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Theranostic nanoparticles for RNA-based cancer treatment

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CONSPECTUS

Certain genetic mutations lead to the development of cancer through unchecked cell growth and division. Cancer is typically treated through surgical resection, radiotherapy, and small-molecule chemotherapy. A relatively recent approach to cancer therapy involves the use of a natural process wherein small RNA molecules regulate gene expression in a pathway known as RNA interference (RNAi). RNA oligos pair with a network of proteins to form an RNA-induced silencing complex which inhibits the translation of messenger RNA into proteins, thereby controlling the expression of gene products. Synthetically produced RNA oligos may be designed to target and silence specific oncogenes to provide cancer therapy.

The primary challenges facing the use of the RNAi pathway for cancer therapy are the safe and efficacious delivery of RNA payloads and their release at pertinent sites within disease-causing cells. Nucleases are abundant in the bloodstream and intracellular environment, and therapeutic RNA sequences often require a suitable carrier to provide protection from degradation prior to reaching their site of action in the body. The use of metal-core nanoparticles (NPs) serving as targeted delivery vehicles able to shield and direct RNA payloads to their intended destinations have recently gained favor. Biological barriers present in the body establish a size prerequisite for drug delivery vehicles; to overcome recognition by the body's immune system and to gain access to intracellular environments, drug carriers must be small (€ 100 nm). Iron-oxide- and gold-core NPs can be synthesized with a high degree of control to create uniform ultra-small drug delivery vehicles capable of bypassing key biological barriers. While progress is being made in size control of liposomal and polymer NPs, such advances still lag in comparison to the exquisite tunability and time-stability of size engineering achievable with metal-core NPs at bulk scales. Further, unlike lipid- and viral-based transfection agents, the biodistribution of metal-core NPs can be traced using noninvasive imaging techniques that capitalize on the interaction of electromagnetic radiation and the inorganic atoms at the core of the NPs. Finally, metal-core NPs have been shown to match the transfection efficiency of conventional RNA-delivery vehicles while also providing less immunogenicity and minimal side effects through the addition of tumor-targeting ligands on their surface.

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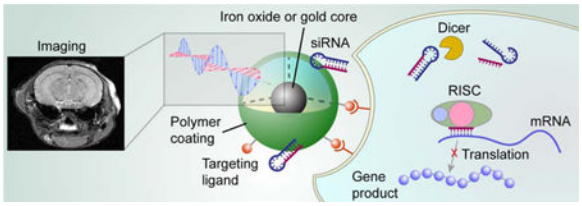
This Account reviews recent advances in the use of iron oxide and gold NPs for RNAi therapy. An overview of the different types of RNA-based therapies is provided along with a discussion of the advantages and current limitations of the technique. We highlight design considerations for the use of iron oxide and gold NP carriers in RNAi, including a discussion of the importance of size and its role in traversing biological barriers, NP surface modifications required for targeted delivery and RNA payload release, as well as auxiliary properties supporting imaging functionality for treatment monitoring. Applications of NPs for combination therapies including the pairing of RNAi with chemotherapy, photothermal therapy, immunotherapy, and radiotherapy are explored through examples. Finally, future perspectives are provided with a focus on the current limitations and the potential for clinical translation of iron oxide and gold NPs in RNAi therapy.

Graphical Abstract



1. INTRODUCTION

Using RNA to treat cancer and other diseases is an exciting concept with both inspiring potential and obstacles yet to be overcome. Many diseases stem from genetic mutations. Genetic defects manifest disease through the failure of cellular systems to produce properly functioning proteins. Discovery and elucidation of the naturally occurring RNA interference (RNAi) pathway provide a potential method of suppressing the adverse effects of genetic mutations.[1] RNAi uses RNA oligos to inhibit the expression of gene products by binding to and cleaving messenger RNA (mRNA) prior to its translation into protein. Degradation of mRNA is accomplished by the RNA-induced silencing complex (RISC), an assembly of proteins including a single-stranded RNA (ssRNA) oligo.[2] The ssRNA component of RISC binds the complex to a complementary strand of mRNA; the RISC then cleaves the mRNA inhibiting expression of the protein for which the mRNA encodes.

The endogenous RNAi pathway typically uses microRNA (miRNA), which are non-coding RNA molecules transcribed from DNA.[3] After transcription, miRNAs are processed by enzymes into shorter ssRNA strands that take part in RISC formation. Additionally, small interfering RNAs (siRNAs) are synthetically produced short double-stranded RNA (dsRNA) molecules that may directly form RISCs in the cytosol without additional enzyme processing. Furthermore, siRNAs bind to target mRNA sequences with high specificity, often silencing a single gene, whereas miRNAs are not exact complements of the mRNA for which they target, and as such are known to hybridize with many distinct mRNA targets representing tens to thousands of genes.

The advantages of using synthetically produced siRNA and miRNA mimics for gene therapy include their ease of synthesis, the ability to tailor nucleotide sequences to bind to a

complementary mRNA target with high specificity, and the high degree of gene silencing achieved through the RNAi pathway. Notwithstanding these exciting prospects, RNA-based therapies face significant impediments that include nuclease activity which degrades unprotected RNA oligos, the inability of siRNAs and miRNA mimics to target specific cells to mitigate deleterious off-target effects, and the inability of negatively charged RNA to cross biological barriers including cell membranes without aid of a transport system.

Non-viral, inorganic nanoparticles (NPs) are emerging as potential miRNA and siRNA delivery vehicles because they provide additional advantages compared to traditional lipid-based and viral vectors, such as tunable size, storage stability, targeting functionality to direct payloads toward the desired site of action and to facilitate crossing biological barriers, excellent pharmacokinetic and toxicity profiles, and the potential to provide concurrent diagnostic capabilities via imaging. Furthermore, iron oxide and gold NPs are well-positioned with respect to regulatory approval from the Food and Drug Administration (FDA) based on the status of extant therapies currently undergoing clinical evaluation or already in clinical use such as the iron oxide NP ferumoxytol in anemia treatment and magnetic resonance imaging (MRI) contrast enhancement.[4]

This Account focuses on the use of iron oxide and gold NPs in RNAi therapy. First, we provide a brief overview of the RNAi process and the types of RNA molecules under investigation for RNAi. Next, we present design considerations salient to the development of metal-core NPs for therapeutic RNA transfection, including the relevance of NP size and polymer coatings to biodistribution and pharmacokinetics, the need for ligating targeting agents to the surface of NPs to increase transfection efficiency of RNA at target sites, and factors influencing RNA payload release from NP carriers. A key benefit of gold- and iron-oxide-core NPs is that they may be engineered to simultaneously exhibit therapeutic and diagnostic properties (theranostics); we showcase the ability of iron oxide NPs to serve as contrast agents in MRI and the capacity of gold NPs to function in X-ray computed tomography (CT) and photoacoustic imaging. Then, we direct our attention to experimental demonstrations of the efficacy of metal-core NPs to deliver RNA oligos and their value in treating disease via the RNAi pathway. We provide examples of RNA therapy combined with iron-oxide- and gold-core NP enabled treatment modalities such as chemotherapy, immunotherapy, and radiotherapy. Finally, we consider the limitations of iron oxide and gold NPs in light of other NP platforms for RNA-based therapies and follow with a discussion of techniques for evaluating the safety of NP platforms and the potential for clinical translation of iron oxide and gold NPs.

