ACCOUNTS
of chemical research

Recent Advances in Nonviral Vectors for Gene Delivery

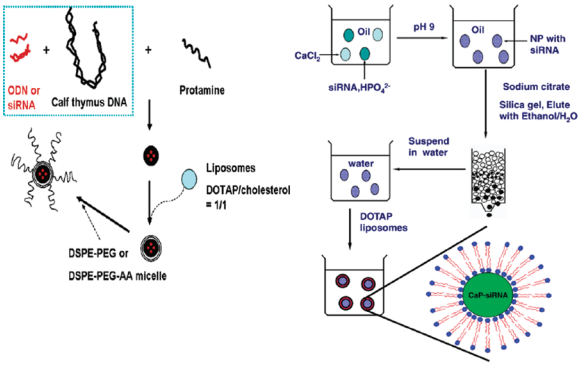
XIA GUO[†,‡] AND LEAF HUANG[*,†]

[†]*Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States, and* [‡]*School of Chemistry and Chemical Engineering, Yangzhou University, Yangzhou, Jiangsu 225002, P. R. China*

RECEIVED ON JUNE 6, 2011

CONSPECTUS

Gene therapy has long been regarded a promising treatment for many diseases, whether acquired (such as AIDS or cancer) or inherited through a genetic disorder. A drug based on a nucleic acid, however, must be delivered to the interior of the target cell while surviving an array of biological defenses honed by evolution. Successful gene therapy is thus dependent on the development of an efficient delivery vector.



Researchers have pursued two major vehicles for gene delivery: viral and nonviral (synthetic) vectors. Although viral vectors currently offer greater efficiency, nonviral vectors, which are typically based on cationic lipids or polymers, are preferred because of safety concerns with viral vectors. So far, nonviral vectors can readily transfect cells in culture, but efficient nanomedicines remain far removed from the clinic. Overcoming the obstacles associated with nonviral vectors to improve the delivery efficiency and therapeutic effect of nucleic acids is thus an active area of current research. The difficulties are manifold, including the strong interaction of cationic delivery vehicles with blood components, uptake by the reticuloendothelial system (RES), toxicity, and managing the targeting ability of the carriers with respect to the cells of interest.

Modifying the surface with poly(ethylene glycol), that is, PEGylation, is the predominant method used to reduce the binding of plasma proteins to nonviral vectors and minimize clearance by the RES after intravenous administration. Nanoparticles that are not rapidly cleared from the circulation accumulate in the tumors because of the enhanced permeability and retention effect, and the targeting ligands attached to the distal end of the PEGylated components allow binding to the receptors on the target cell surface. Neutral and anionic liposomes have been also developed for systemic delivery of nucleic acids in experimental animal models. Other approaches include (i) designing and synthesizing novel cationic lipids and polymers, (ii) chemically coupling the nucleic acid to peptides, targeting ligands, polymers, or environmentally sensitive moieties, and (iii) utilizing inorganic nanoparticles in nucleic acid delivery.

Recently, the different classes of nonviral vectors appear to be converging, and the ability to combine features of different classes of nonviral vectors in a single strategy has emerged. With the strengths of several approaches working in concert, more hurdles associated with efficient nucleic acid delivery might therefore be overcome.

In this Account, we focus on these novel nonviral vectors, which are classified as multifunctional hybrid nucleic acid vectors, novel membrane/core nanoparticles for nucleic acid delivery, and ultrasound-responsive nucleic acid vectors. We highlight systemic delivery studies and consider the future prospects for nucleic acid delivery. A better understanding of the fate of the nanoparticles inside the cell and of the interactions between the parts of hybrid particles should lead to a delivery system suitable for clinical use. We also underscore the value of sustained release of a nucleic acid in this endeavor; making vectors targeted to cells with sustained release *in vivo* should provide an interesting research challenge.

Introduction

Gene therapy has been regarded as a promising and ultimate cure for many acquired and inherited life-threatening diseases, such as AIDS, cancer, genetic disorders, etc. The efficacy of a nucleic acid drug requires that the molecule be delivered to the interior of the target cell.[1] Therefore, to

Published on the Web 08/26/2011 www.pubs.acs.org/accounts
10.1021/ar200151m

achieve successful gene therapy, development of a proper delivery vector is a significant factor.

The vectors for gene delivery are usually divided into two categories: viral and nonviral (or synthetic) vectors. Viruses offer greater efficiency of gene delivery; however, nonviral vectors are preferred due to safety concerns with the viral vectors.[2,3] Synthetic vectors are typically based on cationic lipids or polymers, which can complex with negatively charged nucleic acids to form particles with a diameter in the order of 100 nm. The complex protects the nucleic acid from degradation by nuclease. Moreover, cellular and local delivery strategies have to deal with the need for internalization, release, and distribution in the proper subcellular compartment. In the case of DNA therapy, translocation of the DNA into the nucleus is necessary. In the case of RNA interference (RNAi), siRNA must be delivered to the RNA-induced silencing complex (RISC) in the cytoplasm. Systemic delivery strategies encounter additional hurdles, for example, strong interaction of cationic delivery vehicles with blood components, uptake by the reticuloendothelial system (RES), kidney filtration, toxicity, and targeting ability of the carriers to the cells of interest.[4,5]

Extensive efforts have been focused on overcoming these barriers, and some strategies have been reviewed lately.[4−14] Modifying the surfaces of the cationic nonviral vectors can minimize their interaction with blood components, reduce RES uptake, decrease their toxicity, and increase their binding affinity with the target cells. Binding of plasma proteins (also termed opsonization) is the primary mechanism for RES to recognize the circulating nanoparticles. Macrophages, such as the Kupffer cells in the liver, recognize the opsonized nanoparticles via the scavenger receptor. Liver, spleen, and bone marrow are the major RES organs for nanoparticle clearance. PEGylation (i.e., modifying the surface with poly(ethylene glycol)) is the predominant method used to reduce the opsonization and aggregation of nonviral vectors and minimize the clearance by RES, leading to a prolonged circulation lifetime after intravenous (iv) administration.[4,5] PEGylated nanoparticles are therefore often referred as "stealth" nanoparticles. Nanoparticles that are not rapidly cleared from the circulation will have a chance to encounter the leaky tumor vasculature and accumulate in the tumors, which is known as the enhanced permeability and retention (EPR) effect.[4,5] However, PEG on the surface can decrease the uptake by target cells and reduce the biological activity. Therefore, to attach targeting ligand to the distal end of the PEGylated component is necessary; the ligand is projected beyond the PEG "shield" to allow binding to receptors on the target cell surface.[4] When cationic liposome is used as gene carrier, the application of neutral helper lipid is helpful for the release of the nucleic acid, besides promoting hexagonal phase formation to enable endosomal escape. Some researchers have developed neutral or anionic liposomes for systemic delivery of nucleic acids and obtained therapeutic effect in experimental animal models.[6,7] Designing and synthesizing novel cationic lipids and polymers and covalently or noncovalently binding genes with peptides, targeting ligands, polymers, or environmentally sensitive moieties[8−11] also attract much attention for resolving the problems encountered by nonviral vectors. The application of inorganic nanoparticles (for example, metallic nanoparticles, iron oxide, calcium phosphate, magnesium phosphate, manganese phosphate, double hydroxides, carbon nanotubes, and quantum dots) in gene delivery is an emerging field, too, because they can be prepared and surface-functionalized in many different ways.[12−14]

All these extensive efforts still yield very limited information for an effective gene therapy in the clinic, and obtaining efficient nanomedicines from nonviral vectors is far from evident.[3] Recently, different classes of nonviral vectors appear to be converging; some novel nonviral vectors were formulated, combining the features of different classes of nonviral vectors and, hence, might provide multifunction and multipurpose. Such a strategy may not only avoid the problems associated with stability, toxicity, and protein binding but also facilitate the targeted delivery and release of the nucleic acids from the delivery vehicle within the cell. In this Account, we will focus on these novel nonviral vectors, and the studies where therapeutic effect has been observed *in vivo* will be highlighted.

