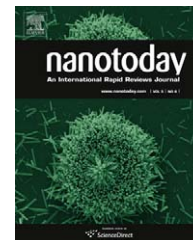




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REVIEW

Functional nanoparticles for molecular imaging guided gene delivery

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Summary Gene therapy has great potential to bring tremendous changes in treatment of various diseases and disorders. However, one of the impediments to successful gene therapy is the inefficient delivery of genes to target tissues and the inability to monitor delivery of genes and therapeutic responses at the targeted site. The emergence of molecular imaging strategies has been pivotal in optimizing gene therapy; since it can allow us to evaluate the effectiveness of gene delivery noninvasively and spatiotemporally. Due to the unique physiochemical properties of nanomaterials, numerous functional nanoparticles show promise in accomplishing gene delivery with the necessary feature of visualizing the delivery. In this review, recent developments of nanoparticles for molecular imaging guided gene delivery are summarized.

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Introduction

Gene therapy has shown potential to treat human diseases that occur from defective genes like cystic fibrosis, macular degeneration, Parkinson's disease, and different types of cancers [1–4]. The development of efficient gene therapy depends on an efficient transfer of therapeutic genes into

a cell to replace or silence defective ones associated with human disease. Viral vectors like adenoviruses and retroviruses are commonly used in gene therapy due to their high efficiency of gene delivery. However, there are several recurring issues that have led to a reconsideration of the use of viral vectors in human clinical trials, such as immunological problems, insertional mutagenesis and limitations in the size of the carried therapeutic genes.

Recently, non-viral particles have been receiving increasing attention in gene therapy, since they can overcome major viral delivery toxicity issues [5]. Common non-viral vectors that allow the genetic material to pass through cellular barriers are extensively discussed elsewhere [6–9]. However, it remains a great challenge to find a carrier that

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will (1) load genetic materials, (2) pass the material through cellular barriers without causing a foreign body immune response, (3) release it into the cell nucleus, and (4) allow the visualization of this entire process without degrading the genetic materials. Other factors affect the effectiveness of gene therapy like the short-lived nature of the therapeutic DNA within the dividing cells and the multigene nature of many disorders where numerous mutations occur on many genes. In addition to such issues, the effectiveness of gene therapy is difficult to study without visualizing the exact transport noninvasively. Therefore there is an urgent need to develop sensitive and noninvasive methods that could be performed to overcome the challenges of gene therapy such as utilizing nano-dimensional materials to carry genes across cellular membrane barriers and exploiting unique optical or magnetic properties for noninvasive and spatiotemporal molecular imaging of gene delivery.

Molecular imaging has flourished over the last decade. Advanced molecular imaging techniques for gene therapy monitoring will enable real-time assessment of the therapeutic process and the refinement of current gene therapy protocols. Probes can allow either direct or indirect spatiotemporal evaluation of gene delivery and gene expression utilizing molecular imaging methods to guide therapeutic gene delivery and monitor the therapeutic response [10,11]. Through non-invasive monitoring of the distribution and kinetics of vector-mediated gene expression, molecular imaging can provide the functionality and most importantly the efficacy of vector and gene delivery systems. Molecular imaging is likely to aid in an improved design of targeted gene transfer methods and the selection and development of safe and efficient gene delivery systems.

The emergence of molecular imaging strategies has been pivotal in optimizing gene therapy with advanced probes [12,13]. Currently, one typical probe for molecular imaging in gene therapy is a unified fusion gene composed of both the therapy and imaging reporter gene whose expression can be imaged using multiple modalities [14]. This strategy is very useful to determine the patterns of gene expression that encode the biological processes of diseases. To date, there have been many imaging reporter genes used in the field of reporter gene imaging, such as herpes simplex virus type 1 thymidine kinase gene for single photon emission computed tomography (SPECT) and positron emission tomography (PET) [14,15], transferrin receptor gene for magnetic resonance imaging (MRI) [16], and fluorescent protein gene for optical imaging [12,15]. Generally, imaging reporter genes are used to study promoter or enhancer elements involved in disease-related gene expression. A promoter of a specific disease biomarker is inserted and the molecular imaging reporter gene is placed under the control of the special promoter fragments. The promoter can be inducible/constitutive and cell-specific and transcription of the reporter gene can be tracked, allowing the study of gene expression. The ideal imaging reporter genes would have the following characteristics: lack of immune response, favorable kinetics, stability and biocompatibility. However, no reporter gene has been found that meets all these criteria at present.

Another strategy to make molecular imaging probes and gene delivery vehicles is based on nanomaterials [17,18]. Emerging nanomaterials provide platforms that have various

sizes and structures that may be used to develop nanoparticles (NPs) with the capability to serve as gene delivery vectors and molecular imaging agents (Fig. 1). At present, there are several types of NPs available for gene therapy and molecular imaging. Such NP-based imaging probes afford many advantages over conventional small-molecule-based approaches [17,19–22]. For example, the ease of functionalizing the NP surface is a clear advantage in designing molecular carrier and probes. Imaging labels (fluorescence tags, radionuclides, and other biomolecules) or a combination of labels for different imaging modalities can be attached to a single NP, which can lead to dramatic signal amplification. Furthermore, targeting motifs, such as antibodies, peptides, aptamers, and small molecules, on the nanoparticle can provide enhanced binding affinity and specificity. The roles of molecular imaging in gene therapy continue to increase because of advances in imaging technologies and concomitant improvements in detection sensitivity and specificity with functional NPs. The combination of different targeting ligands, imaging labels, genetically engineered genes, and many other agents may allow for effective and controlled gene delivery, that could be non-invasively and quantitatively monitored in real time. These multifunctional systems will enhance diagnostic evaluation and gene therapy development and predict clinical outcomes, fulfilling the promise of personalized and advanced medicine. In the subsequent sections we discuss the functional NPs and systems for molecular imaging guided gene delivery and highlight some of the most advanced examples.

Polymer-based nanoparticles

Cationic polymers can form stable polyplexed NPs with DNAs through electrostatic interactions and these polycation/DNA complexes are by far the most widely used non-viral gene delivery vectors. Many factors affect gene transfection efficiency of polymer-based NPs including molecular weight, surface charges, amphiphilicity and the structure and shape of NPs. Several cationic polymers such as polyethyleneimine (PEI), poly-L-lysine (PLL), chitosan and poly(amidoamines) (PAMAM) are used as important vectors for gene delivery. To date, various nanobioconjugation techniques have been reported to enhance gene transfection efficiency and to reduce toxicity of these polymers *in vitro* and *in vivo*. Several comprehensive review articles have summarized the applications of cationic polymer-based gene therapy [23–25]. Imaging techniques allow the visualization of important issues involving polymeric nanoparticles for gene delivery. For example, optical imaging could visualize the degradation of polymers [26], the cellular uptake and secretion of genes and polymers [27,28], and the dissociation of genes and polymers after uptake [28,29]. In this section, a few recent examples of polymer-based gene carriers accompanied by molecular imaging techniques will be introduced.