2. THERANOSTIC IRON-OXIDE- AND GOLD-CORE NPs FOR RNA-BASED THERAPIES

2.1. The RNAi Pathway

RNAi is a naturally occurring biological pathway encompassing many forms of regulatory RNA that have been studied for use as therapeutic agents. RNAi employs a short (approximately 20 base pairs) dsRNA molecule that binds to a protein complex to form a RISC; the RISC unwinds the dsRNA molecule, retains the antisense strand, and degrades the

portion of the RNA able to code for protein (the sense strand). The antisense strand then binds to a complementary mRNA molecule where the RISC facilitates enzymatic cleavage of the mRNA, suppressing translation into protein. The dsRNA molecules involved in RNAi are called miRNA if they originate from the host cell's DNA and are referred to as siRNA if they originate from an exogenous source such as synthetic siRNA or viral RNA.[5] A schematic representation of the RNAi pathway and the different forms of RNA involved is provided in Figure 1.

In therapeutic applications, siRNA is often synthesized as short hairpin RNA (shRNA); shRNAs are siRNA precursors consisting of double stranded sense and antisense RNA with a small loop of ssRNA.[5] miRNA is first transcribed from DNA into primary miRNA (pri-miRNA). Then, both pri-miRNA and shRNA are enzymatically processed by Drosha in the nucleus resulting in precursor miRNA (pre-miRNA). After pre-miRNA exits the nucleus and enters the cytoplasm, the RNAse enzyme Dicer cleaves pre-miRNA into segments of dsRNA roughly 20 base pairs in length that can join in RISC formation and participate in the RNAi pathway. Conversely, siRNA may form into a RISC directly, or, if it is too long, be shortened by Dicer prior to combining into a RISC.[6] miRNAs have low specificity and may silence many genes, and they must be delivered to the nucleus for processing by Drosha; alternatively, siRNAs bind to mRNA in a sequence-specific fashion, minimizing off-target silencing. Furthermore, siRNAs may be synthesized so that they do not require exposure to Drosha or Dicer mechanisms before becoming active in a RISC, and may thereby circumvent unwanted immune responses from endogenous RNA sensors. miRNA mimics, siRNAs, and shRNAs have all been investigated for use as therapeutics for cancer by regulating gene expression. However, these nucleic acids must be packaged in a delivery vehicle to protect them from degradation by nucleases and to enable their entry into the cell interior or nucleus.

2.2. NP Design Considerations for RNAi Therapy

Important design parameters for NP-based therapeutics are hydrodynamic size, particle size distribution (PSD), surface charge, drug-loading capacity, and surface chemistry that accommodates ligation of targeting agents and other functional moieties, which can influence immunogenicity, toxicity, pharmacokinetics, and biodistribution. The hydrodynamic size of NPs is critical because of the relationship between hydrodynamic size and biodistribution. If the hydrodynamic size is too large (Σ100 nm), the NPs will quickly be recognized, removed from blood circulation, and sequestered to the liver and spleen by the reticuloendothelial system (RES); if they are too small (€10 nm) the NPs will not be retained in circulation for a significant period of time due to renal clearance.[7,8] Distinct from an NP's hydrodynamic size is the PSD, which represents the prevalence of each possible hydrodynamic size within a sample of NPs. The overall hydrodynamic size of a sample of NPs is often quoted as the mean of the PSD. Thus, it is important to consider the PSD of drug delivery vehicles since the average hydrodynamic size of a sample of NPs may be less than 100 nm while the NP ensemble may contain a significant number of NPs with a hydrodynamic size greater than 100 nm. If these larger particles are introduced into a biological system, they may trigger an unwanted immune response. Furthermore, NPs with different hydrodynamic sizes will have different physiochemical properties (e.g., magnetic

properties, biodistribution, drug loading capacity, etc.). A highly monodisperse sample of NPs will consist of individual particles with very similar properties, but a polydisperse or broadly distributed sample will have a wide variance in properties between individual NPs which could lead to variable functionality.

The hydrodynamic size of an iron-oxide- or gold-core NP includes its inorganic interior along with any surface coating and the solvent layer that establishes in aqueous environments. Metal-core NPs tend to aggregate in solution without surface coatings, presenting serious safety concerns for in vivo use; thus, metal-core NPs are generally coated with polymer to confer dispersion stability and increase blood circulation times. Polyethylene glycol (PEG) and chitosan are examples of polymer coatings that increase the circulation half-life of NPs and afford biocompatibility. Copolymer coatings may also be employed to benefit from the combined advantages provided by the individual polymers used. For example, a chitosan-PEG copolymer capitalizes on chitosan's functional groups that facilitate ligation chemistry for attachment of functional moieties like targeting agents, and on PEG's ability to provide steric hindrance for improved colloidal stability and avoidance of an innate immune response.[9] Cationic polymers such as polyethylenimine (PEI),[10] poly-L-lysine,[11] and poly-L-arginine[12] are often used as NP coatings for use in gene therapies because the negative charge of the nucleic acid phosphate backbone readily binds to positively charged polymers via electrostatic interactions. However, the positive charge of cationic polymers is not well tolerated in vivo due to activation of the RES and adverse reactions with negatively charged red blood cells which can lead to cell agglomeration, blocked blood flow, and death.[13] To benefit from charged-based complexation of RNA oligos with cationic polymers, additional polymers such as PEG are often used to form a corona surrounding the positively charged polymer, thereby shielding the NP surface. Alternatively, nucleic acids may be covalently bound to gold NPs through thiolation reactions. Oligonucleotides modified with thiol groups bind to gold with high affinity while retaining their gene silencing efficacy.[14] Iron oxide NPs can also be covalently bound to nucleic acids. Oleate-coated iron oxide NPs, and thiol groups were introduced to the NP surfaces by exchanging the oleate layer with dimercaptosuccinic acid.[15] Finally, synthetic peptide nucleic acids, which serve as mimics of RNA and DNA, were grafted to the thiol functional groups, thereby covalently linking oligonucleotides to the iron core.