Multifunctional Hybrid Gene Vectors

Inorganic particles can easily be prepared and surface-functionalized. They exhibit good storage stability and are not subject to microbial attack. Some inorganic nanoparticles have been modified in different ways to develop multifunctional gene delivery systems. Two recent reviews[13,14] discussed the strategy based on magnetic inorganic nanoparticles (such as Fe_3O_4, MnO_2, and so on) for cancer-targeted delivery of nucleic acid and simultaneous diagnosis via magnetic resonance imaging (MRI). Here we will focus on the multifunctional gene delivery systems based on silica or gold nanoparticles.

An efficient gene delivery carrier and imaging agent for HeLa and NIH3T3 cells has been developed based on silica nanotubes (SNT, Figure 1).[15] The tube-structured SNTs are

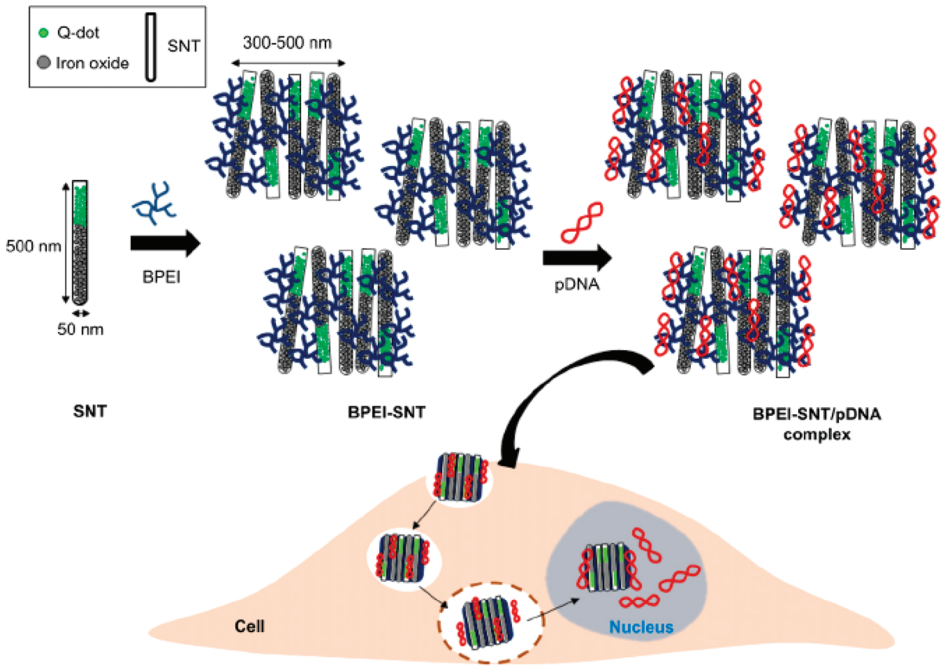


FIGURE 1. Schematic illustration depicting preparation of BPEI–SNT/pDNA complex. Reproduced from ref 15 with permission. Copyright 2011 Elsevier.

endowed with two physically distinct domains: the inner void and the outer surface. Different functionalization of the inner and outer surfaces of SNT could provide a facile and effective method to integrate multifunctionality. By covalently conjugating the outer surface of the SNT with cationic, low molecular weight, branched polyethylenimine (BPEI, MW 1.8K), it could easily load pDNA and transport the cargo into the cells. The inner space of the SNT was filled with a magnetic–fluorescent nanocomposite (iron oxide nanoparticles and green fluorescent quantum dots (CdSe/ZnS)). Since the walls of SNTs are transparent to long-wavelength UV and visible light, the two caged materials in the inner void could be used simultaneously for imaging the cells with internalized SNT by MRI and for monitoring intracellular movement of the SNT by fluorescence. The success of this dual-modality nanoconstruct *in vitro* should be expected to drive further research *in vivo*.

Combination of two or more chemotherapeutic agents with pharmacodynamically synergistic or additive effects is effectively used in a number of cancer therapy protocols.[16] In most cases, a successful drug/gene combination requires delivery of both agents at the same population of tumor cells in a coordinated manner. Because pharmacokinetics and disposition profiles of small-molecule drugs and nucleic acid drugs differ greatly, systems capable of targeted delivery of drug/gene combinations are urgently needed. Bhattarai et al.[17] modified mesoporous silica nanoparticles (MSN) with poly(ethylene glycol) (PEG) and poly(2-(dimethylamino)ethylmethacrylate) or poly(2-(diethylamino)ethylmethacrylate). The particles were then loaded with a lysosomotropic agent chloroquine (CQ, which is often used to enhance transfection of nonviral gene delivery vectors *in vitro*) and complexed with plasmid DNA or siRNA. By using this polycation-modified MSN, CQ was delivered simultaneously with DNA or siRNA *in vitro*, and a significantly increased transfection and silencing activity were observed in B16F10 cells when compared with the case using MSN not loaded with CQ. Considering the fact that when CQ was used alone *in vivo*, achieving the necessary concentrations for enhancing transfection required toxic doses, this study hypothesized that co-delivery of CQ with plasmid DNA or siRNA in a single particle might overcome the need for systemic exposure and eventually allow *in vivo* use of CQ. However, the hypothesis has yet to be tested in animal models.