Yao et al. reported the use of folate grafted PEI₆₀₀-cyclodextrins (folate-PEI₆₀₀-CyD) as an effective polyplex-forming plasmid delivery agent with low toxicity [30]. The toxicity of PEI was reduced by the grafting of β -cyclodextrin as previously reported [31]. To further improve the efficiency of PEI₆₀₀-CyD delivery, folic acid was conjugated as the targeting ligand. Folate-PEI₆₀₀-CyD was

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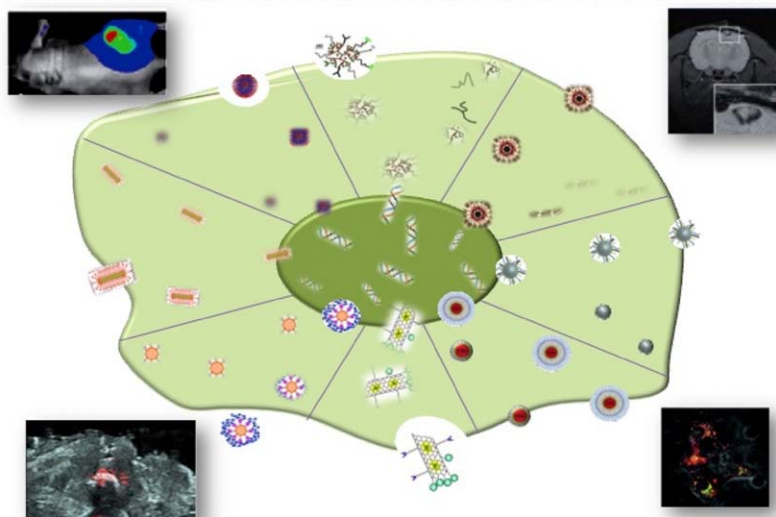


Figure 1 Schematic diagram of functional nanoparticles for molecular imaging guided gene delivery.

five-fold less toxic compared to that of PEI-25 kDa in U87 cells and showed the highest transfection efficiency, about 60–70%, in folic receptor over-expressing U138 and U87 cells. *In vivo* bioluminescence imaging, made possible by the luciferase plasmid (pLuc), showed that the efficiency of folate-PEI₆₀₀-CyD-mediated transfection was 13-fold higher compared to pLuc alone and was comparable to that of adenovirus-mediated luciferase transduction in melanoma-bearing mice.

A polyelectrolyte complex (PEC) micelle-based siRNA delivery system was developed for anti-angiogenic gene therapy and imaging [32]. The charge complexation between PEG-conjugated vascular endothelial growth factor siRNA (VEGF siRNA-PEG) and PEI led to the spontaneous formation of nanoscale PEC micelles, having a characteristic siRNA/PEI PEC inner core with a surrounding PEG shell layer. Intravenous and intratumoral injection of the siRNA containing PEC micelles significantly inhibited VEGF expression at the tumor tissue and suppressed tumor growth in an animal model without showing detectable inflammatory responses in mice. To further confirm the accumulation of PEC micelles in the solid tumor region, the siRNA was labeled with the near-infrared dye Cy5.5 and optically imaged *in vivo*. The siRNA-PEG/PEC micelles predominantly accumulated in the tumor, affirming the feasibility of siRNA-based gene therapy and imaging.

Another PEC for siRNA delivery was designed by low molecular weight chitosan (CS, m.w. 50–150 kDa) derivatized with poly-L-arginine (PLR) and PEG [33]. Among several cationic polymers, CS has been widely studied in delivery systems of negatively charged nucleic acid-based medicine due to its biocompatibility, biodegradability and low cost [34]. The PRL-grafted CS formed PEC, with a size less than 400 nm, showed higher cellular delivery efficiency of siRNA over unmodified CS, PEGylated CS, unmodified PLR, or PEGylated PLR. *In vivo* imaging of transfection efficiency after siRNA treatment was studied by using mice bearing B16F10-RFP (red fluorescent protein) tumors. Optical imaging revealed that the intratumoral administration of

RFP-specific siRNA complexed with PEGylated CS-PLR significantly silenced the expression of RFPs in tumor tissue *in vivo* by 90% as compared to that in untreated tumor tissues.

Multifunctional NPs for simultaneous gene delivery and imaging can also be prepared in a convenient way using amphiphilic biopolymers. Liu et al. constructed bifunctional NPs by encapsulating hydrophobic Re(phen) complexes with a novel amphiphilic block copolymer, PDMAEMA-b-poly(PEGMA)-b-PDMAEMA, for simultaneous cell imaging and gene transfection in living cells [35]. Certain transition metal complexes, such as Ru and Re complexes, may eliminate short-lived autofluorescence and photobleaching by taking advantage of large Stokes shifts, high photostability, low energy absorption and relatively long fluorescence lifetimes [35]. The stable, water soluble and non-toxic NPs with red emission had good interaction with DNA, making them good biolabels for cell imaging and gene transfection.

Huh et al. designed a new nanosized siRNA carrier systems composed of amphiphilic glycol chitosan (GC) and strongly positively charged PEI (Fig. 2) [36]. In order to prepare stable and tumor-homing NPs, each polymer was modified with hydrophobic 5 β -cholanic acid and mixed to form self-assembled GC-PEI NPs via strong hydrophobic interactions of hydrophobic 5 β -cholanic acids in the polymer. The siRNA encapsulated NPs formed compact and stable NP structures (250 nm in diameter) and exhibited rapid time-dependent cellular uptake and gene silencing profiles in B16F10-RFP cells. To determine if the siRNA-GC-PEI NPs specifically target tumors *in vivo*, Cy5.5-labeled siRNA containing NPs were monitored after intravenous injection in mice bearing SCC7 tumors. Cy5.5-siRNA-GC-PEI NPs showed a strong NIR fluorescence signal in tumor tissue within 1 h of injection and the signal persisted for up to two days, indicating the high tumor-targeting ability of NPs. Furthermore, NPs provided a significant inhibition of RFP gene expression of B16F10-RFP tumor-bearing mice, because of the high tumor-targeting ability.

Modern polymer chemistry and imaging science have created new strategies in the design of polymer-based gene

and the positively charged PAMAM dendrimers result in the formation of nanoscale complexes that prevent the degradation of DNA. Generally, the generation (G) and peripheral structure of dendrimers are significant in gene delivery. Low G of dendrimers result in weak electrostatic interactions between the dendrimers and DNA leading to low transfection efficiency. High G dendrimers display high DNA condensation ratio for better transfection but biocompatibility is decreased because a large number of cationic amino groups are introduced on the dendrimers. Therefore, an appropriate balance between transfection efficiency and toxicity must be met for dendrimer-based gene deliveries. To date, several types of dendrimer-based NP systems are being developed for gene delivery and monitoring gene therapy.

Navarro et al. demonstrated that a combination of well-tolerated PAMAM gene delivery carrier with an optimized expression plasmid can result in long-lasting transgene expression in tumor tissue after intravenous vector injection [41]. Polyplexes consisting of a pCMV-Luc (CMV promoter driven luciferase plasmid) condensed with PAMAM G4 and G5 efficiently protected DNA from enzymatic degradation and transfected tumor cells *in vitro*. Intravenous administration of pCpG-hCMV-Luc/PAMAM polyplexes into Neuro2A neuroblastoma tumor-bearing A/J mice showed predominant luciferase reporter gene expression in the tumor and its transfection efficiency was confirmed by bioluminescence imaging. In contrast, low-molecular-weight PEI (LPEI) polyplexes led to high gene expression in the lung and low luminescence signal in the tumor area. To monitor *in vivo* biodistribution, each polyplex was fluorescently labeled with near-infrared (NIR) quantum dots (quantoplexes) and revealed lung accumulation for both PAMAM and LPEI-based formulations, demonstrating that biodistribution and transgene expression of polyplexes do not necessarily correlate with each other.

In another report, a targeting ligand modified PAMAM has been designed and evaluated for efficient brain-targeting gene delivery [42]. A 29 amino acid peptide derived from the rabies virus glycoprotein (RVG29) was modified on PAMAM G5 through bifunctional PEG and complexed with the plasmid, yielding PAMAM-PEG-RVG29/DNA NPs. RVG29 is known to bind to the acetylcholine receptor (AChR) which is widely expressed in the brain including capillary endothelial cells [43]. PAMAM-PEG-RVG29/DNA showed higher blood–brain barrier (BBB)-crossing and gene expression efficiency than PAMAM/DNA NPs in an *in vitro* BBB model and in a small animal model. *In vivo* distribution of NPs was evaluated after systemic injection of fluorophore-labeled PAMAM-PEG-RVG29/DNA and NPs were preferably accumulated in the brain as compared with NPs without a targeting peptide.