NPs bearing RNA oligos enter cells through endocytosis and are subsequently directed through the cell interior inside membrane-bound vesicles. Typically, foreign materials endocytosed into the cell are either removed from the cell when the endosome carrying the material refuses with the plasma membrane, or the material is degraded upon transferring into a lysosome.[16] Thus, endosomal escape is of paramount importance in RNA-based therapies since siRNAs and miRNA mimics must be released from any compartments in order to join the RNAi machinery and carry out their therapeutic activities. Early investigations into RNA-based therapies depended upon spontaneous escape of a small percentage of delivered nucleic acids into the cytoplasm and nucleus. This reliance on chance, of course, is characterized by poor transfection efficiency, and recent efforts for RNA delivery attempt to actively disrupt vesicle barriers by taking advantage of the acidic environments of endosomes and lysosomes or the use of external stimuli. We developed pH-sensitive iron oxide NPs for siRNA delivery by blocking the primary amine groups of the

PEI coating with citraconic anhydride.[17] At neutral pH, the NPs exhibited a large negative surface charge; the surface charge became more positive in acidic environments similar to those found in endosomes and lysosomes. Our results indicate that the amine groups of PEI become unblocked at low pH and the change in surface charge on the NPs destabilizes the membranes of intracellular vesicles allowing siRNA payloads to escape into the cytoplasm. An example of the use of external stimuli for endosomal escape is the application of ultrasound-propelled gold NPs which circumvent endocytosis entirely by mechanically piercing through the cell membrane to gain direct access to the intracellular environment.[18] Recently, another external stimulus technique known as photoporation was to induce gold NPs carrying siRNA to diffuse into T lymphocytes.[19] In this technique, gold NPs located on the surface of T lymphocytes were irradiated with intense, short laser pulses which caused an increase in temperature in the vicinity of the NPs due to the conversion of light to thermal energy via a surface plasmon resonance between the laser light and the NPs. The increased temperature is thought to create small water vapor bubbles which expand, collapse, and create high-pressure shockwaves that form temporary pores in nearby cell membranes to facilitate internalization of NPs by diffusion.

The polymer coatings of inorganic-core NPs also aid in targeted delivery by providing functional groups on which to attach targeting ligands. NPs without targeting agents rely on the enhanced permeability and retention (EPR) effect. The EPR effect describes the passive influx of material from the bloodstream into the tumor microenvironment due to perforations in the asculature servicing the tumor. Further, materials that have diffused into the tumor environment may experience prolonged retention since the lymphatic drainage system of cancer tissue is often compromised. However, the EPR effect alone may not suffice for effective transfection in RNA therapy. Moreover, recent evidence suggests that the EPR effect may not account for much NP accumulation in tumor tissue since tumors are often not well vascularized, especially in humans.[20] Active targeting of drug delivery vehicles may be achieved by molecular and other means. For molecular targeting, a ligand that recognizes and binds to receptors overexpressed on target cells is attached to the NP surface. In our previous work, we used peptides as targeting agents including anti-CD20 single-chain variable fragment-streptavidin fusion protein to target lymphoma,[21] anti-human epidermal growth factor receptor 2 (HER2/neu) monoclonal antibodies to target HER2/neu positive breast cancer cells,[22] and chlorotoxin to target metalloproteinase-2 overexpressed by glioma cells.[23] Attachment of anti-HER2/neu peptides to iron oxide NPs functionalized with a fluorophore facilitates significant targeting of NPs to HER2/neu+ cells as demonstrated in vivo in a breast cancer xenograft mouse model (Figure 2a).[22] Similarly, we used anti-HER2/neu targeting agents to increase NP uptake in breast cancers cells in a transgenic mouse model (Figure 2b).[9]

2.3. Imaging Capabilities Facilitated by Iron-oxide- and Gold-core NPs

Imaging functionalities enabled by the metallic cores of iron-oxide- and gold-core NPs provide key benefits over other drug delivery vehicles. The ability to track NPs in the body allows them to serve as theranostic agents providing both a therapeutic function and diagnostic information about the localization of diseased tissue and delivered therapeutics. Iron oxide NPs provide contrast enhancement in MRI, a noninvasive imaging technique in

widespread clinical use that provides high contrast and spatial resolution of soft tissue while avoiding the use of harmful ionizing radiation. We used MRI to track and compare the biodistribution and pharmacokinetics of iron oxide NPs between nonhuman primates and mice in multiple organ systems throughout the body including the brain (Figure 3a).[24] We have also used MRI to determine the extent of iron oxide NPs delivered to brain tumors via convection enhanced delivery.[25] We suggest these efforts may serve as proof-of-concept results indicating that iron oxide NPs may be used to track the delivery of RNA payloads. Recently, Lu et al. used iron oxide NPs to monitor the targeted delivery of siRNA to neuronal stem cells to aid in the differentiation of the stem cells into neurons to treat damage caused by strokes.[26]

Gold NPs may be used as contrast agents in photoacoustic and X-ray CT imaging. Gold NPs serve as excellent contrast agents in CT imaging due to gold's high X-ray attenuation coefficient. Wei et al. used CT imaging to track the delivery of siRNA to tumors in a xenograft mouse model.[27] Gold NPs enable photoacoustic imaging due to their ability to convert absorbed light into heat. In photoacoustic imaging, gold NPs are irradiated with non-ionizing pulsed laser light which causes the NPs to increase in temperature. This temperature increase causes thermal expansion of material in the immediate vicinity which leads to emission of ultrasonic pressure waves. These ultrasound emissions are detected by a transducer to form an image. Both CT and photoacoustic imaging were used to monitor gold NPs delivering miRNA to tumor cells in mice (Figure 3b and 3c).[28]

3. NP-based RNAi THERAPY

RNA-based therapeutics are gaining popularity due to their potential to target and silence specific disease-causing genes. The first RNA-based therapeutic to receive FDA approval occurred in August 2018; the approved drug uses a lipid NP delivery system and is known as patisiran.[29] Despite this success, patisiran is not a targeted drug as it passively accumulates in the liver to inhibit the production of the protein transthyretin. Iron-oxide- and gold-core NPs offer an alternate solution for delivery of siRNA and miRNA mimics and provide added benefits of enabling diagnostic imaging, conjugation with targeting moieties, and the ability to tailor surface coatings to provide minimal toxicity and immunogenicity.

3.1. Gene Silencing with RNAi

Suppression of oncogene products using the RNAi pathway is a key goal of therapeutic uses of siRNA and miRNA mimics. As a proof-of-principle experiment verifying that metal-core NPs can be used to deliver siRNA to silence gene products in vivo, we developed iron-oxide-core NPs loaded with siRNAs designed to suppress the expression of firefly luciferase (Luc), an enzyme that causes bioluminescence by oxidizing luciferin.[30] To confer tumor targeting, we conjugated a monoclonal antibody against human glypican-3 receptors which are highly expressed in hepatocellular carcinoma (HCC). We tested our NPs in a model of HCC that co-expresses glypican-3 and Luc. Mice bearing HCC tumors were untreated, treated with control siRNA (siScramble), or treated with siRNA designed to knockdown Luc (siLuc). Luminescence imaging of the tumors indicated a significant reduction in Luc

activity for mice receiving NP-delivered siLuc treatments, confirming successful siRNA delivery and gene silencing (Figure 4).