Arg-Gly-Asp peptides (RGD) are ligands for $\alpha_v\beta_3$ integrin receptors. Direct conjugation of RGD to DNA/PEI (polyethylenimine) polyplexes can increase the transfection efficiency *in vitro*[18] and *in vivo*.[19] However, these targeting approaches are not able to differentiate between tumors that express high levels of $\alpha_v\beta_3$ over tumors that express medium level of $\alpha_v\beta_3$ (or over cell types that express low amounts of $\alpha_v\beta_3$).[18,19] To solve this problem, clustered Arg-Gly-Asp peptides were prepared with Au nanoparticles as template.[20] By introducing clustered RGD ligands on the surface of DNA/PEI polyplexes (Figure 2), improved targeting

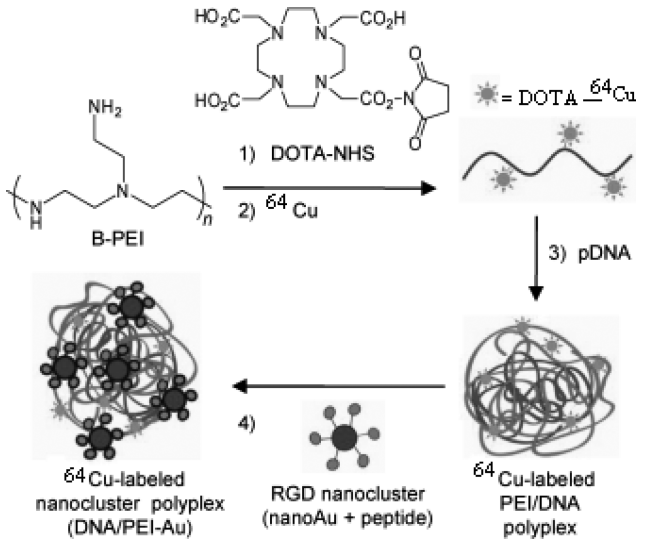


FIGURE 2. ^{64}Cu-labeled RGD nanocluster-modified DNA/PEI polyplexes. Reproduced from ref 21 with permission. Copyright 2011 Wiley-VCH.

of the vector toward U87MG tumors with high levels of $\alpha_v\beta_3$ integrin expression over HeLa tumors with medium $\alpha_v\beta_3$ integrin expression was observed in tumor bearing mice after iv administration.[21] This is an interesting study that exploits the enhanced avidity of multivalent binding.

McMahon et al.[22] synthesized biomimetic high-density lipoprotein (HDL) nanoparticles based upon a gold nanoparticle template (HDL AuNPs) and found that HDL AuNPs could adsorb antisense cholesterylated DNA and regulate target gene expression in PC-3 cells *in vitro*. HDLs are natural phospholipid-rich cholesterol transporters and can deliver adsorbed cholesterylated nucleic acids to cell types targeted by HDL for gene regulation. Thus, the HDL AuNP platform can be expected for the targeted *in vivo* delivery of nucleic acid.

Polymers also played a role in developing multifunctional nucleic acid delivery systems. Using poly(L-lysine) and PEI as templates, Li et al.[23] combined prodrug enzyme therapy, siRNA therapy, and simultaneous diagnosis by making prodrug enzyme, siRNA, MRI reporter, and optical reporter into a single systemic treatment strategy for the ER/PR/Her2-neu negative MDA-MB-231 human breast cancer xenograft model. Prodrug enzyme therapy, where a drug-activating enzyme delivered to the tumor converts a nontoxic prodrug to a cytotoxic drug, is being actively investigated to minimize normal tissue damage. The prodrug enzyme in this report is bacterial cytosine deaminase (bCD), which converts the nontoxic prodrug 5-fluorocytosine (5-FC) to cytotoxic 5-fluorouracil (5-FU), and the siRNA mediates choline kinase-α (Chk-α), an enzyme significantly up-regulated in aggressive breast cancer cells. The combination of siRNA and

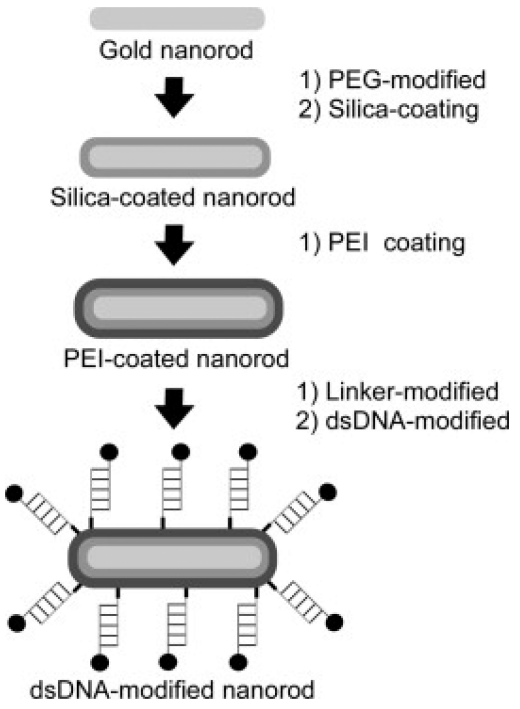


FIGURE 3. Preparation of dsDNA-modified gold nanorods. Reproduced from ref 24 with permission. Copyright 2011 Elsevier.

prodrug enzyme could amplify the selective targeting of cancer cells while minimizing normal tissue damage via iv administration. Meanwhile, noninvasive imaging can demonstrate effective tumor delivery of the siRNA and prodrug enzyme to determine when the prodrug should be administered, as well as detecting target down-regulation by siRNA and prodrug conversion by the enzyme. According to this report, *in vivo* MRI and optical imaging showed efficient intratumoral nanoplex delivery, and a single dose of the siRNA/prodrug enzyme containing nanoplex together with the prodrug resulted in a 6-fold increase of tumor doubling time, suggesting that image-guided combined siRNA and prodrug enzyme treatment should have significant potential to improve therapeutic efficacy and minimize normal tissue damage. This nanoplex strategy could be expected to expand to downregulate multidrug-resistant pathways or repair enzymes and increase the efficiency of chemo- or radiation therapy *in vivo*.

Recently, as alternative strategies to deliver nucleic acids to tumors, a controlled-release system responding to the unique environments of tissues and external stimuli has been investigated. Gold nanorods have strong absorption bands in the near-infrared region, and the absorbed light energy is then converted into heat by gold nanorods, the so-called "photothermal effect". Because the near-infrared light can penetrate deeply into tissues, the surface of the gold

nanorod could be modified with double-stranded DNA for controlled release (Figure 3).[24] When the dsDNA-modified gold nanorods were irradiated by near-infrared light, single-stranded DNA was released due to thermo-denaturation induced by the photothermal effect. The amount of released ssDNA was dependent upon the power and exposure time of light irradiation. Release of ssDNA after light irradiation was also observed in Colon-26 tumors grown in mice when the dsDNA-modified gold nanorods were directly injected into the tumors.[24] Such a controlled-release system of oligonucleotide triggered by the photothermal effect could expand the applications of gold nanorods, which have unique optical characteristics.[24]

Lee et al.[25] fabricated protease-degradable poly(L-lysine) (PLL) and siRNA onto gold nanoparticles (AuNPs), by layer-by-layer fabrication, which is a gentle assembly procedure based on charge–charge interactions between positively and negatively charged polymers. The NPs are multilayered, with the outer surface layer being PLL, they could deliver siRNA into tumor cells, and due to the slow degradation of PLL, the incorporated siRNA could be released gradually and showed extended gene-silencing effects without toxicity. The strategy is yet to be tested in animal models.

Novel Membrane/Core Nanoparticles for Gene Delivery

The pharmacology of a liposomal formulation of nucleic acid is largely determined by the extent to which the nucleic acid is encapsulated inside the liposome bilayer. Encapsulated nucleic acid is protected from nuclease degradation, while that merely associated with the surface of the liposome is not protected. Encapsulated nucleic acids share the extended circulation lifetime and biodistribution of the intact liposome, while those that are surface-associated adopt the pharmacology of naked nucleic acid once they dissociate from the liposome.