Different types of dendrimers were also used for gene delivery and imaging. Polyglycerol (PG)-based dendrimer core shell structures exhibiting low cytotoxicity have been developed to deliver siRNA to tumors *in vivo* [44]. In this study, different PG- and PEI-derived, high molecular weight, dendritic structures were synthesized by combining PG, PEI, glucose and pentaethylenhexamine (PEHA). *In vitro*, PG-amine (aminated PG) and PEI–Glu (glucose conjugated PEI)-based luciferase siRNA-polyplexes successfully accumulated in the cytoplasm in a time-dependent manner and decreased bioluminescence in luciferase-expressing

glioblastoma cells (U87-Luc) and in primary endothelial cells. PG-amine exhibited the best ratio of silencing efficacy and enhanced siRNA transfection efficiency with reduced toxicity compared with other dendritic systems examined. Significant gene silencing of 50% was accomplished *in vivo* within 24 h of siRNA-PG-amine polyplexes treatment. *In vivo* silencing of the luciferase gene by siRNA-PG-amine and delivery of siRNA into tumors were verified by three different ways: (1) bioluminescent imaging in SCID mice bearing U87-Luc tumors, (2) bioluminescent/fluorescent imaging in BALB/C mice bearing DA3-mCherry-Luc tumors, and (3) microscopy imaging of FITC-labeled siRNA in tumor tissues.

Chisholm et al. reported NPs composed of 3G polypropyleneimine dendrimers (PPIG3) that are capable of tumor transfection upon systemic administration in tumor-bearing mice [45]. These NPs were prepared by complexing the PPIG3 dendrimer with a plasmid DNA encoding sodium iodide symporter (NIS) to form colloidal and stable self-assembled NPs with sizes ranging from 30 to 300 nm depending on the DNA concentration. Specific gene transfection efficiency was imaged by using small-animal nano-single-photon emission computed tomography/computer tomography (nanoSPECT/CT) scanner. Using nuclear whole-body imaging and NIS as a reporter gene, they were able to detect a specific and unique radiotracer uptake in tumors of both immunodeficient and immunocompetent mice whereas no signal was detected in normal animal tissue.

Dendrimers are expected to play a key role in biomedical fields in the future as they have shown efficient gene delivery when the ratio of genetic material and dendrimer is achieved. Advances in understanding the role of molecular weight and architecture on the function of dendrimers together with recent progress in molecular imaging will enable the application of these branched polymers as molecular imaging probes for gene therapy. Recently, various types of dendrimer-based inorganic NPs were also developed and this will be discussed in the following sections.

Lipid-based nanoparticles

Lipid-based NPs, such as liposomes or micelles, have been used extensively in the past few decades as gene delivery vehicles [46–48]. Generally, lipid-based NPs interact with negatively charged nucleic acids through electrostatic interactions to form lipoplexes. Many lipid-based gene delivery approaches are currently being tested at the clinical level [46]. Additionally, the lipid coating also ensures good pharmacokinetics and an improved biocompatibility of the NPs for biomedical applications [49]. A relatively new and promising application of lipid-based NPs is their use for molecular imaging of gene delivery [50,51]. The multifunctional character of lipid NP platforms has significant advantages because it allows for the inclusion of a variety of imaging agents ranging from fluorescent molecules to nanocrystals, including QDs, iron oxide NPs, and gold NPs [50,52,53]. These carriers can also be surface modified to carry a variety of other compounds such as targeting peptides by incorporating into or binding to the cationic phospholipids membrane [54,55]. Their use as gene delivery vehicles is made possible by the positively charged surface that can adsorb negatively charged genes [56].

Yagi et al. described a systemically injectable siRNA vehicle, the wrapsome (WS), which contains siRNA and a cationic lipofection complex in a core that is fully enveloped by a neutral lipid bilayer and hydrophilic polymer, PEG [57]. These NPs physically contain the siRNA and protect it from degradation and rapid dissociation from the particle. WS particles were prepared as 100 nm in diameter to maximize the enhanced permeation retention (EPR) effect [58]. *In vivo* efficacy of WS was tested by using KLF5-siRNA, which is known to play a role in tumor angiogenesis. KLF5-siRNA/WS exhibited significant antitumor activity; although, neither WS containing control scrambled-siRNA nor saline containing KLF5-siRNA affected tumor growth. KLF5-siRNA/WS inhibited KLF5 expression within tumors at both mRNA and protein levels without acute or long-term toxicity. Furthermore, NIR whole-body imaging was used to analyze the distribution of siRNA after systemic administration of Cy5-labeled siRNA/WS which confirmed its tumor specificity *in vivo* and *ex vivo*. Another form of lipid-based NPs were developed as siRNA delivery carriers based on the use of a new cationic lipid, N,N'-dioleoylglutamide (DG). DG was synthesized by peptide bond linkage of oleylamine to each carboxylic acid group of glutamic acid. DG-based cationic liposomes and siRNA form stable complexes and show high siRNA transfection efficiency and low cytotoxicity compared with conventional cationic lipid-based liposomes and commercially available Lipofectamine 2000. *In vivo* reduction of target proteins by siRNA complexed to DG was visualized by fluorescence imaging in mice bearing B16F10-RFP tumors.

Ukawa et al. developed multifunctional lipid envelope-type nanoparticles for gene delivery in which plasmid DNA/siRNA is condensed with a polycation, followed by encapsulation by a lipid envelope [59,60]. In this design, pH-sensitive fusogenic peptide (GALA) was introduced into liposomal membranes using a cholesteryl moiety for anchoring. The intracellular trafficking of dissociation, as well as the endosome escape process of the nanof ormula after receptor-mediated endocytosis was successfully revealed by a quantitative imaging analysis involving nuclear isolation, real-time PCR, and confocal laser scanning microscopy. The incorporation of cholesteryl GALA (chol-GALA) into the lipid envelope enhanced membrane fusion with the endosome, and then increased the transfection activity and gene knockdown activity of the encapsulated plasmid DNA or siRNA. Interestingly, additional coating of GALA-modified liposomes with 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer resulted in enhanced transfection activity of encapsulating plasmid DNA in primary hepatocytes [60]. The presence of an MPC polymer-coating supported cellular uptake and the subsequent cytoplasmic dissociation of DNA from the lipid envelope by assisting the endosome-fusogenic function of GALA. Such MPC/GALA-modified lipid nanoparticles appear to be a great potent carrier for a liver-targeting gene delivery system.

The relative ease of preparation, the flexibility, and most importantly, the biocompatibility of lipid-based NPs make them useful tools as multifunctional NP. Recently, lipid-based inorganic NPs were developed as siRNA gene carriers and imaging agents. Oliver et al. reported a novel liposome formulation containing a lipidic gadolinium contrast agent [61]. The gadolinium liposome was found to be an effective vehicle for transport of plasmid DNA into cells, which

shows a promising application for molecular imaging of gene therapy. The relative ease of preparation, the flexibility and, most importantly, the biocompatibility of lipid-based NPs make them useful tools as multifunctional NP. Ongoing developments in bionanotechnology may lead to the development of lipid-based NPs for molecular imaging of gene delivery with better imaging properties, improved stability and a more defined size and structure.

Iron oxide nanoparticles

Iron oxide NPs (IONPs) have a long history of investigation and have shown remarkable potentials in biomedical research, including MRI contrast enhancement, drug delivery, hyperthermia, and cell separation/labeling [62–68]. The popularity of IONPs is mainly because they: (1) provide an MR-based read-out, in particular on T_2^* -weighted images; (2) can be magnetically manipulated and change their magnetic properties; (3) can be biologically degraded, metabolized and integrated into serum Fe pool to form hemoglobin or to enter other metabolic processes; (4) have a large surface area for carrying drugs and genes. These features give IONPs great advantages for *in vivo* molecular imaging and drug/gene delivery.