With respect to silencing oncogenes, both iron oxide and gold NPs have been used in animal models to deliver siRNA to HCC,[31–33] colon cancer,[34] lung cancer,[35] melanoma,[36] and glioblastoma.[37–39] Likewise, both NP varieties have been used to deliver miRNA mimics to knockdown the expression of oncogene products with promising results.[40–43]

3.2. Combinatorial RNAi Therapy

3.2.1. Enhancing Immune Responses Against Cancer Using RNAs—The introduction of foreign RNA oligos into the body often triggers an immune response. While delivered therapeutics are generally engineered to minimize the initiation of an immune response, recent efforts have attempted to leverage this phenomenon into coaxing the immune system to attack cancer. Exogenous RNA is typically recognized by the immune system when the RNA is endocytosed; within the endosome, toll-like receptors sense foreign RNA and signal the type I interferon (IFN) response. RNA oligos that successfully escape the endosome may still be recognized by the cytoplasmic RNA sensors of melanoma differentiation-associated protein 5 and retinoic acid-inducible gene 1 protein; these proteins also initiate the IFN response upon sensing exogenous RNA.[44] The type I IFN pathway is an anti-viral response that inhibits the translation of mRNA from exogenous RNA sources. However, the type I IFN pathway also stimulates the effector function of lymphocytes including T cells, B cells, and natural killer cells. It is the activation of these lymphocytes that RNA-based immunomodulation attempts to exploit for cancer treatment.

Recently, Cobaleda-Siles et al. used iron oxide NPs to deliver the synthetic dsRNA analog polyinosinic-polycytidylic acid to lymph nodes to activate an antitumor immune response via toll-like receptors.[45] Their results showed the NP platform increased cytokine production in immune cells. Zhang et al. loaded synthetic oligonucleotide cytosine-phosphate-guanine (CpG) onto iron oxide NPs and injected them intratumorally in a xenograft breast cancer model.[46] Tumors receiving CpG showed increased lymphocyte infiltration; further, mice receiving CpG treatment showed decreased tumor growth and inhibited metastasis (Figure 5).

3.2.2. RNAi and Chemotherapy—RNA-based therapies may enhance the efficacy of extant chemotherapeutics by silencing genes known to be sources of drug resistance. Doxorubicin, vincristine, and cisplatin all showed increase in cell killing when combined with siRNA-mediated knockdown of the MXD3 gene in a neuroblastoma cell line.[47] PEG-PEI coated iron oxide NPs were developed for the simultaneous delivery of miRNA and gemcitabine to pancreatic cancer; their results showed inhibited metastasis and improved apoptosis in cancer cells receiving co-delivery of miRNA and gemcitabine as opposed to cells receiving a single therapeutic.[41] By pairing RNA-based therapies with chemotherapeutics, it may be possible to increase both tumor cell killing by sensitizing cancer cells to chemotherapeutics and minimize off-target side effects by decreasing the overall required chemotherapeutic dose needed for effective treatment.

3.2.3. RNAi and Radiotherapy—Along with surgical resection and chemotherapy, the application of ionizing radiation is an essential part of the treatment arsenal in the fight against oncological disease. Some DNA repair enzymes have been implicated in radiation resistance. We developed an iron oxide NP delivery system to silence apurinic endonuclease 1 (Ape1), an enzyme involved in the DNA base excision repair pathway, using siRNA.[48] These NPs were shown to achieve knockdown of Ape1 expression in over 75% of medulloblastoma and ependymoma cells, reducing Ape1 activity by 80%; further, this decrease in Ape1 activity resulted in increased DNA damage after radiotherapy. We followed these in vitro experiments with an in vivo study with similarly impressive results.[49] Systemically delivered iron oxide NPs carrying siRNA to knockdown Ape1 expression were found to extend the overall survival 2-fold over traditional radiation alone in a genetic mouse model of glioblastoma (Figure 6). Such combinations of standard cancer therapeutics with siRNA and miRNA mimics are very promising for the future of cancer treatment.

4. LIMITATIONS OF IRON-OXIDE- AND GOLD-CORE NPs

NP formulations under investigation as RNA delivery vehicles include viral vectors, liposomes, polymeric particles, and other inorganic NPs. With all of these choices of drug delivery platform, the main challenge with siRNA therapy remains: transport of sufficient payload to diseased tissue while minimizing off-target toxicity. While the NP systems mentioned above protect RNA therapeutics from extracellular environments and promote traversing of biological barriers, each formulation has drawbacks. Viral vectors have been used to deliver siRNA to cancer cells and are efficient with respect to payload transfection. However, viral vectors are limited physiologically by their propensity to induce adverse immune responses and are limited economically due to the inability to reliably produce viral vectors at scale.[50] Among non-viral siRNA delivery vehicles, the most widely investigated category of carriers is cationic liposomes. The positive charge of the liposomes promotes complexation with negatively charged nucleic acids forming lipoplexes. The previously discussed FDA-approved siRNA lipoplex, patisiran, is a systemically delivered siRNA carrier that passively accumulates in its target organ, the liver. While lipoplexes seem well-suited for targeting the liver, spleen, and kidneys in this fashion, we believe that directing siRNA to genes outside the RES may be more effectively delivered with a metal-core NP with associated targeting ligands given the deleterious immune responses triggered by chronic administration of liposomes.[20] Nucleic acids encapsulated in cationic polymers have also entered Phase I clinical studies, but these polyplexes have been plagued by toxicity issues with a lack of concomitant efficacy.[51] Inorganic NPs other than iron oxide and gold include those based on silica,[52] calcium phosphate,[53] CdSe and ZnSe quantum dots,[54] and carbon nanotubes,[55] among others. As with iron oxide- and gold-core NPs, the limitations of inorganic NPs are defined by the potential toxicity of their constituents and the ability of their polymer coatings to aid the NPs in avoiding recognition by the host's immune system, remaining in circulation for a sufficient period of time, and traversing biological barriers. Iron oxide cores are readily degraded and incorporated into the host's natural iron stores,[7] and gold is biologically inert. Thus, the primary factors restricting clinical translation of these metal-core NPs are the pharmacokinetic, biodistribution, and safety profiles conferred onto the NPs by their polymer coatings and functional ligands.

With regard to the clinical translation of iron oxide and gold NP systems, a primary hurdle to overcome is that they must prove to be more efficacious than existing therapies. A typical route of comparing efficacy between NP platforms and conventional drugs consists of cell-based in vitro studies followed by in vivo investigations in relevant animal models. Currently, lipoplexes and polyplexes have greater standing in terms of clinical relevance with respect to siRNA delivery. As such, metal-core NPs should be compared to liposomal and polymer delivery vehicles when performing efficacy studies.