Liposomal encapsulation of small molecule drugs may be achieved by either "passive" or "active loading".[26] Unlike small molecule drugs, nucleic acids cannot cross intact lipid bilayers, predominantly due to the large size and hydrophilic nature of the nucleic acid. Therefore, nucleic acids are entrapped within liposomes with conventional passive loading technologies, such as ethanol drop method (as in SALP),[26] reverse-phase evaporation method, and ethanol dilution method (as in SNALP).[26] These methods rely on the electrostatic interaction between nucleic acid and cationic lipid; the formation of liposomes and the encapsulation efficiency of nucleic acid are sensitive to changes in the ionic strength, cationic lipid, and PEG lipid content, and the scalability and reproducibility are not satisfactory.[26]

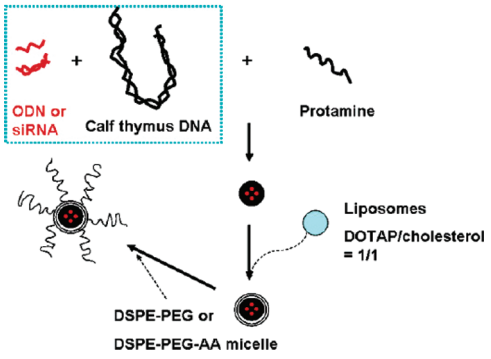


FIGURE 4. Illustration of preparation of PEGylated LPD. Reproduced from ref 30 with permission. Copyright 2006 John Wiley and Sons.

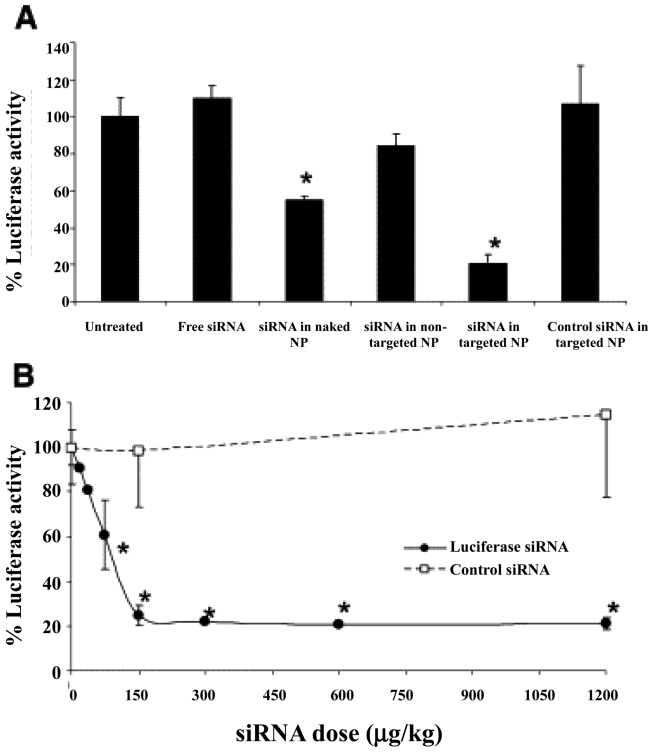


FIGURE 5. *In vivo* luciferase gene silencing effect of different siRNA formulations at a dose of 150 μg/kg (A) and of the targeted NPs at various doses (B). B16F10 tumor bearing mice were iv injected with different siRNA formulations. Data = mean ± SD ($n = 3–8$); ∗ indicates $p < 0.05$ compared with the untreated control. Reproduced from ref 29 with permission. Copyright 2008 Elsevier.

Recently, a viruslike structure with condensed nucleic acid located inside the lipid membranes was developed.[27,28] It was initially prepared by condensing DNA with protamine into a compact complex, followed by coating with cationic liposome to obtain LPD (liposome–polycation–DNA) nanoparticles. The compact complex formed by DNA and protamine

constitutes the core of the LPD. Compared with cationic liposome/DNA complex, LPD offers better protection of plasmid DNA against enzymatic digestion and gives a higher level of gene expression in mice via intravenous administration. The formulation was also modified for selectively delivering siRNA to receptor positive tumor cells *in vitro* and *in vivo* (Figures 4 and 5). siRNA was mixed with a carrier DNA, calf thymus DNA, before complexing with protamine, and PEG conjugated lipids were inserted into the outer lipid membrane after complex formation to further stabilize the formulation. A targeting ligand (anisamide, a sigma-1 receptor ligand) was conjugated to the distal end of PEG for targeting sigma receptor expressing tumor cells.[29−33] The PEG on the LPD surface was arranged in a brush mode and, thus, prevented serum opsonization and improved the chance to reach the tumor via EPR effect. The targeting ligand increased the delivery efficiency and tissue specificity.[34] Moreover, by adjustment of the ratio of protamine to siRNA and calf-thymus DNA, the core could be coated with anionic liposomes (formed by DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, and cholesterol) to form LPD-II.[35] Both LPD and LPD-II can systemically deliver doxorubicin (Dox) and siRNA to multiple drug resistance (MDR) tumors simultaneously. Although the same amount of siRNA and Dox delivered by targeted LPD and LPD-II showed similar levels of apoptosis induction and therapeutic efficacy in NCI/ADR tumors, LPD-II showed a lower toxicity profile, which might suggest a larger therapeutic window and potential clinical application for cancer therapy.[35]

Metallic ions (such as Ca^{2+} and Mg^{2+}) were also used to mediate and optimize lipoplex formation. Mozafari et al.[36,37] once constructed a nonviral and noncationic gene transfer vector by incorporating plasmid DNA to the liposomes formed by DPPC (dipalmitoylphosphatidylcholine)/DCP (dicetylphosphate)/CHOL (cholesterol) liposomes by the electrostatic mediation of Ca^{2+} ions. It is possible to get a high DNA entrapment capacity and high transfection efficiency in CHO-K1 and 16HBE14o- cells using the anionic nanolipoplex, but two or three aggregated/semifused vesicles were observed as a result of their complexation with DNA mediated by Ca^{2+}. To avoid this drawback and still employ the affinity of calcium to the phosphate groups in nucleic acids, novel membrane/core nanoparticles for siRNA delivery were developed lately.[38] In this strategy, the core of LPD is replaced with the acid-sensitive nanosized calcium phosphate (CaP), prepared by using water-in-oil microemulsions in which siRNA was entrapped. The CaP core was then coated with DOTAP (1,2-dioleoyl-3-trimethylammonium-propane chloride salt)/cholesterol liposome membrane (Figure 6).