Like other inorganic NPs, surface modification processes are necessary to stabilize IONPs for strong binding enhancement of the therapeutic gene and to control the release mechanism. Without optimized surface modification the gene may not strongly bind to IONPs, resulting in the instant release of the therapeutic gene during the delivery. Positive charges are the most suitable surface charges for IONP applications in gene delivery. Thus, a large amount of negatively charged DNA molecules can bind on the positively charged surface of the particles by utilizing electrostatic interactions. A popular choice for this approach is to use cationic polymers such as PEI, PLL, and chitosans. Various PEI-coated IONPs were reported for *in vitro* magnetic NP-mediated non-viral gene delivery [69,70]. However, the *in vivo* applications of these NPs have been limited due to concerns over cellular toxicity.

To overcome these limitations, various polymers have been used to coat or conjugate to the IONPs. PEGylated PEI has been synthesized and applied to IONPs for targeted gene delivery and imaging. Chen et al. reported a T cell specific ligand, the CD3 single chain antibody (scAb_{CD3}), conjugated to PEG-g-PEI stabilized IONPs for gene delivery to T cells for immunosuppression [71]. Based on a reporter gene assay, scAb_{CD3}-PEG-g-PEI functionalized IONPs led to 16-fold gene transfection enhancement in rat T lymphocyte HB8521 cells with low cytotoxicity, demonstrating effective T-lymphocyte-targeted immunotherapy. Furthermore, this targeting process in cells was successfully imaged by MRI. Self-assembled chitosan was also used as an effective coating to stabilize IONPs. A hepatocyte-targeted gene delivery and imaging system has been developed by using ^{99m}Tc -labeled IONPs-loaded chitosan-linoleic acid NPs (^{99m}Tc -SCLNs) [72]. After intravenous injection of NPs, SCLNs were specifically driven to the hepatocytes by linoleic acid, which accumulate in hepatocytes and play a central role in the liver. Furthermore, SCLN complexes containing enhanced green fluorescence protein (pEGFP) plasmid

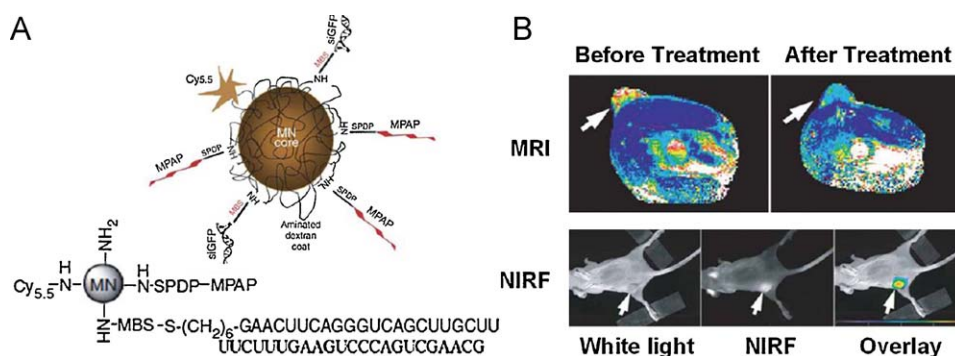


Figure 3 Functional iron oxide nanoparticles (IONPs) for molecular imaging of gene delivery. (A) Schematic illustration of the multifunctional nanoparticles consisting of a magnetic nanoparticle labeled with Cy5.5, membrane translocation peptides (MPAP), and short-interfering ribose nucleic acid (siRNA) molecules. (B) *In vivo* magnetic resonance imaging (MRI) of mice bearing subcutaneous tumors before and after treatment. High-intensity optical signal in the tumor confirmed the delivery of the nanoparticle. Adapted with permission from ref. [19].

expressed GFP in hepatocytes after administration in mice and the selective accumulation of NPs in hepatocytes could be monitored. Recently, Kievit et al. engineered a novel copolymer, CP-PEI, to coat onto IONPs by combining the biocompatibility of chitosan and the steric stabilization of PEG with the large positive charge of PEI [73]. The NP-CP-PEI was designed for stable binding, protecting, and delivering of DNA for gene expression while maintaining IONP properties and high biocompatibility. *In vivo*, the NP-CP-PEI demonstrated a high level of GFP expression in a C6 xenograft mouse model and showed enhanced MR contrast in cells.

Multifunctional probes based on IONPs for multimodality imaging and delivery of genes have been developed as well [19,74,75]. Noninvasive dual-modality imaging was accomplished using multifunctional IONPs for simultaneous *in vivo* imaging and transfer of siRNAs into tumors by both MRI and fluorescence optical imaging [19]. The resulting probe consisted of aminated dextran-coated IONPs labeled with Cy5.5 and two different linkers: membrane translocation peptides (MPAP, for intracellular delivery) and siRNA molecule targeting GFP (siGFP) (Fig. 3). Tracking results *in vivo* of these multifunctional IONPs by MR and NIR fluorescence imaging demonstrated that they could simultaneously deliver siRNAs and monitor therapeutic efficacy.

In another example oligodeoxynucleotide (ODN) was attached to the surface of the IONPs by employing cleavable linkers [76,77]. In this study, a phosphorothioate-modified ODN (sODN) complementary to the target mRNA was labeled with rhodamine or FITC on the 5'-end and biotin on the 3'-end, allowing the biotinylated ODN to conjugate to IONPs via an avidin–biotin linkage. NPs were delivered by intracerebroventricular infusion to the cerebral ventricle of mice. Superparamagnetic iron oxide (SPIO) nanoparticles with sODN in the living brain could be imaged with MRI. Another design of IONPs multifunctional imaging and gene delivery was reported by Agrawal et al. In this study, dendrimer-conjugated magnetofluorescent nanoworms (dendriworms) as carriers and imaging agents for siRNA delivery were engineered [78]. To prepare dendriworms, cysteamine core PAMAM G4 dendrimers were reduced and conjugated via a heterobifunctional linker to cross-linked iron oxide nanoworms which were amine-modified and NIR dye labeled.

Dendriworms were able to deliver cargo into cells with negligible toxic effects on cells and delivered siRNA 2.5-fold more efficiently than commercial cationic lipids. In addition, anti-EGFR dendriworms led to specific and significant suppression of EGFR expression in an EGFR-driven transgenic model of glioblastoma and enabled fluorescent tracking of siRNA delivery *in vivo*.

Quantum dots

Quantum dots (QDs) are colloidal nanosized semiconductor particles. These NPs can be excited over a wide range of wavelengths and emit specific sharp bands with limited photobleaching that can be fine-tuned during the synthesis technique [79,80]. In addition to the advantages that these NPs have for molecular imaging, QDs can also be used as intracellular carriers for various biomolecules such as proteins, antibodies, and genes. QDs have been used extensively as cell labels, tissue imaging agents, FRET donors, and sensing agents. The wide variety of QD applications in the biomedical field has been summarized elsewhere [81–85].

To improve the knockdown efficacy, various delivery techniques are used to deliver QDs into the nucleus. Srinivasan et al. designed QD-decorated plasmid DNA for long-term intracellular and intranuclear tracking studies [86]. Numerous phospholipid-coated, CdSe/ZnS core/shell QDs were conjugated to plasmid DNA via a peptide nucleic acid (PNA)-N-succinimidyl-3-(2-pyridylthio) propionate linker. These QD–DNA conjugates were capable of expressing enhanced green fluorescent protein in the presence of Lipofectamine 2000 and monitored by fluorescence microscopy, while the cellular uptake of QD–DNA conjugates was monitored in real-time at the same time. Overall, transfection was 62% more efficient than unconjugated plasmid DNA.