In conjunction with efficacy studies, an equally important hurdle to clinical translation is that posed by safety considerations. Specifically, any new medical treatment must be shown to be sufficiently biocompatible: the NP platform must display the ability to perform its desired function while not engendering a detrimental response from the host. An evaluation of an NPs platform's safety profile should include basic physiochemical characterization followed by in vitro and in vivo analyses of both acute and chronic toxicity. Safety studies begin with dose escalation studies while monitoring for adverse events such as signs of abnormal cell function in vitro or health issues in vivo (e.g., weight loss, fever, diarrhea, etc.). Pharmacokinetic studies in animals consist of drawing blood at multiple time points after NP administration to determine the blood plasma concentration of the introduced therapeutic over time. These blood draws can also be evaluated for signs of liver or kidney toxicity by monitoring the blood for increased liver enzymes, creatinine, and blood urea nitrogen. Likewise, dose-dependent analyses of serum chemistry should be monitored for deviations in normal blood cell counts (i.e., monitor levels of hematocrit, hemoglobin, white blood cells, neutrophils, and platelets). These metrics, along with other biochemical components of physiological relevance, can be used to determine preclinical safety of an NP platform.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Iron-oxide- and gold-core drug delivery vehicles can be synthesized to yield consistently homogeneous nanoscale particles suitable for bypassing biological barriers and effectively delivering RNA oligonucleotides for participation in the RNAi pathway. Polymer coatings can be tailored to prolong blood circulation, avoid recognition by the innate immune system, and provide functional groups facilitating ligation chemistry for the attachment of tumor-targeting peptides, optical imaging probes, and therapeutic agents. RNA oligos can be loaded onto metal-core NPs through electrostatic interactions between positively charged moieties composing the NP and negatively charged phosphate groups located in nucleic acids or through covalent bonding with thiol groups, among other techniques. NPs can be targeted to specific cells by taking advantage of surface receptors overexpressed on cells of interest. To facilitate the release of the RNA payload, NPs can be engineered to respond to intracellular environments such as the low pH of lysosomes or triggered by externally applied stimuli such as pulsed laser light. Beneficially, iron oxide and gold NPs facilitate in vivo tracking of NPs through MRI (iron oxide NPs), and photoacoustic and X-ray CT imaging (gold NPs). Both iron oxide and gold NPs have demonstrated efficacious silencing of oncogenes in small animal models of various cancers through the delivery of both siRNA and miRNA mimics. Further, when RNAi therapy is combined with auxiliary treatments enabled by these metal-core NPs such as chemotherapy delivery, immunotherapy, or

radiotherapy, a synergistic response is seen with even more pronounced tumor killing efficacy. Finally, iron-oxide- and gold-core NPs can be synthesized by methods that are scalable and produce highly stable drug delivery vehicles conducive to long-term storage and economical production.

The future of RNA-based therapies for cancer and other genetic diseases is bright. However, translational barriers persist for iron-oxide- and gold-core drug delivery platforms including the minimization of immunogenic response, effective targeting to deliver RNA-based therapeutics to organs other than the liver and to minimize harmful side effects, endosomal escape mechanisms to improve the efficiency of RNA delivery, and proof of mitigation of long-term toxicity under persistent therapeutic use. Nevertheless, iron oxide and gold NPs are well-positioned with respect to gaining FDA approval if efficacy and nontoxicity can be shown for RNA-based therapies. Clinical trials on the delivery of spherical nucleic acids loaded onto gold NPs to treat glioblastoma began in 2017.[56] Many iron oxide NP formulations have been approved by the FDA for use in iron replacement therapy and MRI; existing FDA approval may lower the barrier of entry for other iron oxide NP formulations with uses beyond anemia therapy and MRI contrast enhancement.

Further, the metal-core of iron oxide and gold NPs with small size and uniform size distribution serves as a stable foundation on which subsequent molecules can build upon. Different polymer coatings can be added based on the specific therapeutic application. Similarly, the same iron oxide or gold NP platform can be used to deliver different RNA oligos. Distinct siRNAs that target a unique aspect of the cancer to be treated such as an oncogene that promotes metastasis or a gene the encourages angiogenesis can be synthesized and loaded onto the same base NP delivery vehicle. A single drug platform capable of carrying many RNA oligos greatly reduces the cost and time burdens necessary for obtaining FDA approval and assists in the development of personalized medicine where siRNA fragments can be synthesized to target patient-specific genes. Furthermore, the ultra-small and uniform size of iron oxide NPs makes them especially suitable for treating metastatic cancers because they are better able to distribute throughout a tumor network even if it is poorly vascularized. Finally, RNA-based therapies are not specific for cancer, but may be leveraged to combat many genetic diseases.

Although exciting, at the current stage of development, multifunctional iron oxide and gold NPs are simply proofs-of-concept. Rather than continue to add on bells and whistles to base NP designs, more pressing issues require immediate attention if such NPs are ever to reach the clinic. Can an iron oxide or gold NP be designed that meets the requisite RNA delivery, toxicity, and efficacy standards, regardless of what other advantageous attributes it may possess? Even though viral and lipid NP transfection agents have well-known toxicity issues, they are the most employed delivery methods for RNA-based therapies in clinical trials. Serious efforts are needed to develop better polymer coatings to reduce toxicity and immune responses, and more effective targeting methods are needed to overcome the poor delivery efficiency currently observed for in vivo experiments of NP-mediated RNA delivery.

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Miqin Zhang is a Kyocera professor in the Department of Materials Science and Engineering and professor of Neurological Surgery at the University of Washington. She received her Ph.D. in 1999 from the University of California at Berkeley. Her research focuses on nanomaterials for cancer diagnosis and treatment, biodegradable scaffolds for tissue engineering, and biosensors for detection of chemical and biological agents.