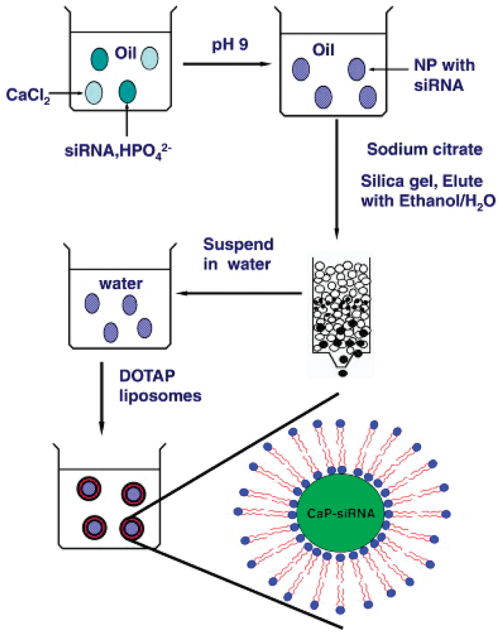


FIGURE 6. The formation process of liposome/calcium/phosphate (LCP) nanoparticles. Reproduced from ref 38 with permission. Copyright 2010 Elsevier.

The resulting new formulation is called liposome/calcium/phosphate or LCP. The LCP nanoparticles were further modified by post-complexation insertion of PEG with or without anisamide. The targeted LCP nanoparticles silenced about 70% and 50% of luciferase activity for H460 cells in culture and those grown in a xenograft model, respectively. Since CaP rapidly dissolves in the acidic pH, endocytosed CaP should disassemble in the endosomes and release its cargo into the cytoplasm. Furthermore, calcium phosphate is an inorganic component of biological hard tissues, that is, bone, teeth, and tendons, where it exists as carbonated hydroxyapatite. Therefore, the replacement of DNA (or siRNA)−protamine complex by calcium phosphate/DNA (or siRNA) complex may also decrease the immunotoxicity. Although calcium phosphate is a well used nonviral vector for *in vitro* transfection, its rapid aggregation hinders the application *in vivo*.[13,39−43] Poly(ethylene glycol)-*block*-poly(methacrylic acid) was once used to stabilize CaP crystals, but only *in vitro* gene silencing efficacy was observed.[44] Therefore, wrapping the CaP core with a PEGylated lipid membrane not only stabilizes the core but also promises a potential application of CaP for clinical trials.

PEI was also used to condense nucleic acid, followed by modification with liposomes.[45,46] Although a decreased toxicity and enhanced biological activity were shown compared with the case using nonlipidated PEI, the toxicity of PEI should be still the hurdle for its application *in vivo*.

Ultrasound-Responsive Gene Vectors

Low-intensity ultrasound in combination with microbubbles has recently acquired much attention as a safe method of gene delivery. Ultrasound shows tissue-permeabilizing effect. Ultrasound-triggered delivery allows the control of the deposition of the drug from outside the patient's body using suitable force fields.[47,48] It is noninvasive and site-specific and could make it possible to destroy tumor cells after systemic delivery, while leave nontargeted organs unaffected.

In ultrasound-triggered drug delivery, tissue-permeabilizing effect can be potentiated using ultrasound contrast agents, gas-filled microbubbles.[49−51] The use of microbubbles as gene vectors is based on the hypothesis that destruction of DNA-loaded microbubbles by a focused ultrasound beam during their microvascular transit through the target area will result in localized transduction upon disruption of the microbubble shell while sparing nontargeted areas. However, the therapeutic effect of ultrasound-targeted microbubble destruction (UTMD) is relative to the size, stability, and targeting function of microbubbles. Recently, some groups improved the properties of DNA binding microbubbles by using lipid-stabilized microbubbles. UTMD has been used to deliver genes to cells *in vitro* and *in vivo*, to treat diabetes,[50] cardiovascular disease,[51] and carcinoma[52,53] in experimental animal models. Negishi et al.[54] combined poly(ethylene glycol)-modified bubble liposomes and ultrasound exposure and developed a safe and efficient gene delivery system for skeletal muscle via intraperitoneal administration. More recently, Un et al.[55] targeted the bubble lipoplexes with mannose and developed a DNA vaccination for metastatic and relapsed melanoma, by transfection of pUb-M, coexpressing ubiquitylated gp100 and TRP-2 (Figure 7). They reported that the vaccine effects against melanoma were sustained for at least 100 days after iv administration.

Besides ultrasound-mediated delivery, magnetic targeting delivery could be used for drug targeting. However, there are fundamental limitations to the use of magnetic drug targeting. Sufficient magnetic force must be exerted on the nanomagnetic carriers at the target site before they are cleared from circulation.[56,57] Thus, magnetic nanoparticles are usually entrapped in gene vectors for imaging the delivery of nucleic acid,[13−15,23] as discussed above. Recently, Vlaskou et al.[58] generated nucleic acid carriers that combined responsiveness to both ultrasound and magnetic fields, that is, magnetic and acoustically active lipospheres (MAALs). The lipospheres were obtained upon shaking a mixture of soybean oil, a cationic lipid, magnetic nanoparticles

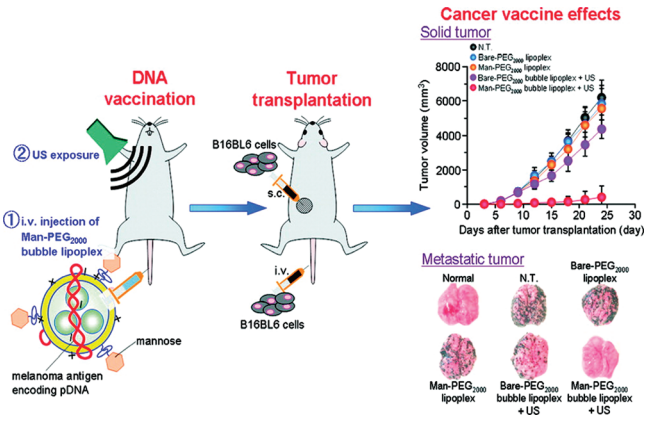


FIGURE 7. Cancer vaccine effects against solid tumors by DNA vaccination using Man-PEG_{2000} bubble lipoplexes and US exposure. Reproduced from ref 55 with permission. Copyright 2011 American Chemical Society.

(iron oxide nanoparticles), a nucleic acid, and aqueous buffer in a perfluoropropane atmosphere in a sealed vial. Although the combined application of magnetic field and ultrasound had no synergistic effect in terms of liposphere capture in the lungs, a synergistic effect of magnetic field and ultrasound was observed in site-specific plasmid deposition in a dorsal skinfold chamber model in mice after injection into the carotids. This study may indicate that gene delivery mediated by ultrasound irradiation could be improved if effective means of accumulating and retaining ultrasound microbubbles at the target sites are available.

It seems that gene therapy using microbubbles as vectors and ultrasound to direct local transfer of genes to the target site is minimally invasive and is, in theory, easily adapted to serial treatments. It may become a promising strategy that could circumvent limitations of viral gene delivery systems. However, to make it an effective therapeutic method in the clinic will require further improvements of the formulation as well as the use of more advanced ultrasound-transducing devices. The biocompatible shell to encapsulate the ultrasound contrast agent is mainly based on lipid compositions that are used for the preparation of liposomes. Detailed research on screening lipids and optimizing formulation is necessary.