Other functionalizing techniques have been used to improve cell and nucleus internalization. By using a PEGylated QD core as a scaffold, siRNA and a FITC-labeled tumor targeting peptide FITC-F3 were conjugated to the particle surface [87]. F3 peptide is a 31 amino acid peptide that has been shown to target tumor cells *in vitro* and in tumor-bearing mice when systemically administered as a

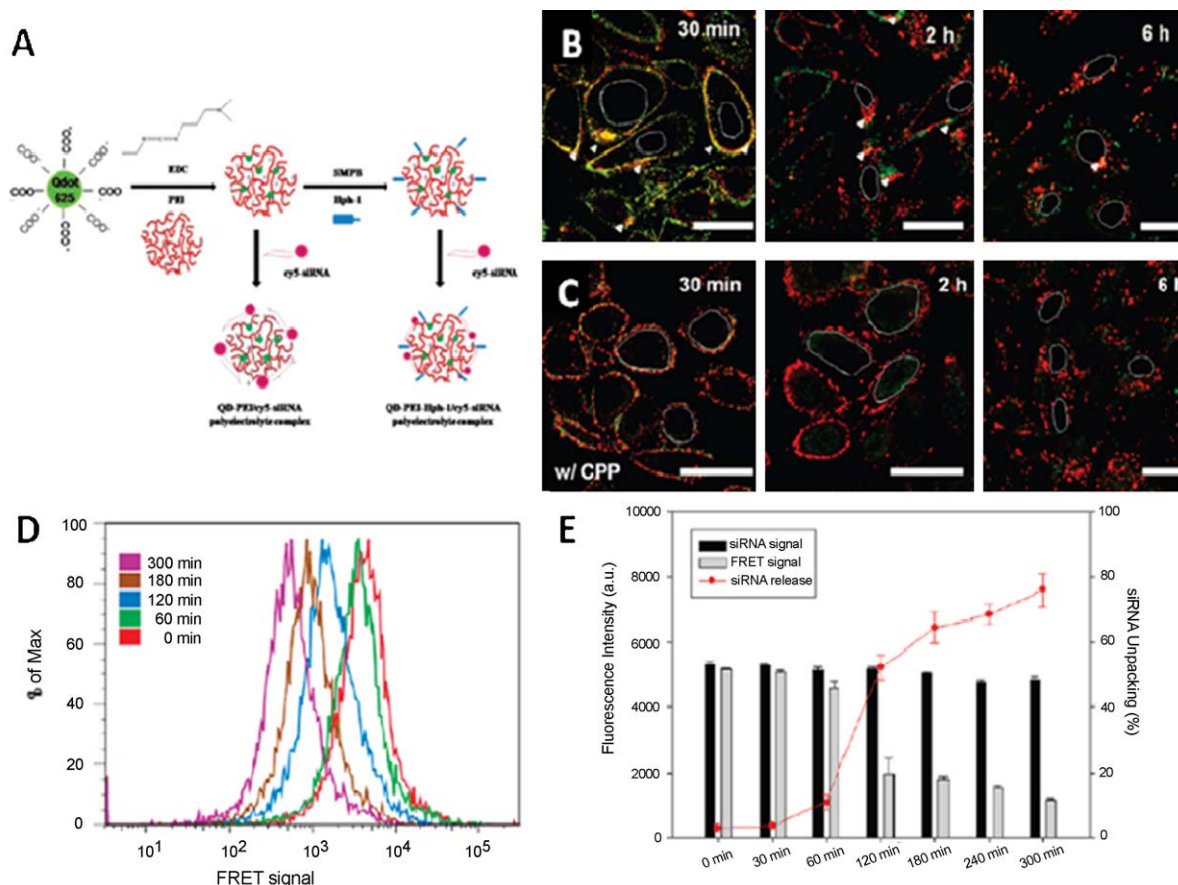


Figure 4 (A) Schematic image of the PEI conjugated QDs (QD-PEI) and endocytosis inhibitor conjugated QD-PEI (QD-PEI-Hph-1). Intracellular uptake observed by confocal imaging of (B) Cy5-siRNA to QD-PEI and (C) Cy5-siRNA to QD-PEI-Hph-1. (D) Intensity of FRET signals when QD-PEI is incubated *in vitro*. (E) The FRET intensity over incubation time as compared to the fluorescence intensity of the Cy5-siRNA alone. Scale bar is 10 μm . Adapted with permission from ref. [86].

free peptide or conjugated with PEGylated QDs [88]. In an EGFR model system, prepared F3/siRNA-QDs produced significant knockdown of EGFR signal (29%) after delivery to cells and release of siRNA from their endosomal entrapment [87]. Delivery of siRNA containing QDs was successfully imaged and tracked by fluorescent microscopy. Recent works have focused on preventing the use of additional chemical treatments for cellular internalization to minimize its toxicity. Tan et al. showed that QDs can be encapsulated into polymer NPs and used as self-tracking carriers for human epidermal growth factor receptor 2 (HER-2) siRNA [89]. When positively charged chitosan NPs are used in conjunction with the fluorescent probes, the particles are internalized into cells, gene delivery is easily monitored *in vitro*, and the HER-2 gene is successfully silenced [89]. Another form of polymer encapsulated QDs were developed for effective and safe siRNA delivery via a proton-absorbing polymeric coating (proton sponges) [90]. With a balanced composition of positively and negatively charged functional groups on the QD surface, such as carboxylic acid and tertiary amine, these NPs can be designed to specifically release the trapped siRNAs into the cytoplasm. Proton-sponge layer coated QDs allowed for siRNA adsorption and demonstrated a 10–20 fold improvement in gene silencing efficiency. Without the use of

Lipofectamine 2000, a six-fold reduction in cellular toxicity was achieved because of the increased rate of endosomal escape. Cellular uptake of these particles could be visualized in real-time due to the fluorescent signal of the QDs while ultrastructural localizations could be determined by electron microscopy by detecting the presence of semiconductors [90].

Fluorescence resonance energy transfer (FRET) is an interaction between the electronically excited states of two fluorophores, in which energy is transferred by long-range dipole-dipole coupling from a donor fluorophore that is in an electronic excited state to an acceptor chromophore. FRET is very sensitive to nanometer-scale changes in donor-acceptor separation distances and their relative dipole orientations, which provides a powerful tool to probe a great variety of biological processes. QDs have size-dependent narrow emission, broad absorption windows and strong resistance to photobleaching, making them well-suited for multi-colored imaging applications. These properties would facilitate effective wavelength separation between donor and acceptor fluorescence and therefore allow the use of QDs as very attractive donors in FRET-based applications. Several comprehensive reviews have summarized their biological applications [91–93].

A FRET-based technique was applied to image the release of genetic materials from QDs [94]. QDs were first covalently conjugated with positively charged PEI and then complexed by electrostatic interactions with Cy5-labeled vascular endothelial growth factor siRNA (Cy5-VEGF siRNA) to prepare nanosized PECs (Fig. 4A). A FRET effect occurred between the dye on the VEGF siRNA (the acceptor) and the QD (the donor) when the two components were electrostatically bound and a visible fluorescence overlap was observed. Significantly enhanced delivery was observed with the use of PEI-coated-QDs as opposed to the PEI alone. From confocal imaging analysis, the intracellular uptake and release of the siRNA was observed during delivery from the different fluorescent signals emitted by the two components during detachment (Fig. 4B and C). The FRET effect not only provided real-time imaging of the gene delivery, but also provided a quantitative evaluation of the siRNA release from the nanoparticle by the use of flow cytometry analysis to monitor when the siRNA and QD were tightly bound and when the two components would start to detach and unpack into the nucleus and hence exhibit two distinct fluorescence spectra (Fig. 4D and E).

Other organic and inorganic nanoparticles

Carbon nanotubes (CNTs), gold NPs, and silica NPs have inherently interesting properties that could be potential candidates for gene delivery and tracking. Although not common nanoparticles for gene delivery applications, these organic and inorganic NPs are becoming more popular for imaging and drug/gene delivery applications. Collectively, these particles exhibit simple surface functionalization, unique acoustic signals, and loadability.