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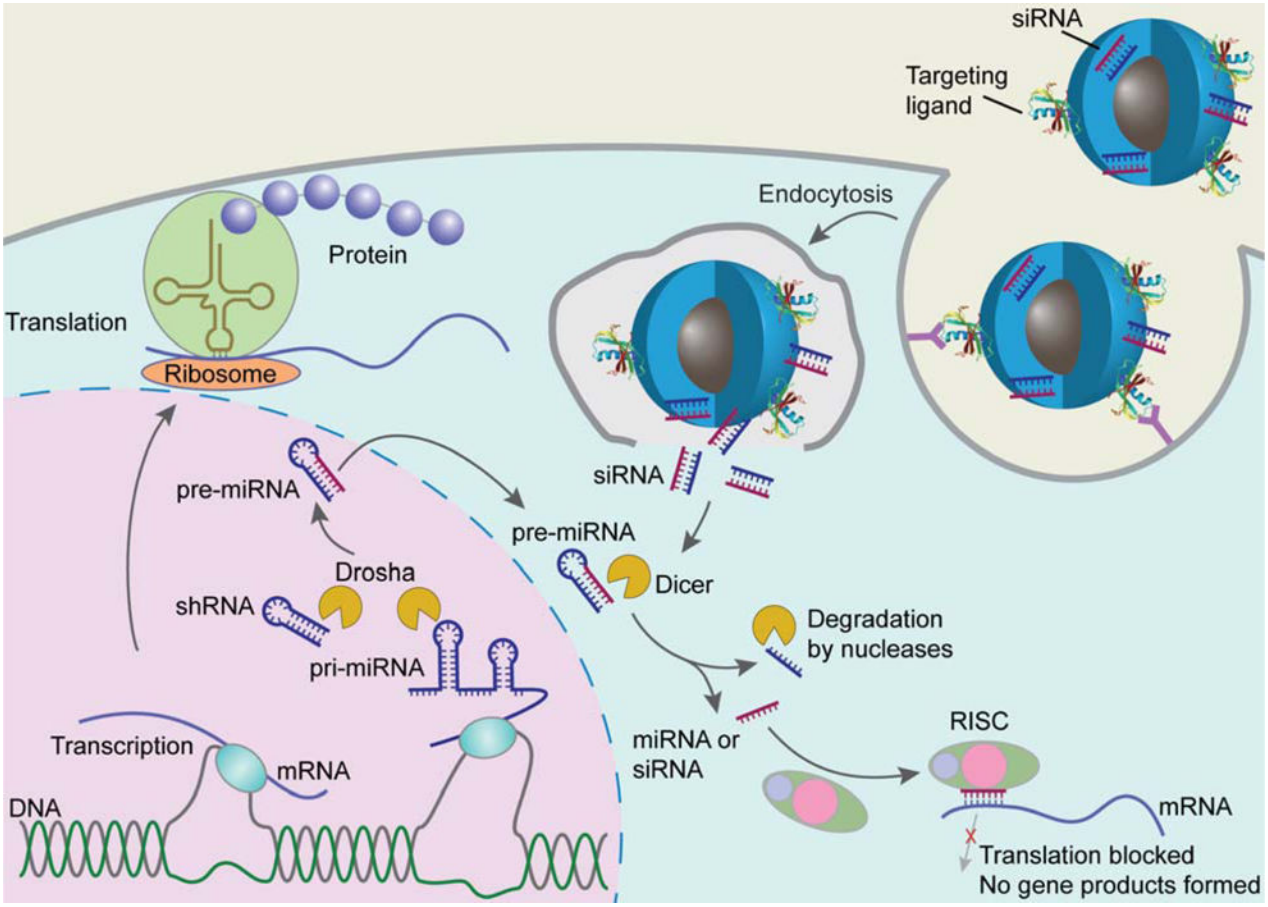

Figure 1.

Schematic representation of the types of RNA involved in transcription, translation, and gene regulation via the RNAi pathway.

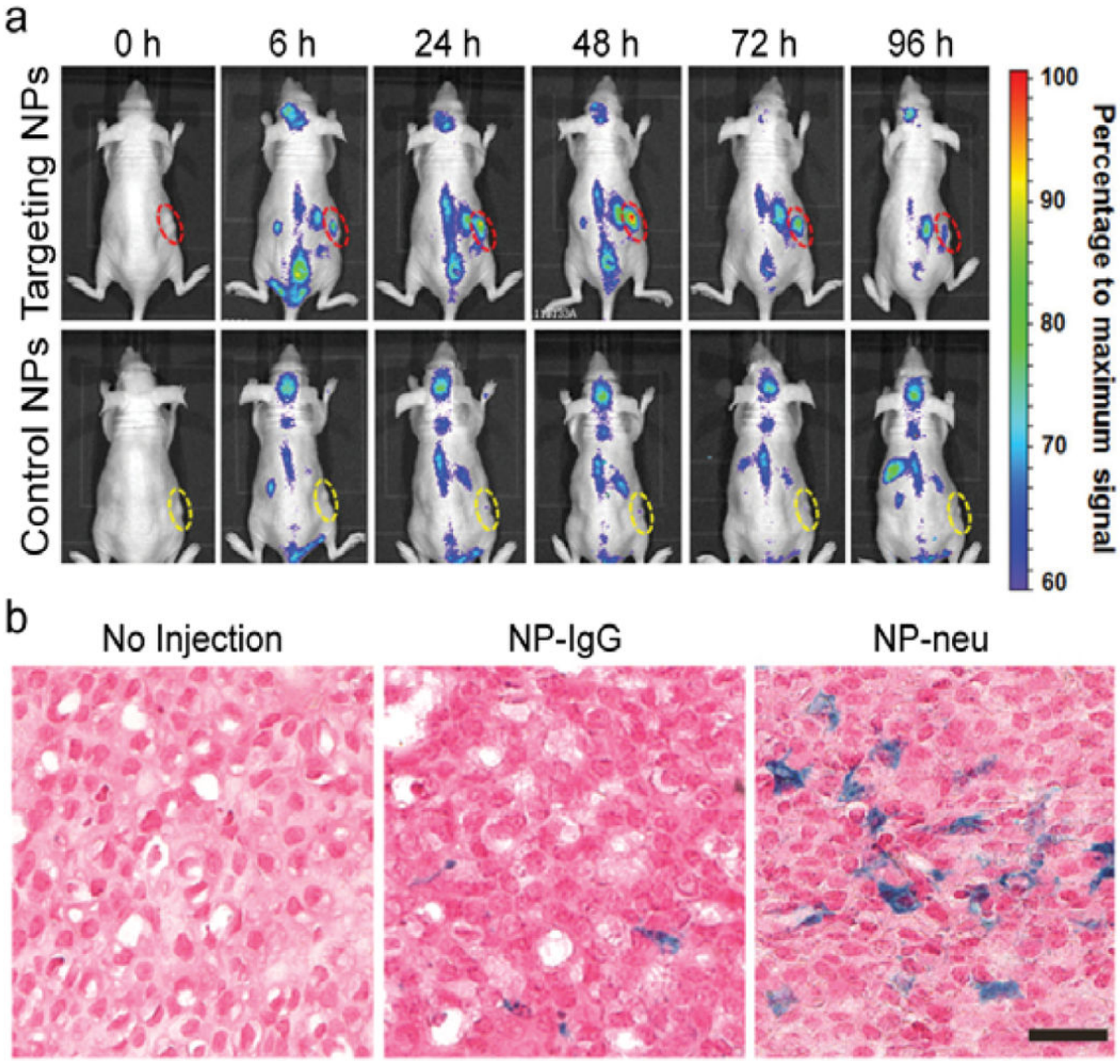

Figure 2.