Prospective and Perspective

Employing chemical and biological strategies to prepare multifunctional vectors to overcome hurdles associated with efficient cellular nucleic acid delivery has proven to be beneficial. It has provided exciting new nanomedicine-based strategies for gene therapy. Unfortunately, progress into clinical trials has been slow. There is insufficient knowledge of the physicochemical and biological properties

during the various phases of the transfection process. Leal[59] recently reported the development of cationic liposome−siRNA complexes with a novel cubic phase nanostructure that exhibited efficient silencing with low toxicity. This finding underscores the importance of understanding membrane-mediated interactions between cationic liposome−siRNA complex nanostructure and cell components in developing cationic liposome-based gene silencing vectors. Better understanding of the fate of the nanoparticles inside the cell and of the interactions between the parts of a hybrid particle will lead to a delivery system suitable for clinical use. In addition, different cell lines show a different selectivity toward the hydrophilicity of the particle's surface when it comes to the uptake of nanoparticles. A thorough study of the interaction between cells and vectors is necessary. Escaping the rapid uptake of nanoparticles by the reticuloendothelial system is also a necessary requirement for efficient tumor uptake of the encapsulated nucleic acid.

To date, the delivery of siRNA has predominantly utilized agents that were developed for plasmid DNA delivery. DNA and RNA are different in physicochemical properties, for example, the size, the affinity of cation, the stiffness of the strand (resistance to condensation), etc. Plasmid DNA needs to be transported into the nucleus for gene expression, while siRNA only needs to be transferred across the plasma membrane to reach its target in the cytoplasm. The optimal carriers may be different for these two applications and delivery reagents should be specifically developed for siRNA delivery. In addition, RNA is relatively unstable. There are only a few reports so far about the sustained delivery of siRNA.[60−62] How to make vectors targeted to cells with sustained release *in vivo* should be an interesting research challenge.

Inorganic nanoparticles offer many ways to prepare systems with a defined particle size, surface functionalization, nucleic acid protection, and biocompatibility. Because it is possible to fine-tune their nanostructure, for example, by coating them with different layers or by loading internal nanopores, their use as carriers could be extended beyond the current applications. Combination of inorganic nanoparticles with other classes of nonviral vectors should be an interesting and promising research field.

BIOGRAPHICAL INFORMATION

Xia Guo is a Full Professor of School of Chemistry and Chemical Engineering, Yangzhou University, P.R. China and currently is a visiting scholar in University of North Carolina at Chapel Hill. She obtained her Ph.D. in 1998, and her research is mainly in the field of colloids and interfaces.

Leaf Huang, Ph.D., is the Fred Eshelman Distinguished Professor and Chair, Division of Molecular Pharmaceutics in the Eshelman School of Pharmacy, University of North Carolina at Chapel Hill. His research has been in the area of gene therapy and targeted drug delivery. He has pioneered the liposome nonviral vector and has produced the vector for the first nonviral clinical trial in 1992. He has authored or coauthored more than 300 peer-reviewed papers and more than 120 reviews and book chapters. He is the inventor or co-inventor of 16 U.S. and foreign patents and cofounded 5 biotech start-ups in the past.

The authors are supported by NIH Grants CA129835, CA149363, CA151652, and CA129421 and by National Natural Scientific Foundation of China (Nos. 21073155 and 20603031) and Jiangsu provincial natural scientific foundation (No. BK2010308).

FOOTNOTES

*To whom correspondence should be addressed. E-mail: leafh@email.unc.edu. Phone: (919) 843-0736. Fax: (919) 966-0197.

REFERENCES

1 Verma, I. M; Somia, N. Gene Therapy-Promises, Problems and Prospects. *Nature* **1997**, *389*, 239–242.

2 Cartier, N.; Hacein-Bey-Abina, S.; Bartholomae, C. C.; Veres, G.; Schmidt, M.; Kutschera, I.; Vidaud, M.; Abel, U.; Dal-Cortivo, L.; Caccavelli, L.; Mahlaoui, N.; Kiermer, V.; Mittelstaedt, D.; Bellesme, C.; Lahlou, N.; Lefrere, F.; Blanche, S.; Audit, M.; Payen, E.; Leboulch, P.; l'Homme, B.; Bougneres, P.; Von Kalle, C.; Fischer, A.; Cavazzana-Calvo, M.; Aubourg, P. Hematopoietic Stem Cell Gene Therapy with a Lentiviral Vector in X-linked Adrenoleukodystrophy. *Science* **2009**, *326*, 818–823.

3 Braeckmans, K.; Buyens, K.; Naeye, B.; Vercauteren, D.; Deschout, H.; Raemdonck, K.; Remaut, K.; Sanders, N. N.; Demeester, J.; De Smedt, S. C. Advanced Fluorescence Microscopy Methods Illuminate the Transfection Pathway of Nucleic Acid Nanoparticles. *J. Controlled Release* **2010**, *148*, 69–74.

4 Xu, L.; Anchordoquy, T. Drug Delivery Trends in Clinical Trials and Translational Medicine: Challenges and Opportunities in the Delivery of Nucleic Acid-Based Therapeutics. *J. Pharm. Sci.* **2011**, *100*, 38–52.

5 Malam, Y.; Lim, E. J.; Seifalian, A. M. Current Trends in the Application of Nanoparticles in Drug Delivery. *Curr. Med. Chem.* **2011**, *18*, 1067–1078.

6 Peer, D. Induction of Therapeutic Gene Silencing in Leukocyte-Implicated Diseases by Targeted and Stabilized Nanoparticles: A Mini-Review. *J. Controlled Release* **2010**, *148*, 63–68.

7 Stokman, G.; Qin, Y.; Rácz, Z.; Hamar, P.; Price, L. S. Application of siRNA in Targeting Protein Expression in Kidney Disease. *Adv. Drug Delivery Rev.* **2010**, *62*, 1378–1389.

8 Gao, W.; Chan, J. M.; Farokhzad, O. C. pH-Responsive Nanoparticles for Drug Delivery. *Mol. Pharmaceutics* **2010**, *7*, 1913–1920.

9 Krebs, M. D.; Alsberg, E. Localized, Targeted, and Sustained siRNA Delivery. *Chem.—Eur. J.* **2011**, *17*, 3054–3062.

10 Wang, J.; Lu, Z.; Wientjes, M. G.; Au, J. L.-S. Delivery of siRNA Therapeutics: Barriers and Carriers. *AAPS J.* **2010**, *12*, 492–503.

11 Saranya, N.; Moorthi, A.; Saravanan, S.; Devi, M. P.; Selvamurugan, N. Chitosan and Its Derivatives for Gene Delivery. *Int. J. Biol. Macromol.* **2011**, *48*, 234–238.

12 Posadas, I.; Guerra, F. J.; Ceña, V. Nonviral Vectors for the Delivery of Small Interfering RNAs to the CNS. *Nanomedicine* **2010**, *5*, 1219–1236.

13 Sokolova, V.; Epple, M. Inorganic Nanoparticles as Carriers of Nucleic Acids into Cells. *Angew. Chem., Int. Ed.* **2008**, *47*, 1382–1395.

14 Prijic, S.; Sersa, G. Magnetic Nanoparticles as Targeted Delivery Systems in Oncology. *Radiol. Oncol.* **2011**, *45*, 1–16.