Carbon nanotubes

Since the first publication of CNTs in 1991, these carbon nanomaterials have been widely explored for their many potential biomedical applications [95–97]. CNTs are one dimensional seamless cylindrical graphene sheets where single-walled carbon nanotubes (SWNTs) are composed of a single graphene sheet and multi-walled carbon nanotubes (MWNTs) are made up of multiple concentric SWNTs. Diameters of SWNTs can be as low as 0.4 nm while for MWNTs the diameter can be around 100 nm. Lengths typically range from hundreds of nanometers up to tens of micrometers. The unique feature of CNTs is the graphene wall that is easily functionalized with various biomolecules, imaging agents, and drugs. Gene delivery is made possible when these insoluble nanomaterials are either covalently functionalized by oxidation of the CNTs in acidic conditions and 1,3-dipolar cycloaddition reaction or noncovalently functionalized with hydrophobic or π – π stacking between the CNT and another non-polar ring such as the backbone of DNA. Ammonium-functionalized CNTs can bind plasmid DNA by electrostatic interactions and penetrate the cell membrane through a nanoneedle model as visualized by TEM [98]. In this way, CNTs are seen as exceptional nanomaterials for gene and drug delivery because they can be taken up by mammalian cells in an endocytosis-independent pathway. Both SWNTs and MWNTs have also been found to form stable complexes

with plasmid DNA and allow for the successful delivery of genes [99,100]. Due to the versatility of the CNT wall, fluorescent markers and biomolecules can be bound to study the cellular uptake efficiency. In this way, CNTs can be covalently linked with fluorescein or biotin to form a fluorescent biotin–avidin complex to study *in vitro* uptake [101].

As *in vitro* studies of DNA delivery have demonstrated successful cellular uptake and versatile functionalization of CNTs, siRNA bound to CNTs can be used for gene silencing. CNT-PEG-siRNA have been synthesized with efficient uptake and inhibition of the gene coding for lamin A/C protein in HeLa cells [102]. The knockdown efficiency was dependent on the siRNA linkage to CNT and a disulfide bond linkage allowed for the gene knockdown levels to reach about 70%. *In vivo* drug delivery of the anticancer drug such as paclitaxel and doxorubicin has been exemplified by PEGylated SWNTs with specific tumor targeting and minimal toxicity [103], but very few examples of *in vivo* gene delivery has been reported using CNTs [104–108]. Notably, pristine SWNTs were noncovalently bound to siRNA, which served as the delivery agent to silence hypoxia-inducible factor 1 alpha (HIF-1 α) as well as the dispersing agent for SWNTs [104]. The complex was intratumorally administered into mice bearing pancreatic cells with a HIF-1 α /luciferase reporter. Using this model, bioluminescence was used to image luciferase reporter gene activity *in vivo* and SWNTs demonstrated a high photon flux indicating the activity of HIF-1 α . Therefore, if the activity of HIF-1 α decreased then the luciferin bioluminescence signal had decreased intensity. As seen in Fig. 5, the bioluminescence signal after luciferin is injected to the tumor is high but with the addition of SWNTs complexed to HIF-1 α siRNA, the bioluminescence signal of the mouse tumor decreases corresponding to significant HIF-1 α inhibition. Recent works have shown the effective gene delivery of insulin 2 gene to mice using another carbon nanomaterial – fullerene, namely by tetra(piperazino)fullerene epoxide [109]. Individual SWNTs exhibit unique intrinsic properties such as NIR photoluminescence, strong Raman scattering, and photoacoustic signals that could be used to track gene delivery. Future works could enhance the interesting properties of CNTs for efficient gene delivery tracking.

Silica nanoparticles

Silica NPs have served as excellent drug and gene delivery agents since they are easily chemically and biologically modified with biomolecules and detection agents necessary for gene therapy. For efficient cellular delivery, silica NPs need to be modified with an anchoring group and charge transfer functional group to allow for DNA binding by electrostatic interactions. PLL is bound to silica NPs by electrostatic interactions to bind antisense ODN and to enhance the endocytotic cellular uptake of the genetic materials [110]. To study the delivery of ODNs and the procedure of cellular uptake, the nucleotide was labeled with FITC and monitored with fluorescence microscopy. The endosome/lysosome encapsulation was short-lived because PLL is able to destabilize the membrane allowing an efficient ODN escape from the acidic environment. By using ring opening

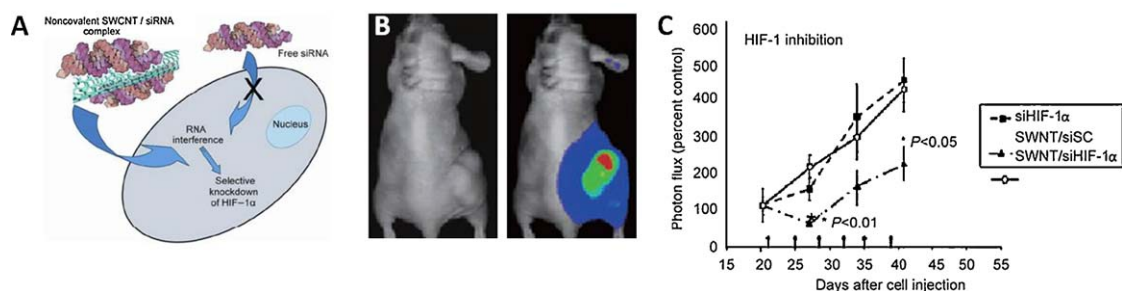


Figure 5 (A) Schematic diagram of SWNT complexed with non-targeted siRNA (SWNT/siSC). (B) Tumor bearing mice were imaged 5 min after the addition of luciferin. Then the tumors were injected with siRNA targeting HIF-1 alpha (siHIF-1a), SWNT/siSC, and SWNT complexed with targeted siRNA for HIF-1 alpha (SWNT/siHIF-1a). (C) Photon flux was imaged during the treatments illustrating the HIF-1 inhibition and therefore the delivery effectiveness of siRNA. Adapted with permission from ref. [104].

NCA polymerization and click chemistry, PLL was functionalized to silica NPs at a graft density of one chain per 1 nm², a very effective technique that can be applied for future efficient gene delivery [111].

Silica NPs modified with sodium chloride and the inorganic fluorescent dye [Ru(II)(bpy)₃]²⁺ also showed *in vitro* transfection efficiency greater than 70% [112]. *In vivo* studies demonstrated that these DNA-carrying NPs were able to pass through the blood–brain, blood–prostate, and blood–testis barriers without any significant toxicity. A unique feature of silica particles is the ability to be organically modified in order to self-assemble micelles in which the core of the particles can be loaded with various

biomolecules, and either hydrophilic or hydrophobic dyes [113]. Using this approach, Roy et al. loaded the organically modified silica NPs with ethidium monoazide (EMA) [114]. Another dye, ethidium homodimer-1 (EthD-1), was embedded between the DNA and the surface of the silica NPs. A FRET occurred between EMA and the EthD-1 when the DNA was bound to the surface of the micelle. Fluorescence microscopy was used to visualize the silica NPs entering into the cytoplasm and the DNA being delivered into nucleus as it detached from the silica micelle. DNA was electrostatically bound to the triethoxyvinylsilane found on the surface of the silica NPs, which protected the DNA from enzymatic digestion during intracellular trafficking. These organically