Active targeting by peptide ligands increases cellular uptake and improves distribution of NPs within tumors. (a) Fluorescence imaging of mice receiving targeted NPs (fluorophore and tumor-targeting antibody) and non-targeted NPs (fluorophore only) demonstrates significant targeting of NPs to flank tumors indicated by red dashed ellipses (top row); yellow dashed ellipses (bottom row) indicate flank tumors with little fluorescence due to a lack of targeting agent. Time labels on top indicate the elapsed time after NP injection. (b) Histological images of tumor tissue harvested from iron oxide NP treated transgenic breast cancer mice 48 hours after injection. Iron was stained using Prussian blue, and cell nuclei were counterstained with nuclear fast red. Mice injected with NPs conjugated with anti-

HER2/neu ligands (NP-neu) showed more pronounced and broadly distributed NP uptake in tumor than mice injected with NPs conjugated to immunoglobulin G antibody (NP-IgG). Scale bar corresponds to 20 μm. Panel a was adapted with permission from ref [22]. Copyright 2015 The Royal Society of Chemistry. Panel b was adapted with permission from ref [9]. Copyright 2012 American Chemical Society.

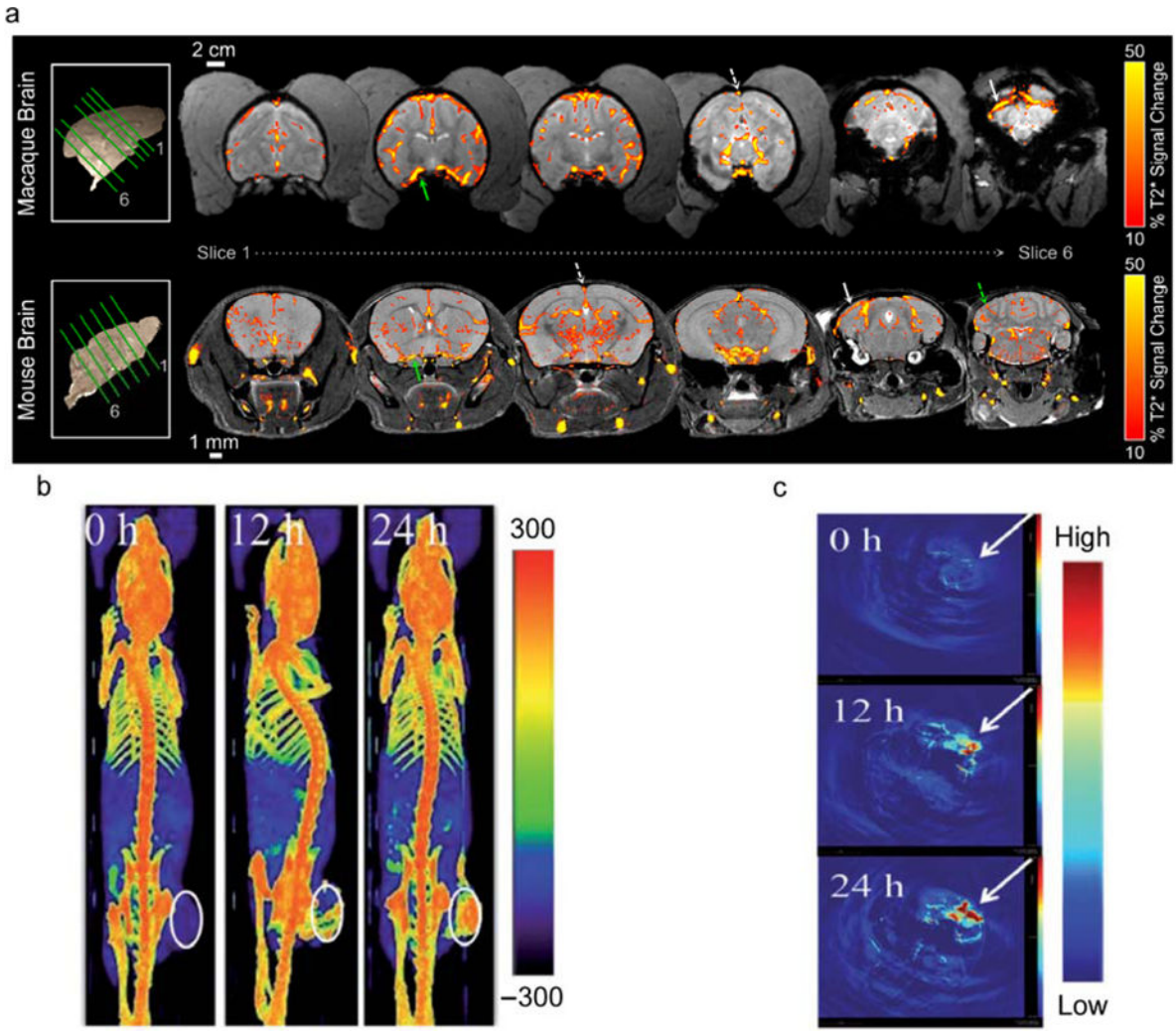

Figure 3.

Imaging iron-oxide- and gold-core NPs for tracking of biodistribution and drug delivery. (a) Contrast enhancement in MRI allows for the visualization of iron oxide NPs throughout the brain. A comparison of the distribution of iron oxide NPs in the brains of macaques (top row) and mice (bottom row) is accomplished by comparing the change in T_2*-weighted signal before and 30 minutes after NP injection. (b) CT and (c) photoacoustic imaging enabled by gold NPs in mice bearing flank tumors. The white circles in (b) and the white arrows in (c) indicate tumor locations. Panel a was reprinted with permission from ref [24]. Copyright 2017 American Chemical Society. Panels b and c were adapted with permission from ref [28]. Copyright 2017 WILEY-CVH Verlag GmbH & Co.