15 Namgung, R.; Zhang, Y.; Fang, Q. L.; Singha, K.; Lee, H. J.; Kwon, I. K.; Jeong, Y. Y.; Park, I.-K.; Son, S. J.; Kim, W. J. Multifunctional Silica Nanotubes for Dual-Modality Gene Delivery and MR Imaging. *Biomaterials* **2011**, *32*, 3042–3052.

16 Jia, J.; Zhu, F.; Ma, X.; Cao, Z. W.; Li, Y. X.; Chen, Y. Z. Mechanisms of Drug Combinations: Interaction and Network Perspectives. *Nat. Rev. Drug Discovery* **2009**, *8*, 111–128.

17 Bhattarai, S. R.; Muthuswamy, E.; Wani, A.; Brichacek, M.; Castañeda, A. L.; Brock, S. L.; Oupicky, D. Enhanced Gene and siRNA Delivery by Polycation-Modified Mesoporous Silica Nanoparticles Loaded with Chloroquine. *Pharm. Res.* **2010**, *27*, 2556–2568.

18 Kunath, K.; Merdan, T.; Hegener, O.; Haberlein, H.; Kissel, T. Integrin Targeting Using RGD-PEI Conjugates for in Vitro Gene Transfer. *J. Gene Med.* **2003**, *5*, 588–599.

19 Schiffelers, R. M.; Ansari, A.; Xu, J.; Zhou, Q.; Tang, Q.; Storm, G.; Molema, G.; Lu, P. Y.; Scaria, P. V.; Woodle, M. C. Cancer siRNA Therapy by Tumor Selective Delivery with Ligand-Targeted Sterically Stabilized Nanoparticle. *Nucleic Acids Res.* **2004**, *32*, No. e149.

20 Ng, Q. K. T.; Sutton, M. K.; Soonsawad, P.; Xing, L.; Cheng, H.; Segura, T. Engineering Clustered Ligand Binding into Nonviral Vectors: $\alpha_v\beta_3$ Targeting as an Example. *Mol. Ther.* **2009**, *17*, 828–836.

21 Ng, Q. K. T.; Su, H.; Armijo, A. L.; Czernin, J.; Radu, C. G.; Segura, T. Clustered Arg−Gly−Asp Peptides Enhances Tumor Targeting of Nonviral Vectors. *ChemMedChem* **2011**, *6*, 623–627.

22 McMahon, K. M.; Mutharasan, R. K.; Tripathy, S.; Veliceasa, D.; Bobeica, M.; Shumaker, D. K.; Luthi, A. J.; Helfand, B. T.; Ardehali, H.; Mirkin, C. A.; Volpert, O.; Thaxton, C. S. Biomimetic High Density Lipoprotein Nanoparticles for Nucleic Acid Delivery. *Nano Lett.* **2011**, *11*, 1208–1214.

23 Li, C.; Penet, M.-F.; Wildes, F.; Takagi, T.; Chen, Z.; Winnard, P. T.; Artemov, D., Jr.; Bhujwalla, Z. M. Nanoplex Delivery of siRNA and Prodrug Enzyme for Multimodality Image-Guided Molecular Pathway Targeted Cancer Therapy. *ACS Nano* **2010**, *4*, 6707–6716.

24 Yamashita, S.; Fukushima, H.; Akiyama, Y.; Niidome, Y.; Mori, T.; Katayama, Y.; Niidome, T. Controlled-Release System of Single-Stranded DNA Triggered by the Photothermal Effect of Gold Nanorods and Its in Vivo Application. *Bioorg. Med. Chem.* **2011**, *19*, 2130–2135.

25 Lee, S. K.; Han, M. S.; Asokan, S.; Tung, C.-H. Effective Gene Silencing by Multilayered siRNA-Coated Gold Nanoparticles. *Small* **2011**, *7*, 364–370.

26 MacLachlan, I. *Liposomal Formulations for Nucleic Acid Delivery*; Taylor and Francis Group, LLC: London, 2007; pp 242−249.

27 Li, S.; Rizzo, M. A.; Bhattacharya, S.; Huang, L. Characterization of Cationic Lipid-Protamine-DNA (LPD) Complexes for Intravenous Gene Delivery. *Gene Ther.* **1998**, *5*, 930–937.

28 Li, S.; Huang, L. In Vivo Gene Transfer via Intravenous Administration of Cationic Lipid-Protamine-DNA (LPD) Complexes. *Gene Ther.* **1997**, *4*, 891–900.

29 Li, S.; Chono, S.; Huang, L. Efficient Gene Silencing in Metastatic Tumor by siRNA Formulated in Surface-Modified Nanoparticles. *J. Controlled Release* **2008**, *126*, 77–84.

30 Li, S.; Huang, L. Surface-Modified LPD Nanoparticles for Tumor Targeting. *Ann. N.Y. Acad. Sci.* **2006**, *1082*, 1–8.

31 Li, S.; Huang, L. Targeted Delivery of Antisense Oligodeoxynucleotide and Small Interference RNA into Lung Cancer Cells. *Mol. Pharmaceutics* **2006**, *3*, 579–588.

32 Li, S.; Chen, Y.; Hackett, M.; Huang, L. Tumor-Targeted Delivery of siRNA by Self-Assembled Nanoparticles. *Mol. Ther.* **2008**, *16*, 163–169.

33 Chen, Y.; Bathula, S. R.; Yang, Q.; Huang, L. Targeted Nanoparticles Deliver siRNA to Melanoma. *J. Invest. Dermatol.* **2010**, *130*, 2790–2798.

34 Li, S.; Huang, L. Nanoparticles Evading the Reticuloendothelial System: Role of the Supported Bilayer. *Biochim. Biophys. Acta* **2009**, *1788*, 2259–2266.

35 Chen, Y.; Bathula, S. R.; Li, J.; Huang, L. Multifunctional Nanoparticles Delivering Small Interfering RNA and Doxorubicin Overcome Drug Resistance in Cancer. *J. Biol. Chem.* **2010**, *285*, 22639–22650.

36 Mozafari, M. R.; Reed, C. J.; Rostron, C. Prospects of Anionic Nanolipoplexes in Nanotherapy: Transmission Electron Microscopy and Light Scattering Studies. *Micron* **2007**, *38*, 787–795.

37 Mortazavi, S. M.; Mohannadabadi, M. R.; Khosravi-Darani, K.; Mozafari, M. R. Preparation of Liposomal Gene Therapy Vectors by a Scalable Method without Using Volatile or Detergents. *J. Biotechnol.* **2007**, *129*, 604–613.

38 Li, J.; Chen, Y.; Tseng, Y.; Mozumdar, S.; Huang, L. Biodegradable Calcium Phosphate Nanoparticle with Lipid Coating for Systemic siRNA Delivery. *J. Controlled Release* **2010**, *142*, 416–421.

39 Sokolova, V.; Kovtun, A.; Prymak, O.; Meyer-Zaika, W.; Kubareva, E. A.; Romanova, E. A.; Oretskaya, T. S.; Heumann, R.; Epple, M. Functionalisation of Calcium Phosphate Nanoparticles by Oligonucleotides and Their Application for Gene Silencing. *J. Mater. Chem.* **2007**, *17*, 721–727.

