made to polymeric nanoparticles to add PEGylated phospholipids to avoid recognition and clearance and achieve passive targeting [59].

Future prospects

Before siRNA therapies are applied as a general approach to treat human diseases, several issues should to be addressed. The most crucial is that therapeutic siRNA should reach their intended targets, or, they may cause damage to nontargeted cells. Aptamers, antibodies and peptides are useful for the cell-type-specific delivery of siRNA, and these should be further utilized.

The combination of ultrasound-targeted microbubble destruction, siRNA, viral vectors, and

nanoparticles is a noteworthy and important system for establishing a novel and noninvasive gene-targeted delivery system. Understanding these mechanisms of gene delivery may help to explain the development of targeting and provide new potential strategies of targeted delivery. Multitargeting and multiplexing combinatorial strategies are likely to significantly contribute to the future of gene-targeted delivery.

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