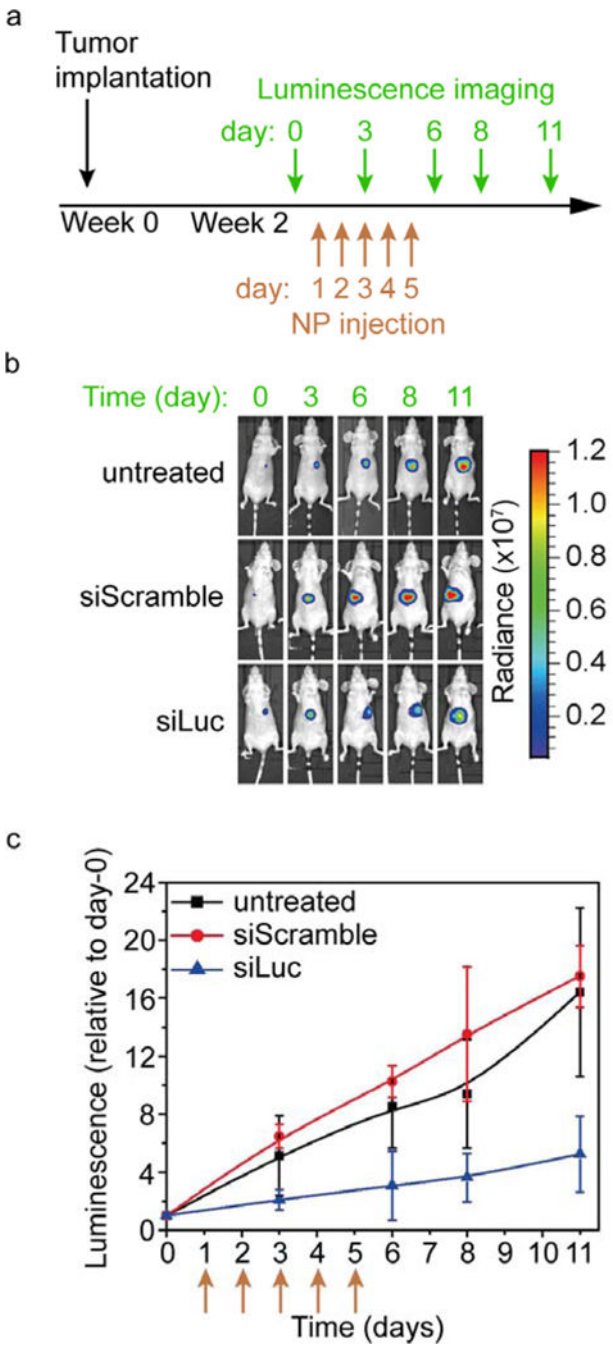

Figure 4.

In vivo gene silencing mediated by iron oxide NP delivery of siRNA. (a) Timeline of tumor implantation and treatment. Two weeks after injection with HCC cells, mice received five daily injections of NPs. Luminescence imaging was initiated one day prior to the first NP injection. (B) Representative luminescence images of mice bearing HCC tumors. Untreated mice and mice treated with scramble siRNA served as controls. (c) Quantitative luminescence of mice from untreated and treated groups ($n = 4$). Luminescence was normalized to day 0 and the line graph presents the mean ± standard deviation. Arrows

indicate NP injection time points. Adapted with permission from ref [30]. Copyright 2015 WILEY-CVH Verlag GmbH & Co.

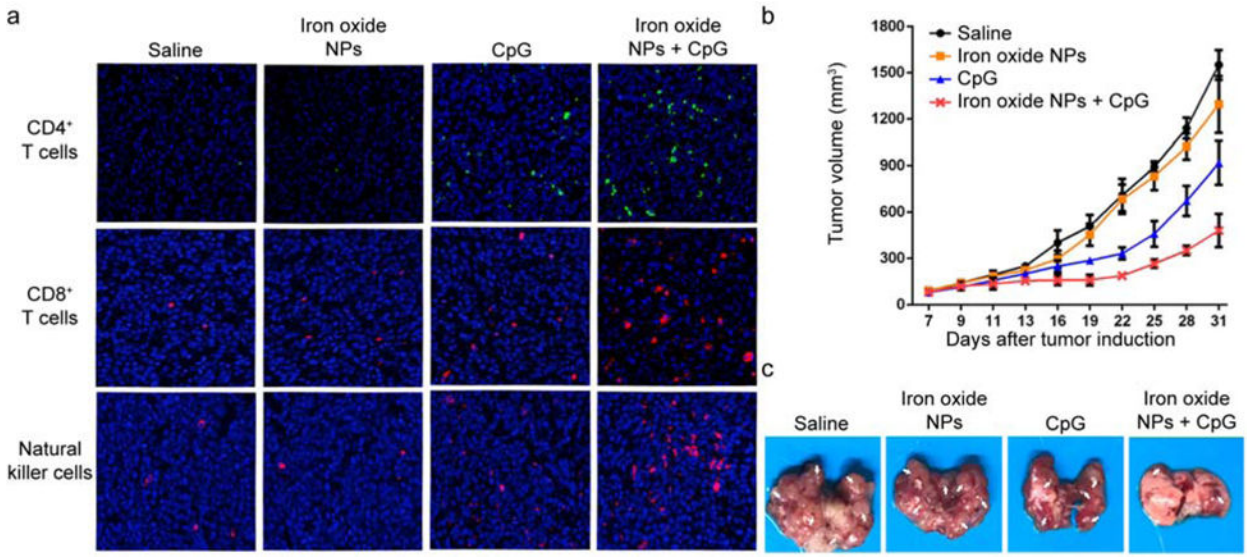

Figure 5.

RNA-based antitumor immunomodulation. (a) Breast tumor tissue harvested from mice treated with saline, iron oxide NPs, CpG, and iron oxide NP delivery of CpG. Representative immunofluorescence images showing infiltrating immune cells in tumor tissues of each group. (b) Tumor growth curves for each treatment group. (c) Brightfield imaging of the lungs after treatment; white arrows indicate lung metastases. Adapted with permission from ref [46]. Copyright 2018 SpringerNature.

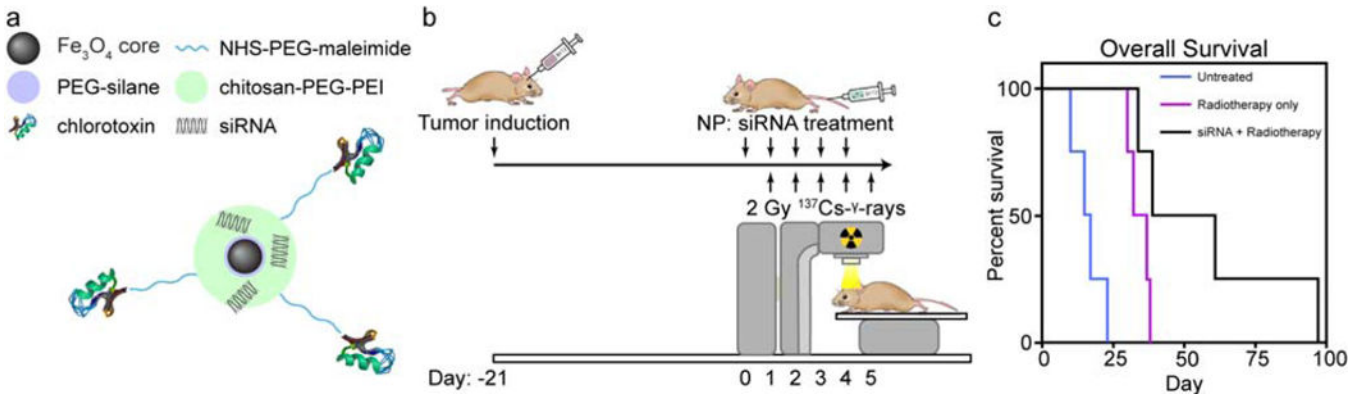

Figure 6.

Combinational siRNA and radiotherapy for cancer treatment. (a) Iron oxide NP design schematic showing the iron oxide core, chitosan-PEG-PEI coating, siRNA loading, and chlorotoxin targeting agent. (b) Treatment timeline for glioblastoma tumor induction, NP injections, and gamma irradiation. (c) Kaplan-Meier curves for overall survival. Adapted with permission from ref [49]. Copyright 2017 Elsevier.