40 Olton, D.; Li, J.; Wilson, M. E.; Rogers, T.; Close, J.; Huang, L.; Kumta, P. N.; Sfeir, C. Nanostructured Calcium Phosphates (NanoCaPs) for Non-Viral Gene Delivery: Influence of the Synthesis Parameters on Transfection Efficiency. *Biomaterials* **2007**, *28*, 1267–1279.

41 Maitra, A. Calcium Phosphate Nanoparticles: Second-Generation Nonviral Vectors in Gene Therapy. *Expert Rev. Mol. Diagn.* **2005**, *5*, 893–905.

42 Eufinger, H.; Rasche, C.; Lehmbrock, J.; Wehmoller, M.; Weihe, S.; Schmitz, I.; Schiller, C.; Epple, M. Performance of Functionally Graded Implants of Polylactides and Calcium Phosphate/Calcium Carbonate in an Ovine Model for Computer Assisted Craniectomy and Cranioplasty. *Biomaterials* **2007**, *28*, 475–485.

43 Sokolova, V. V.; Radtke, I.; Heumann, R.; Epple, M. Effective Transfection of Cells with Multi-Shell Calcium Phosphate-DNA Nanoparticles. *Biomaterials* **2006**, *27*, 3147–3153.

44 Kakizawa, Y.; Furukawa, S.; Ishii, A.; Kataoka, K. Organic-Inorganic Hybrid-Nanocarrier of siRNA Constructing through the Self-assembly of Calcium Phosphate and PEG-Based Block Aniomer. *J. Controlled Release* **2006**, *111*, 368–370.

45 Schäfer, J.; Höbel, S.; Bakowsky, U.; Aigner, A. Liposome-Polyethylenimine Complexes for Enhanced DNA and siRNA Delivery. *Biomaterials* **2010**, *31*, 6892–6900.

46 Ko, Y. T.; Bhattacharya, R.; Bickel, U. Liposome Encapsulated Polyethylenimine/ODN Polyplexes for Brain Targeting. *J. Controlled Release* **2009**, *133*, 230–237.

47 Mayer, C. R.; Geis, N. A.; Katus, H. A.; Bekeredjian, R. Ultrasound Targeted Microbubble Destruction for Drug and Gene Delivery. *Expert Opin. Drug Delivery* **2008**, *5*, 1121–1138.

48 Polyak, B.; Friedman, G. Magnetic Targeting for Site-Specific Drug Delivery: Applications and Clinical Potential. *Expert Opin. Drug Delivery* **2009**, *6*, 53–70.

49 Chen, S.; Shimoda, M.; Wang, M.; Ding, J.; Noguchi, H.; Matsumoto, S.; Grayburn, P. A. Regeneration of Pancreatic Islets in vivo by Ultrasound-Targeted Gene Therapy. *Gene Ther.* **2010**, *17*, 1411–1420.

50 Villanueva, F. S. Ultrasound Mediated Destruction of DNA-loaded Microbubbles for Enhancement of Cell-based Therapies: New Promise Amidst a Confluence of Uncertainties? *J. Am. Coll. Cardiol. Cardiovasc. Imaging* **2009**, *2*, 880–882.

51 Fujii, H.; Sun, Z.; Li, S. H.; Wu, J.; Fazel, S.; Weisel, R. D.; Rakowski, H.; Lindner, J.; Li, R. K. Ultrasound-Targeted Gene Delivery Induces Angiogenesis after a Myocardial Infarction in Mice. *J. Am. Coll. Cardiol. Cardiovasc. Imaging* **2009**, *2*, 869–879.

52 Carson, A. R.; Mctiernan, C. F.; Lavery, L.; Hodnick, A.; Grata, M.; Leng, X.; Wang, J.; Chen, X.; Modzelewski, R. A.; Villanueva, F. S. Gene Therapy of Carcinoma Using Ultrasound-Targeted Microbubble Destruction. *Ultrasound Med. Biol.* **2011**, *37*, 393–402.

53 Chen, Z.; Liang, K.; Liu, J.; Xie, M.; Wang, X.; Lu, Q.; Zhang, J.; Fang, L. Enhancement of Survivin Gene Downregulation and Cell Apoptosis by a Novel Combination: Liposome Microbubbles and Ultrasound Exposure. *Med. Oncol.* **2009**, *26*, 491–500.

54 Negishi, Y.; Matsuo, K.; Endo-Takahashi, Y.; Suzuki, K.; Matsuki, Y.; Takagi, N.; Suzuki, R.; Maruyama, K.; Aramaki, Y. Delivery of an Angiogenic Gene into Ischemic Muscle by Novel Bubble Liposomes Followed by Ultrasound Exposure. *Pharm. Res.* **2011**, *28*, 712–719.

55 Un, K.; Kawakami, S.; Suzuki, R.; Maruyama, K.; Yamashita, F.; Hashida, M. Suppression of Melanoma Growth and Metastasis by DNA Vaccination Using an Ultrasound-Responsive and Mannose-Modified Gene Carrier. *Mol. Pharmaceutics* **2011**, *8*, 543–554.

56 Häfeli, U. O.; Gilmour, K.; Zhou, A.; Lee, S.; Hayden, M. E. Modeling of Magnetic Bandages for Drug Targeting: Button vs. Halbach Arrays. *J. Magn. Magn. Mater.* **2007**, *311*, 323–329.

57 Hayden, M. E.; Hafeli, U. O. 'Magnetic Bandages' for Targeted Delivery of Therapeutic Agents. *J. Phys.: Condens. Matter* **2006**, *18*, No. S2877.

58 Vlaskou, D.; Mykhaylyk, O.; Krötz, F.; Hellwig, N.; Renner, R.; Schillinger, U.; Gleich, B.; Heidsieck, A.; Schmitz, G.; Hensel, K.; Plank, C. Magnetic and Acoustically Active Lipospheres for Magnetically Targeted Nucleic Acid Delivery. *Adv. Funct. Mater.* **2010**, *20*, 3881–3894.

59 Leal, C.; Bouxsein, N. F.; Ewert, K. K.; Safinya, C. R. Highly Efficient Gene Silencing Activity of siRNA Embedded in a Nanostructured Gyroid Cubic Lipid Matrix. *J. Am. Chem. Soc.* **2010**, *132*, 16841–16847.

60 Ge, J.; Jacobson, G. B.; Lobovkina, T.; Holmberg, K.; Zare, R. N. Sustained Release of Nucleic Acids from Polymeric Nanoparticles Using Microemulsion Precipitation in Super-critical Carbon Dioxide. *Chem. Commun.* **2010**, *46*, 9034–9036.

61 Hardin, J. O.; Milam, V. T. Controlled Release of Active DNA from Uncrosslinked Matrices. *Soft Matter* **2011**, *7*, 2674–2681.

62 Kulkarni, M. M.; Greiser, U.; O'Brien, T.; Pandit, A. A Temporal Gene Delivery System Based on Fibrin Microspheres. *Mol. Pharmaceutics* **2011**, *8*, 439–446.