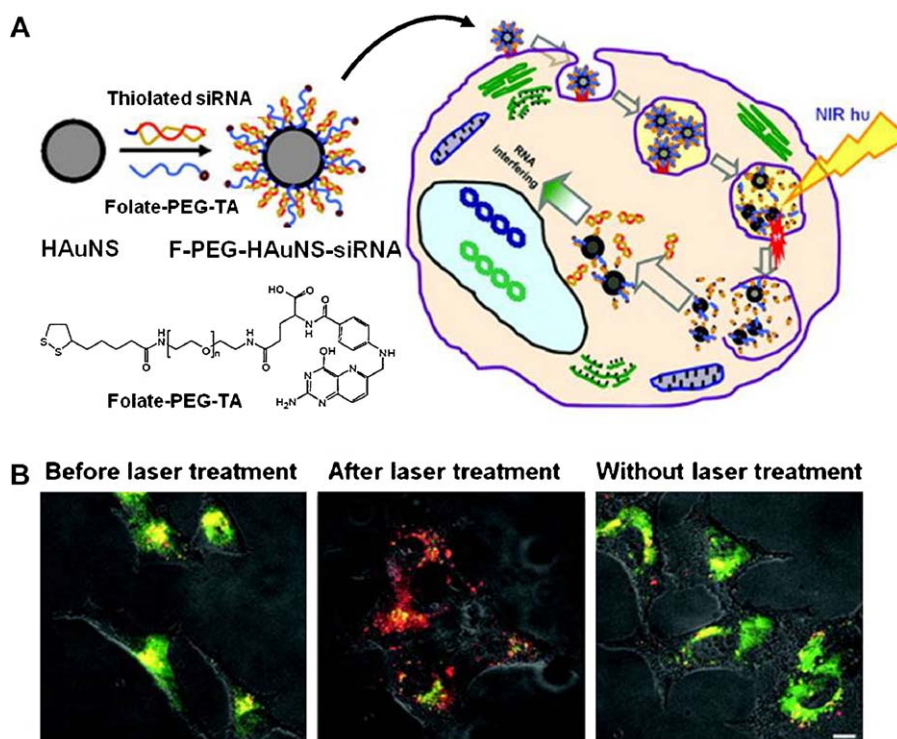


Figure 6 Scheme for bioconjugation of HAuNS-siRNA and photothermal-induced siRNA release. (A) Scheme for the synthesis of F-PEG-HAuNS-siRNA and their proposed intracellular itinerary following NIR light irradiation. (B) Photothermal-induced endolysosomal escape of Dy547-labeled siRNA. Adapted with permission from ref. [125].

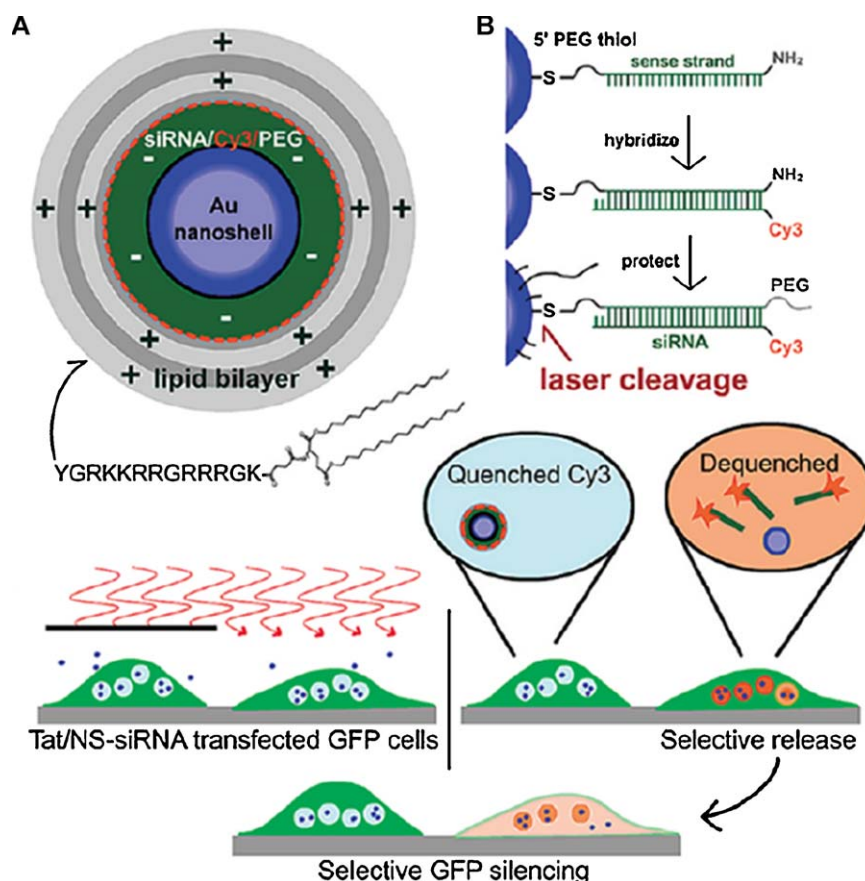


Figure 7 (A) Diagram of Tat-lipid-coated siRNA used for transfection and selective release of siRNA and (B) schematic of the siRNA construct.

Adapted with permission from ref. [126].

modified silica NPs were used as *in vivo* genetic vectors to deliver the nucleus-targeting plasmid expressing EGFP into the neuronal cells of the subventricular zone in the mouse brain [115]. When the silica NPs carry the plasmid into the cell, GFP is expressed which can be captured using an *in vivo* confocal fluorescence imaging system. The *in vivo* imaging and immunostaining show a successful gene transfection of the EGFP within the subventricular zone.

Gold nanoparticles

The rapid advancement of nanotechnology over the past decade has opened opportunities for the design of functional gold nanoparticles (AuNPs) for photothermal therapy, biosensing, molecular imaging, and gene therapy. Recently, AuNPs have been considered as excellent gene delivery systems due to their unique properties and excellent abilities to bind/bioconjugate biological ligands, DNA and siRNA through surface bonding [116–120]. Lee et al. reported biologically functional cationic phospholipid-gold NPs that simultaneously exhibit carrier capabilities, demonstrate improved colloidal stability, maintain plasmonic properties, and show good biocompatibility under physiological conditions [121].

AuNPs exhibit unique optical properties due to strong and tunable surface plasmon absorption in the NIR range, which can cause photothermal effects to trigger a variety of biological activities, such as NIR laser-induced release of therapeutic genes from the AuNPs. Chen et al. reported the remote control of gene expression in HeLa cells using AuNPs excited with NIR irradiation [21]. The GFP reporter gene was attached to the surface of AuNPs by linking of thiolated DNA through Au–S bonds. In addition, AuNPs were further labeled with Streptavidin-Alexa Fluor 647 to image AuNPs in living cells. When femtosecond NIR irradiation was applied to the AuNP–DNA conjugates, the shape of AuNPs was changed, and the gene was expressed. The transformation of shape might be induced by a release of DNA from AuNPs, as similar phenomenon was described by the Niidome group [122,123]. Wijaya et al. also showed the use of gold nanorods to selectively release multiple DNA oligonucleotides [124]. Therefore, a controlled release system for gene delivery based on AuNPs offers great potency in gene therapy. Recently, Lu et al. reported NIR light-inducible NF- κ B down regulation through folate receptor-targeted hollow gold nanospheres carrying siRNA recognizing NF- κ B p65 subunit (Fig. 6) [125]. The targeted AuNPs exhibited significantly high tumor uptake in a mouse model of cervical cancer as imaging by micro-PET/computed tomography. Controlled cytoplasmic delivery of siRNA was

possible by the AuNP photothermal effect after NIR light irradiation. Efficient down regulation of NF- κ B p65 was achieved only in tumors irradiated with NIR light. Heat or cavitation-induced endosomal membrane disruption and siRNA diffusion into the cytosol can be monitored by Cy3 fluorescence.

Braun et al. reported a novel lipid-based gold NP that provides temporally and spatially controlled cellular delivery of siRNA for gene silencing, through a direct endosomal release mechanism activated by pulsed laser treatment (Fig. 7) [126]. The NIR laser was able to release Cy3-labeled siRNA, which was conjugated to the surface of gold NPs. Fluorophores in close proximity to gold NP can be efficiently quenched by nanoparticle-based surface energy transfer (NSET) mechanism [127]. The Cy3 dye is partially quenched when near the gold, laser-mediated release and delivery of siRNA can be monitored after dequenching of Cy3. In addition, a Tat peptide-lipid coating allows the use of low NP concentrations and laser-dependent endosomal RNA release. The photothermal transfection technique may be used in the rational design of targeted gene delivery systems for successful gene therapy and imaging.

Advancements in nanotechnology continue to introduce inorganic and organic nanomaterials for molecular imaging and gene delivery. Here AuNPs, silica NPs, and CNTs have been introduced as future gene delivery materials. The unique intrinsic properties of these nanomaterials are utilized to image gene delivery and future works can combine such materials for synergistic effects.

Conclusions and future perspectives

NPs are particularly useful for molecular imaging of gene delivery due to their unique physicochemical properties. With advances in nanotechnology, functional NPs deserve significant research efforts as they can be integrated for quantitative, noninvasive imaging and targeted gene therapy within one entity. The ultimate goal is that functional NPs allow for efficient, specific *in vivo* delivery of genes without systemic toxicity, and the dose delivered as well as the therapeutic efficacy can be accurately measured noninvasively and spatiotemporally.

However, looking into the future, it is imperative to have a better understanding of the basic principles involved in designing and applying NPs for diagnosis, treatment, or the combination of imaging and therapy in different clinical situations. Many factors need to be optimized to design advanced NPs for molecular imaging of gene delivery, among which are biocompatibility, pharmacokinetics, *in vivo* targeting efficacy, and cost-effectiveness. Foremost, minimizing the potential toxicity of NPs is critically important. In order to validate the potential of therapeutic gene delivery with NPs, *in vitro* cytotoxicity and nanoparticle effects have been examined to a large extent. Generally, the toxicity has a strong dependency on the physicochemical properties of NPs, such as size, surface charge, and surface coating materials, in addition to the dosage of NPs and the duration of exposure. However, with regards to potential cytotoxic effects of NPs, many apparently contradicting results have been obtained due to the different cell types/animal models tested, the variety in type of materials utilized, and the vari-

ability in concentrations of the NPs used. The modifications to reduce cytotoxicity may also compromise the functionality of the NPs. For example, modification of the NPs with PEG may improve aqueous dispersion, prevent aggregation, but it will also significantly reduce cellular uptake [93].

Second, the specificity of NPs for selected cells and tissues is critical in both diagnostic imaging and gene therapy. Antibodies, peptides, aptamers and small organic molecules, have been used in NP systems as targeting agents for specific biomarkers on target cells. The development of novel noninvasive imaging in combination with biomarker targeted NPs has the potential for early detection of diseases and determination of the responses to gene therapy. However, linking biomarkers with disease behavior and personalized treatment remain a significant challenge due to the heterogeneous nature of most diseases. While targeting agents enable NP binding affinity/specificity, the type of ligand and method of NP attachment can significantly affect its targeting ability [128]. For instance, a functional group modification of the NPs during conjugation may change their chemical properties by embedding part of the ligand and binding site in NPs therefore decreasing the targeting capabilities. Knowledge of how to tune and exactly control the ratio between imaging labels and targeting units on functional nanomaterials can lead to improved system design and predictions of structure, function, and activity of the generated NPs [129,130]. In addition to ligand bioactivity, these molecules also may affect NP stability and immunogenicity. For example, antibodies/proteins derived from non-human animal sources can create the possibility of unwanted immune responses.

Third, the generation of multifunctional NPs for multimodality imaging will extend the limits of current molecular diagnostics and permit accurate diagnosis as well as the development of targeted gene delivery. A single probe helps to ensure the same pharmacokinetics and colocalization of signal for each modality and it also can avoid putting the additional stress on the body's blood clearance mechanisms that can accompany administration of multiple doses of probes [18,131]. However, future work will have to address the issue of sensitivity and determine the detection thresholds for the different modalities. For example, PET is a highly sensitive imaging modality that requires the introduction of only a trace amount of probes, whereas a relatively high amount of probes needed for MRI in current systems limits the unique sensitivity of the PET. Thus, the detection sensitivities for different imaging modalities should be further considered and optimized. Additionally, the biodistribution of therapeutics exhibits changes on time scales of seconds to minutes. To ensure that a subject is being imaged in the same physiologic state and to correlate changes over time in the different modalities' signals in response to an intervention, data must be acquired simultaneously or at least in very rapid succession. In designing an integrated scanner for simultaneous imaging, an obvious challenge relates in which the different modalities can interfere with each other, leading to major artifacts and/or image degradation.

Finally, gene therapy imaging to monitor therapeutic effects requires monitoring the changes of transduced/transfected target cells in terms of their location and number over time [132]. Unfortunately, till now, imag-

ing techniques are typically used to visualize the delivery of fluorescently labeled siRNAs or carriers at the targeted site. However, it is not clear how a therapeutic response can be effectively monitored, as labeled siRNA or nanoparticles will fluoresce inside or outside target cells without transfection and gene expression. Therefore, more sophisticated imaging techniques need to be developed to monitor therapeutic responses induced by efficiently delivered therapeutic genes. In this point of view, utilizing reporter gene-based imaging techniques in combination with nanoplatforms introduced in this article can be an alternative choice. The expression of a transferred gene *in vivo* can be visualized indirectly by using imaging reporter gene, especially optical reporter genes. For the evaluation and optimization of gene therapy based on reporter gene imaging, the development of a novel gene carrier with controllable gene expression system coupled to a imaging reporter gene that can be monitored noninvasively and simultaneously is critical [133,134]. Ideally, plasmid DNA should first be compacted into functional nanoparticles to form stable formula for targeted gene delivery and capable of providing protection against enzymatic, and thereby, the development of gene carrier may provide improved interpretation of therapeutic responses and thus allow optimization of novel gene therapeutic strategies.

Over a decade ago Dr. Verna said, "There are only three problems in gene therapy, delivery, delivery and delivery" [135]. Now in the new millennium, as gene therapy moves beyond viral vectors and into the nanotechnology field, there are at least three additional problems – toxicity, good manufacturing practice (GMP), and regulatory affairs. To date, the United States Food and Drug Administration (FDA) has not approved human gene therapy for clinical use. However, as nanoparticle gene therapy research continues to develop with standardized nanomaterial characterization and clinical trial efficacy, FDA is actively involved in overseeing the fast growth of such research. Being the sole regulatory affair administration for human drug approval, the FDA has partnered with numerous agencies to keep up with gene delivery and nanotechnology research. One such partnership is to include the National Institutes of Health (NIH), to launch the Genetic Modification Clinical Research Information System, providing transparent information of all ongoing clinical research for gene delivery. Other Public–Private Partnerships have also been set up to promote the understanding of the biological interactions of nanoscale materials. One critical partnership include FDA–National Cancer Institute (NCI)–National Institute of Standards and Technology (NIST) to establish the Nanotechnology Characterization Laboratory to speed the development of effective medical products with nanoengineered products. However, the regulatory hurdles to gene therapy, such as safety and efficacy, remain roadblocks in the clinical application of gene therapy. The future of functional NP design for molecular imaging and gene delivery mainly depends on multidisciplinary cooperation between molecular biologists, chemists, physicists, materials scientists, and imaging specialists. With continuous efforts by multidisciplinary approaches, the use of such nanoplatforms will shed new light on molecular diagnostics, gene therapy, and personalized medicine.

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chemotherapeutics, gene therapeutics, and imaging tags.



ultrasensitive diagnostics towards her PhD thesis.

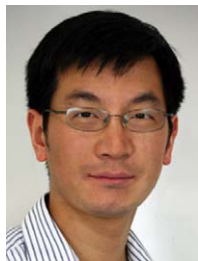


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